Staphylococcal exotoxins deliver activation signals to human T-cell clones via major histocompatibility complex class II molecules

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Communicated by Judah Folkman, May 3, 1991

ABSTRACT We investigated whether staphylococcal exotoxins (SEs), in addition to their capacity to induce T-cell activation restricted by the T-cell receptor (TCR) β -chain variable region, can deliver an activation signal to human T-cell clones through major histocompatibility complex (MHC) class II molecules. Eleven human T-cell clones (9 $\alpha\beta$ TCR and 2 $\gamma\delta$ TCR clones) of different antigenic specificities were tested for their capacity to proliferate in response to toxic shock syndrome toxin 1 (TSST-1) and two SEs, SEA and SEB. In the absence of accessory cells, only 4 $\alpha\beta$ TCR clones were stimulated to proliferate, each by a single SE, and to mobilize intracellular free Ca^{2+} in response to that SE, events indicative of TCR engagement and, presumably, recognition restricted by the β -chain variable region. In the presence of accessory cells, each of the 11 T-cell clones was stimulated to proliferate by any one of the three SEs tested. This apparently TCR-unrestricted SE-mediated polyclonal proliferation of T-cell clones occurred in the absence of an increase in intracellular free Ca^{2+} and was not dependent on the presence of MHC class II expression on accessory cells. In contrast, SE-mediated polyclonal proliferation did not occur in 3 $\alpha\beta$ TCR clones derived from an MHC class II-deficient patient. Furthermore, all of the three SEs induced the proliferation of 4 natural-killer-cell clones, suggesting that expression of TCR/CD3 complex is not essential for SE-mediated polyclonal proliferation of activated lymphocytes. These results indicate that MHC class II molecules transduce activation signals to human T- and natural-killer-cell clones.

Staphylococcal exotoxins (SEs) bind to human major histocompatibility complex (MHC) class II molecules (1–3) and also stimulate the proliferation of T cells in a manner restricted by the T-cell receptor (TCR) β -chain variable region (V β) (4). Binding of SE to MHC class II molecules on monocytes results in interleukin (IL) 1 and tumor necrosis factor release (5, 6). Binding of SE to MHC class II molecules on B cells delivers a costimulatory signal that acts synergistically with anti- μ chain antibodies to cause B-cell proliferation (7).

Because activated human T cells express MHC class II molecules, we investigated whether SE can deliver an activation signal to MHC class II⁺ T cells. The data presented demonstrate that SEs deliver activation signals via MHC class II molecules to MHC class II⁺ human T-cell clones. These signals act synergistically with TCR/CD3-mediated signals and with signals delivered by accessory cells to induce T-cell proliferation.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs). OKT3 is a mouse IgG2a anti-CD3 mAb. 3B12 is a mouse IgG1 mAb that recognizes the three human MHC class II molecules DR, DP, and DQ.

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Both mAbs were purified from ascites fluid by affinity chromatography on a protein A-Sepharose column (Pharmacia).

$\alpha\beta$ TCR T-Cell Clones and Lines. The human $\alpha\beta$ TCR T-cell clones G8, 827, and H10 were accessory-cell-dependent T-cell clones and were specific for tetanus toxoid (TT) presented in association with, respectively, HLA-DR5, -DR3, and -DR5 (8, 9). Clone F6 had dual reactivity with HLA-DR4 and TT in the context of HLA-DR3 (10). Clone A1 was an HLA-DP alloreactive clone (8). Clone S12 and S14 were derived from peripheral blood mononuclear cells (PBMCs) stimulated with OKT3, an anti-CD3 mAb. Clones P4 and P13 were derived from PBMCs stimulated with phytohemagglutinin (PHA). Clone SP-B21 is TT-specific and was established from a patient with severe combined immunodeficiency who was immunologically reconstituted after transplantation with fetal liver and thymus (11, 12). Clone SP-B21 recognizes TT (Calbiochem) in the absence of any accessory cell (M. G. Roncarolo and H.S., unpublished data). Clones F6 and A1 were subcloned twice at 0.3 cell per well. In addition, the clonality of F6 (13), G8 (data not shown), 827 (9), and SP-B21 (data not shown) was established by Southern blot analysis with a TCR β -chain constant region (C β) probe. Only the V β family expressed by clones SP-B21 and 827 could be ascertained; these two clones reacted (>99% of cells), respectively, with the anti-V β 8 mAb MX-11 (14) and the anti-V β 5 mAb ICI (15). The other 11 clones failed to react with a panel of mAbs directed to V β 5, V β 6, V β 8, and V β 12 (T Cell Sciences, Cambridge, MA). T-cell clones were maintained in RPMI 1640 medium (M. A. Bioproducts), supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, and recombinant IL-2 (20 units/ml), and were stimulated once a week as appropriate with TT (30 $\mu\text{g}/\text{ml}$) and autologous irradiated (7500 rad; 1 rad = 0.01 Gy) Epstein-Barr virus (EBV)-transformed B cells (EBV-B cells, clones G8 and H10) or with irradiated autologous EBV-B cells and PHA (10 $\mu\text{g}/\text{ml}$; clones P4 and P13) or with anti-CD3 and autologous irradiated monocytes (clones S12 and S14) or with allogeneic irradiated monocytes (clones F6 and A1). Clones 827 and SP-B21 were stimulated biweekly with irradiated PBMCs, irradiated JY cells, and PHA (0.1 $\mu\text{g}/\text{ml}$; Wellcome) in Yssel's medium (12) and expanded with recombinant IL-2 (20 units/ml). An alloreactive MHC class II⁺ T-cell line was derived from PBMCs of a normal donor stimulated with irradiated allo-EBV-B cells and maintained in RPMI 1640 medium containing IL-2 supernatant. The MHC class II⁻ line SA was derived from PBMCs of a patient deficient in MHC class II molecules (16) and stimulated with PHA (10 $\mu\text{g}/\text{ml}$).

