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Cytokine Induction by *Mycoplasma arthritidis*-Derived Superantigen (MAS), but not by TSST-1 or SEC-3, is Correlated to Certain HLA-DR Types

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Superantigens bind to major histocompatibility complex (MHC) class II molecules on antigen presenting cells and T cells in a Vβ-restricted manner. Both cell types are activated resulting in cytokine production. Although the MHC-II binding site for superantigens has been well described, little is known as to whether this binding complex has an influence on cytokine induction. In order to assess superantigen induced cytokine production and its correlation to HLA-DR types, the authors stimulated peripheral blood from 40 subjects with superantigens toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin C-3 (SEC-3) and *Mycoplasma arthritidis*-derived superantigen (MAS), and measured cytokine levels thereafter. The HLA-DR type was determined in each subject. A statistical evaluation was carried out between the highest superantigen cytokine induction and the presence of certain HLA-DR types. Whereas MAS presented a statistical association between the highest cytokine production with HLA-DR4, DR7 and DR12, no such associations were observed for TSST-1 and SEC-3. These results demonstrate that T cell stimulation, and consequently its cytokine production by MAS but not by TSST-1 and SEC-3, depends on the presenting HLA-DR type. Because the diverse HLA-DR specificities are given according to the variability of the β chain of the HLA-DR molecule, the data suggest the participation of the human MHC-II β chain in the MAS/MHC-II binding.

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INTRODUCTION

Superantigens are considered to be among the most potent activators of the immune system. They are products of bacteria, including one mycoplasma or viruses and bind to major histocompatibility complex (MHC) class II and T cells through a specific interaction with the Vβ region of the T-cell receptor (TCR) [1]. The staphylococcal enterotoxins (SE) cause food poisoning and toxic shock syndrome [2], and they induce cytokine secretion in monocytes and T cells [3, 4]. This has been suggested to be related to the toxic symptoms [5]. *Mycoplasma arthritidis*-derived superantigen (MAS) is the unique superantigen produced by a mycoplasma, and like other superantigens MAS induces a high level of cytokine production in human and murine T lymphocytes [6, 7]. Whereas murine T cells respond better to MAS than to staphylococcal superantigens, human T cells respond much better to staphylococcal superantigens [8]. This difference appears to be due to differences in the MHC–superantigen interaction, since lymphocytes from transgenic mice expressing human MHC molecules respond better to staphylococcal superantigens than to MAS [9]. Some authors have suggested the participation of MAS in the pathogenesis of rheumatoid arthritis [10, 11].

The structure of the complex HLA-DR/superantigen has been defined by crystallography for toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin B (SEB). It was shown that both superantigens bind to the α chain of the DR1 molecule [12, 13]. In contrast, staphylococcal enterotoxins A (SEA) and E

(SEE) bind to α and β chains of the HLA-DR molecule [14]. Allelic polymorphism of the HLA-DR molecule affects the binding of SEA and SEE, but not the binding of TSST-1 and SEB [15]. The amino acid sequences of SEB and staphylococcal enterotoxins C-1, 2 and 3 (SEC-1, SEC-2 and SEC-3) are very similar and they share biological and immunological properties [16]. *Mycoplasma arthritidis*-derived superantigen interacts with the murine I-E α chain, which corresponds to the human α chain of the HLA-DR molecule; it also binds to the human HLA-DR molecule, particularly to the conserved α chain, and for that reason it has been suggested that responses to MAS in humans may be DR type-independent [17].

In order to assess the influence of the HLA-DR polymorphism on superantigen cytokine induction, the present study measured the cytokine production induced by three superantigens, TSST-1, SEC-3 and MAS, in peripheral blood from 40 subjects and determined the HLA-DR types of each subject. Thereafter the cytokine production by each superantigen was statistically correlated with the different HLA-DR types.

MATERIALS AND METHODS

Subjects. A total of 40 voluntary subjects, 22 women and 18 men, were included in the study. The mean age was 43 ± 13. Informed consent was given by all subjects.

HLA-DR type identification. We isolated DNA according to the salting-out procedure [18]. Genotyping for HLA-DR was performed using a two-step nested PCR-SSP amplification procedure as described by Bein *et al.* [19]. The PCR products for HLA-DR were electrophoresced on a 2% agarose gel and the HLA-DRB1 types were defined using the WHO nomenclature, 1994 [20].

Whole blood assay. Between 1 and 2 h after blood collection, the blood samples were cultured in a whole blood assay as described previously [21]. Test tubes (5 ml, Greiner, Solingen, Germany) were filled with 850 μl RPMI-1640 medium supplemented with L-glutamine (2 mM) and penicillin/streptomycin (100 U/100 μg/ml) (Biochrom, Berlin, Germany). Subsequently, 100 μl of blood was introduced in each tube. Cytokine induction was stimulated using the superantigen MAS obtained from cultures of *Mycoplasma arthritidis*, Jasmin strain, partially purified and lipopolysaccharide (LPS)-free as previously described [7], at a final concentration of 250 ng/ml and the staphylococcal superantigens TSST-1 and SEC-3 (Serva, Heidelberg, Germany) at the same final concentration. All tubes were covered and samples were incubated in a humidified atmosphere of 5% CO_2 and 37°C for 6–24 h (tumour necrosis factor (TNF)-α), 24–48 h (interleukin (IL)-10) and 48–72 h (IL-1β, IL-2, IL-6 and interferon (IFN)-γ). Cell-free supernatants were harvested after incubation and stored at −80°C until used.

Determination of cytokines. The cell-free supernatants were collected and the cytokine concentrations were determined by quantitative enzyme-linked immunosorbent assay (ELISA). The ELISA kit for IL-2 was kindly provided by Dr H. Galatti, Hofmann LaRoche (Basel, Switzerland). The ELISA kits for IL-1β, IL-6 and TNF-α were obtained from R&D (Wiesbaden, Germany), for IL-10 from Biosource (Ratingen, Germany), and for IFN-γ from Holland Biotechnology (Leiden, the Netherlands). Colour intensity was measured photometrically in an ELISA reader (Anthos Labtec, Salzburg, Austria). Individual concentrations could be determined from a standard curve.

Statistics. The Mann–Whitney *U*-test was used for statistical evaluation of the cytokine concentrations stimulated by different superantigens and the Pearson correlation test for the correlation among different cytokines. The analysis of the association between different HLA-DR types and cytokine production was carried out by multiple stepwise linear regression (STATISTIX V4.0). For the HLA-DR, nine dummy variables were used, taken as reference HLA-DR8. For the cytokines, only values ≥ 75th percentile were used for the statistic. Low statistical significance was defined as $P < 0.05$ (two-tailed) and high significance as $P < 0.01$ (two tailed).

Table 1. Cytokine concentration means after MAS, TSST-1 and SEC-3 stimulation

Cytokine (pg/ml)	Incubation time	MAS	TSST-1	SEC-3
IL-1β	48 h	184.3	73.12	92.78
	72 h	187.8	99.67	147.5
IL-2	48 h	177.2	492.2	601.5
	72 h	170.3	455.4	570.7
IL-6	48 h	4814	1328	1386
	72 h	4649	1299	1257
IL-10	24 h	48.25	92.85	130.8
	48 h	15.05	97.93	134.2
IFN-γ	48 h	348.1	2253	3315
	72 h	873.1	3538	4305
TNF-α	6 h	18.03	39.88	39.46
	24 h	25.13	86.96	126.1

After two different incubation times, every cytokine was measured by ELISA. While TSST-1 and SEC-3 showed no significant differences in their cytokine induction, both revealed significantly ($P < 0.01$) higher production of IL-2, IL-10, IFN-γ and TNF-α than MAS. In contrast, MAS showed significantly ($P < 0.01$) higher IL-1β and IL-6 production than both other superantigens.