Abbreviations: SE, staphylococcal exotoxin; TSST-1, toxic shock syndrome toxin 1; MHC, major histocompatibility complex; mAb, monoclonal antibody; TCR, T-cell receptor; PHA, phytohemagglutinin; EBV, Epstein-Barr virus; $[\text{Ca}^{2+}]_i$, intracellular free calcium concentration; V β , β -chain variable region; TT, tetanus toxoid; PBMC, peripheral blood mononuclear cell; IL, interleukin; NK, natural killer.

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irradiated EBV-B cells, and recombinant IL-2. MHC class II⁻ clones TSA2, TSA6, and TSA21 were isolated from this line by limiting dilution at 0.3 cell per well and maintained in culture as the parent MHC class II⁻ line.

$\gamma\delta$ TCR and Natural-Killer (NK) Clones. Two $\gamma\delta$ TCR clones, WM-14 [from cord blood (17, 18)] and ES-443 [from PBMCs (19)], were derived by limiting dilution from CD4⁻CD8⁻ T cells that were isolated by cell sorting and stimulated with the EBV-transformed cell line JY. WM-14 expresses V δ 3 (18); its antigen specificity is unknown. ES-443 expresses V δ 1 as shown by its reactivity with the mAb δ TCS1 (T Cell Sciences) and is specific for HLA-A2 (19).

The NK clones were isolated from human peripheral blood by selecting CD3 ϵ ⁻CD16⁺CD56⁺ and CD3 ϵ ⁻CD16⁻CD56⁺ lymphocytes by cell sorting and then cloning by flow cytometry (20). All four NK clones (NL-1, NL-9, 13, and 4) were shown to have their TCR genes in the germ-line configuration. The cells were cultured in Yssel's medium supplemented with PHA (0.1 μ g/ml), recombinant IL-2 (20 units/

ml), irradiated PBMCs, and irradiated JY cells as described (12). Feeder cells rapidly deteriorated and disappeared after 3–5 days. After day 5, no cell expressing CD3, CD4, CD8, CD14, or CD19 could be detected in the culture by flow cytometry. NK cells were stimulated with SEs (1 μ g/ml) at day 6 after feeding.

Proliferation Assay. Prior to stimulation with SE, T cells were depleted from accessory cells either by adherence on a Sephadex G-10 column (21) in 5% (vol/vol) type AB⁺ human serum/Hanks' balanced salt solution or on a Percoll gradient (22). Depletion of accessory cells was confirmed by the absence of a proliferative response to PHA (10 μ g/ml). T cells were distributed in a 96-microwell culture plate (40 $\times$ 10³ cells per well) (Nunc) and stimulated with toxic shock syndrome toxin 1 (TSST-1) or one of two SEs, SEA or SEB, at 1 μ g/ml (Toxin Technology, Madison, WI) for 96 h. Autologous irradiated (7500 rad) EBV-B cells (40 $\times$ 10³ cells per well) were used as accessory cells as indicated. MHC class II⁻ EBV-B cells used as accessory cells in some

FIG. 1. SE-mediated proliferation of $\alpha\beta$ TCR and $\gamma\delta$ TCR T-cell clones. T-cell clones were depleted of feeder cells and stimulated with a SE (1 μ g/ml, as indicated) in the absence (–) or presence (+) of autologous irradiated MHC class II⁺ accessory cells (EBV-B cells). [³H]Thymidine incorporation was measured at day 4 of culture. Results are expressed as mean of triplicate determinations. The SD was always <15% of the mean.

FIG. 2. Effect of SEs on $[Ca^{2+}]_i$ in T-cell clones. Clone A1 (MHC class II⁺) was loaded with quin-2 tetrakis(acetoxymethyl) ester and stimulated with SEB (A) or SEA (B) at 2.5 μ g/ml. Results similar to B were obtained with TSST-1. Clone P4 (MHC class II⁺) was stimulated with SEB (C) or SEA (D). Results similar to C were obtained with TSST-1. Clone TSA21 (MHC class II⁻) was stimulated with SEB (E) or SEA (F). Stimulation of clone TSA21 with TSST-1 did not mobilize intracellular Ca^{2+} , comparable to F. Stimulations were done in presence of MHC class II⁺ accessory cells (EBV-B cells).

experiments were derived from two patients deficient in MHC class II molecules, Ramia (23) and SA (16). An MHC class II⁺ T-cell line and the MHC class II⁻ T-cell line SA were depleted in feeder cells by Percoll gradient and stimulated at day 8 after feeding. Prior to stimulation, anti-CD3 (OKT3) and anti-MHC class II (3B12) mAbs were crosslinked to a 96-well microtiter plastic plate, by incubation at 37°C in 0.1 M sodium carbonate (pH 9.6). After 3 h, plates were washed three times with Hanks' solution. Cultures were harvested after the addition of 1 μ Ci of [³H]thymidine (New England Nuclear; 6.7 Ci/mol; 1 Ci = 37 GBq) during the final 18 h of culture. Results are expressed as cpm of [³H]thymidine incorporated into DNA. The SD of triplicates was <15% of the mean for all samples.

Measurement of Intracellular Free Calcium Concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was determined on T-cell clones loaded with quin 2 tetrakis(acetoxymethyl) ester as described (24).

RESULTS

Fig. 1 demonstrates that, in the absence of accessory cells, SE-mediated proliferation was observed in 4 of the 11 $\alpha\beta$ - or $\gamma\delta$ TCR clones tested (the $\alpha\beta$ TCR clones A1, G8, S12, and P4). In each case, proliferation was stimulated by only a single SE. In the presence of accessory cells (EBV-B cells), each of the 9 $\alpha\beta$ TCR clones and both $\gamma\delta$ TCR clones studied were stimulated to proliferate by all of the three SEs tested, TSST-1, SEA, and SEB.

The single-SE restricted proliferation of clones A1, G8, S12, and P4 in the absence of accessory cells suggested that the TCR of these clones recognized that SE in a V β -restricted manner (4). Because TCR/CD3-complex engagement is known to result in an increase in $[Ca^{2+}]_i$, we performed Ca^{2+} flux studies with three of these T-cell clones (A1, S12, and P4). SEB, which caused accessory-cell-independent proliferation of clones A1 and S12, induced an intracellular Ca^{2+} flux in clones A1 (Fig. 2A) and S12 (data not shown). Similarly SEA, which caused accessory-cell-independent proliferation of clone P4, induced an increase in $[Ca^{2+}]_i$ in clone P4 (Fig. 2D). Furthermore, preincubation with SEB (clones A1 and S12) or SEA (clone P4) completely inhibited the capacity of anti-CD3 mAb to induce an increase in $[Ca^{2+}]_i$ (data not shown). These results strongly suggest that the TCR of clones A1 and S12 was engaged by SEB and the TCR of clone P4 was engaged by SEA.

Clones A1, G8, S12, and P4 failed to proliferate in response to crosslinked anti-CD3 mAb in the absence of accessory cells (data not shown). This observation suggested that the SE-stimulated accessory-cell-independent proliferation of clones A1, G8, S12, and P4 involved, in addition to signaling by the TCR, SE-mediated signaling by the MHC class II molecules. This hypothesis was supported by the observation that 3B12, an anti-MHC class II molecule mAb, acted synergistically with OKT3, an anti-CD3 mAb, to cause the proliferation of T-cell lines and clones derived from normal subjects but not from a patient with an MHC class II deficiency (Table 1). Thus, MHC class II molecules are able to transduce signals that act synergistically with signals delivered by the TCR/CD3 to cause the proliferation of MHC class II⁺ T cells.