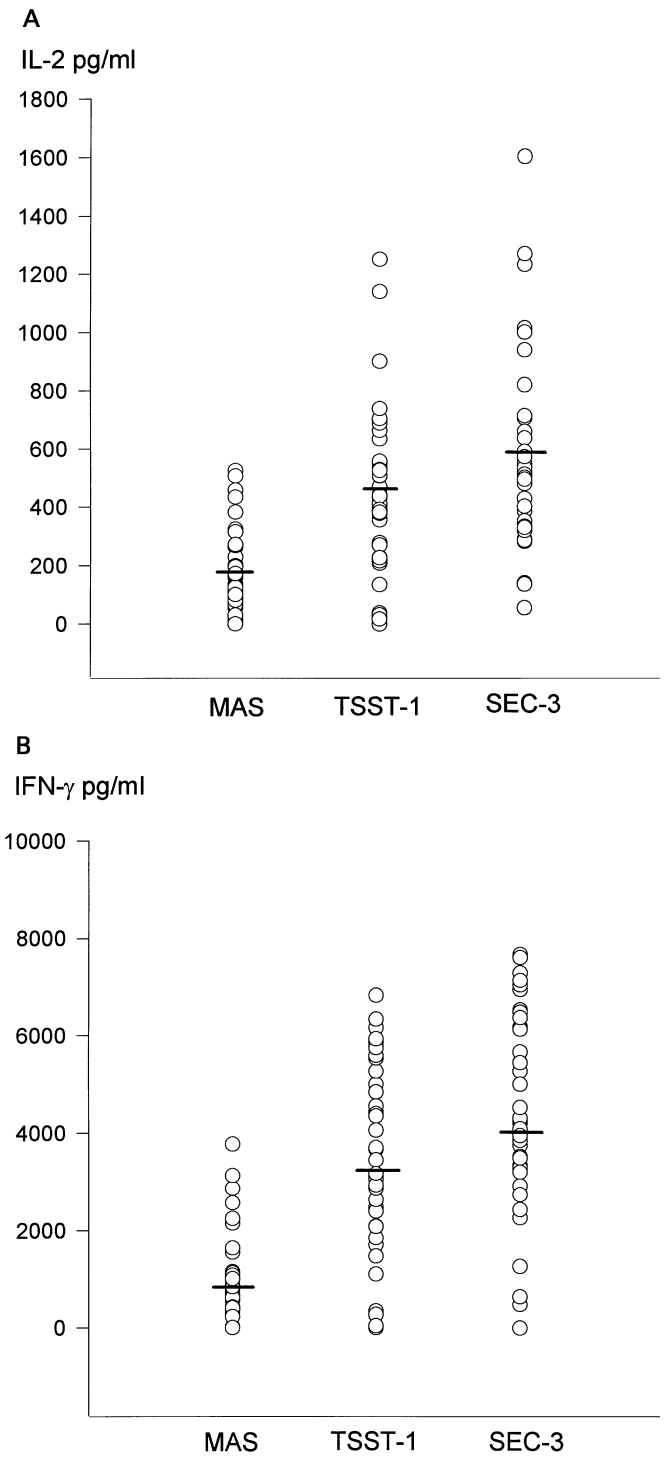
RESULTS

The cytokine induction by the tested superantigens (see Table 1) revealed no significant differences between TSST-1 and SEC-3 but such differences were found between staphylococcal superantigens and MAS. Whereas TSST-1 and SEC-3 showed a significantly higher production of the lymphokines IL-2, IL-10 and IFN-γ than MAS, MAS revealed a significant major production of the monokines IL-1β and IL-6. In contrast, TNF-α was significantly more highly induced by TSST-1 and SEC-3 than by MAS. For both these superantigens TNF-α was significantly correlated with IL-10 and IFN-γ ($P < 0.01$), whereas for MAS TNF-α was correlated with IL-1β and IL-6, which suggests a different TNF-α induction among different superantigens.

For all three superantigens we found remarkable interindividual differences in cytokine induction (see Fig. 1). *Mycoplasma arthritidis*-derived superantigen, which is known to stimulate murine lymphocytes much more than human, could

induce IL-2 and IFN-γ levels in certain subjects above the mean level induced by TSST-1 and SEC-3, both typical potent human T-cell activators. Most of the subjects with high IL-2 and IFN-γ production after MAS stimulation also showed high IL-1β and IL-6 concentrations.

In order to assess the influence of the HLA-DR polymorphism related to the cytokine induction by these three superantigens, we used multiple stepwise linear regression to analyze superantigen induced cytokine values over the 75th percentile with the HLA-DR types of each subject. The analysis revealed a statistical association ($r^2 = 0.4885$) between the highest cytokine induction by MAS and the types DR4 ($P < 0.001$), DR7 ($P < 0.05$) and DR12 ($P < 0.05$), but no statistical significances for the superantigens TSST-1 and SEC-3 ($r^2 = 0.10$ and $r^2 = 0.15$, respectively). Figure 2 shows the SEC-3, TSST-1 and MAS induced IL-1β and IFN-γ levels after 48 h related to the different HLA-DR types. Whereas after MAS stimulation the types DR4, DR7 and DR12 showed higher IL-1β and IFN-γ mean levels (over the whole cytokine mean) than the rest of the types (both cytokine mean levels under the whole cytokine mean), such a difference in the cytokine mean levels between the different HLA-DR types could not be seen after TSST-1 and SEC-3 stimulation. Similar results were observed for the rest of the cytokines measured (data not shown). These statistical results implicate an HLA-DR polymorphism influence on the cytokine response to MAS stimulation, but not on the stimulation by TSST-1 and SEC-3, and consequently different MHC class II binding among different superantigens.



DISCUSSION

Superantigens stimulate a large fraction of T cells upon their interaction with the TCR and the MHC class II molecule. Although T cells from several species are able to respond to superantigens, human T lymphocytes respond better to staphylococcal superantigens whereas murine T cells respond better to MAS [8]. In the present study, TSST-1 and SEC-3 induced significantly higher IL-2, IFN-γ and IL-10 concentrations than MAS, which reveals their stronger human T cell stimulatory effect. On the other hand, MAS induced significantly higher amounts of IL-1β and IL-6 than TSST-1 and SEC-3, which implies that MAS, a typical murine T cell superantigen, stimulates human monocytes more strongly than these staphylococcal superantigens and suggests different cell activation among different superantigens. Interestingly, significantly higher levels of TNF-α are induced by TSST-1 and SEC-3 than by MAS. The failure of MAS to induce high levels of TNF-α in human peripheral blood mononuclear cells has been described previously [7] and was shown to be specific for MAS and not common to all superantigens. The correlation of TNF-α with IL-

Fig. 1. Individual IL-2 (A) and IFN-γ (B) levels induced by MAS, TSST-1 and SEC-3 after 72 h. In both (A) and (B) the cytokine levels, as well as the cytokine mean for each subject, are represented by symbols and black lines, respectively. For all three superantigens there are remarkable interindividual differences in IL-2 and IFN-γ induction. *Mycoplasma arthritidis*-derived superantigen, which is known to stimulate murine lymphocytes much more than human, induces lower lymphokine production than TSST-1 and SEC-3 in most of the subjects; in certain subjects IL-2 and IFN-γ levels after MAS stimulation are over the mean level induced by TSST-1 and SEC-3, both typical human T-cell potent activators. Most of those subjects with high IL-2 and IFN-γ production after MAS stimulation also showed high IL-1β and IL-6 concentrations.

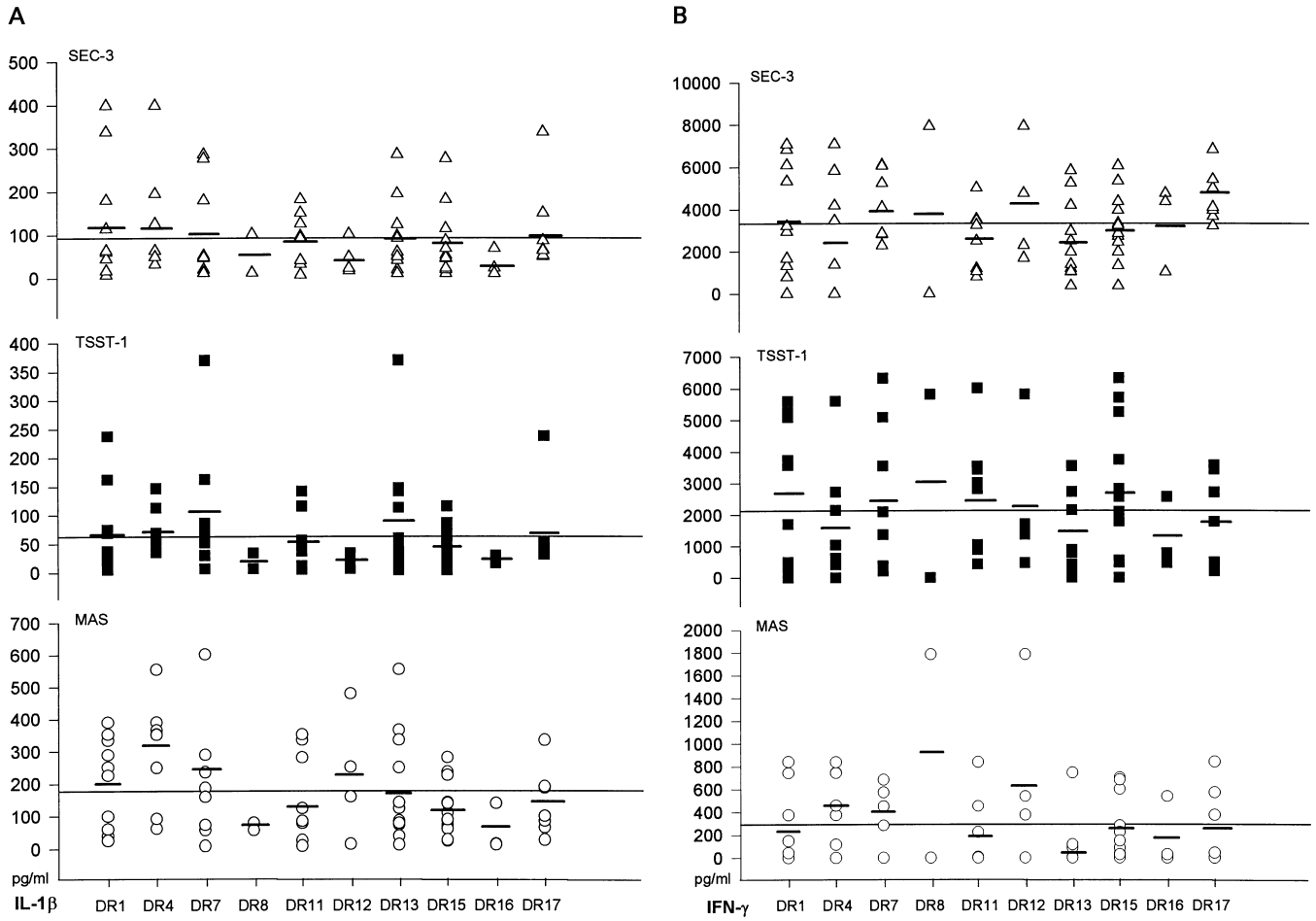


Fig. 2. SEC-3, TSST-1 and MAS induced IL-1β (A) and IFN-γ (B) after 48 h related to HLA-DR types. In both figures IL-1β and IFN-γ concentrations were given by each HLA-DR type. In subjects with homozygous HLA-DR types the cytokine concentration was given twice. The different cytokine concentrations are represented by symbols (△, ■, ○) for SEC-3, TSST-1 and MAS, respectively. The IL-1β and IFN-γ concentration mean for each type and the whole cytokine mean for each superantigen are also represented. Whereas after MAS stimulation the types DR4, DR7 and DR12 showed higher IL-1β and IFN-γ mean levels (over the whole cytokine mean) than the rest of the types (cytokine mean levels under the whole cytokine mean), no such difference could be shown between the different IL-1β and IFN-γ means by each HLA-DR type after TSST-1 and SEC-3 stimulation.

2, IFN-γ and IL-10 and not with IL-1β and IL-6 by TSST-1 and SEC-3, suggests that TNF-α is produced by T cells and supports recent studies which report TNF-α production by T cells stimulated with TSST-1 [22].

The present data also show the influence of the HLA-DR polymorphism on cytokine induction by MAS but not by TSST-1 or SEC-3, and reveal the existence of different HLA-DR binding sites for MAS, TSST-1 and SEC-3. The fact that T cell stimulation by MAS and its subsequent cytokine production depends on the presence of certain HLA-DR types, e.g. DR4, DR7 and DR12, and because the diverse HLA-DR specificities are given according to the variability of the β chain of the HLA-DR molecule, our results implicate the participation of the β chain in the MAS–HLA-DR binding and not only binding to the α chain as previously described [17]. This could take place in a similar way to SEA and SEE. Both bind to α and β chains of the HLA-DR molecule [14] where the interaction of the β chain is zinc-dependent [23], since zinc regulates MAS cytokine induction as well [24]. Furthermore the binding of MAS to the α chain has already been described [17]. Recently Bernatchez *et al.* [25] have shown by competition with SEA mutants that MAS dimerizes class II molecules by interacting with their α and β chains, which also supports our present data. However, not all individuals presenting the HLA-DR types DR4, DR7 or DR12 showed high cytokine production after MAS stimulation, which suggests a different binding affinity for MAS to the MHC-II molecule. Furthermore, preliminary studies with murine CTL and human B lymphoblastoid cell lines with different homozygous HLA-types revealed MAS induced CTL proliferation in the presence of only certain alleles of a defined HLA-DR type (unpublished observations).

While TSST-1 binds only to the α chain [12], specific binding of TSST-1 has been observed on all human HLA-DR types [26] and it could be an explanation as to why there was no statistical

association between the HLA-DR polymorphism and the production of cytokines after TSST-1 stimulation. Concerning SEC-3, although its molecular binding to the HLA-DR molecule has not been described till now, it seems to bind only to the α chain of the HLA-DR molecule, because the cytokine production was independent of the HLA-DR polymorphism and consequently independent of the binding to the HLA-DR β chain. Its molecular binding to the HLA-DR probably takes place in a similar way to SEB [13] because both superantigens share biological and immunological properties [16].

The different HLA-DR binding sites among these three superantigens may be a further explanation for the high capacity of MAS to induce IL-1 and IL-6 in contrast to TSST-1 and SEC-3. Recently Mehindate *et al.* have showed the requirement of the cross-linking of MHC-II α and β chains for the monocyte activation by superantigen [27].

Finally, some authors have involved MAS in the pathogenesis of rheumatoid arthritis [10, 11]. The influence of the HLA-DR polymorphism on the MAS cytokine production might be the linkage between the genetic predisposition of rheumatoid arthritis and the outcome of this disease.

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