The SE-stimulated proliferation of all 11 T-cell clones and each of the three SEs tested in the presence of accessory cells suggested that SE-mediated signals delivered by MHC class II molecules acted synergistically with accessory-cell signals to cause polyclonal T-cell proliferation. In contrast to V β -restricted SE-mediated TCR activation, SE-mediated polyclonal activation was not associated with a demonstrable increase in $[Ca^{2+}]_i$. SEA and TSST-1 failed to cause an increase in $[Ca^{2+}]_i$ in clones A1 (Fig. 2B) and S12 (data not

Table 1. Anti-CD3 and anti-MHC class II mAbs act synergistically to induce activated human T-cell proliferation

T-cell source	MHC class II	$[^3H]$ Thymidine, cpm $\times 10^{-3}$		
		Anti-CD3	Anti-MHC class II	Anti-CD3/anti-MHC class II
Normal line	+	0.3	0.5	43.2
Patient line	-	0.3	0.3	0.3
Clone 9	+	0.4	0.3	31.7
Clone TSA6	-	0.3	0.3	0.4

Plastic 96-well microtiter plates were coated with anti-CD3 mAb OKT3 (40 ng/ml) and/or anti-MHC class II mAb 3B12 (10 μ g/ml) in carbonate buffer for 3 h at 37°C. Plates were washed three times with Hanks' solution. T cells were incubated (50×10^3 cells per well) in RPMI 1640 medium containing 10% type AB⁺ serum in triplicate at 37°C for 3 days. [³H]Thymidine incorporation into DNA was measured during the last 18 h of a 96-h proliferation assay. The SD of triplicates was always <15% of the mean.

shown), although these two SEs induced the proliferation of the two clones in the presence of accessory cells. Similarly, stimulation of clone P4 with TSST-1 or SEB did not induce an increase in $[Ca^{2+}]_i$, although clone P4 would proliferate in response to these two SEs in an accessory-cell-dependent manner (Fig. 2C). Furthermore, prior exposure to SEA or TSST-1 (clones A1 and S12) or to SEB or TSST-1 (clone P4) did not abort the increase in $[Ca^{2+}]_i$ caused by subsequent stimulation with anti-CD3 mAb (data not shown).

The hypothesis that SEs transduce an activation signal to T cells from MHC class II molecules was further supported by the study of three T-cell clones derived from a patient with an MHC class II deficiency (16). Fig. 3 shows that, in contrast to normal clones, three clones derived from the MHC class II-deficient patient failed to polyclonally proliferate in the presence of accessory cells to all of the three SEs tested. Two of these clones (TSA2 and TSA21) were restricted in their proliferation to a single SE (SEA and SEB, respectively). Stimulation of clone TSA21 with SEB but not with the other SEs triggered an increase in $[Ca^{2+}]_i$ (Fig. 2E and F), suggesting that SEB engaged the TCR of clone TSA21. Because clones TSA2 and TSA21 failed to express MHC class II molecules, their TCR-restricted SE-mediated proliferation, in contrast to that of normal clones A1, G8, S12, and P4, required the presence of accessory cells that expressed MHC class II molecules.

SE binding to MHC class II molecules on accessory cells results in lymphocyte function-associated antigen 1 (LFA-1)-dependent cell adhesion (16) and in cytokine production by monocytes (5, 6). We examined whether MHC class II expression by accessory cells was required for the delivery of the accessory-cell signal. Fig. 4 demonstrates that EBV-B cells

FIG. 3. SE-mediated proliferation of $\alpha\beta$ TCR MHC class II⁻ T-cell clones. MHC class II⁻ T-cell clones were depleted of feeder cells and stimulated with a SE (1 μ g/ml; as indicated) in the absence (-) or presence (+) of irradiated MHC class II⁺ accessory cells. $[^3H]$ Thymidine incorporation was measured at day 4 of culture. Results are expressed as means of triplicate determinations. The SD are always <15% of the mean.

derived from an MHC class II-deficient patient and expressing no detectable MHC class II molecules supported the SE-mediated proliferation of T-cell clones, albeit to a lesser degree than that of normal EBV-B cells. The requirement for an accessory-cell signal in addition to that delivered by MHC class II molecules for SE-mediated polyclonal T-cell proliferation was illustrated by an additional clone we studied, clone SP-B21. Clone SP-B21 is known to be an accessory-cell-independent clone in that it undergoes TT-mediated proliferation in the absence of accessory cells. In contrast to the other 11 MHC class II⁺ clones tested (Fig. 1), clone SP-B21 was stimulated to proliferate by all three SEs in the absence of accessory cells (medium, 1100 cpm; TSST-1, 4400 cpm; SEA, 22,200 cpm; SEB, 24,600 cpm). These results support the proposal that a second signal is required for T-cell proliferation in response to MHC class II engagement by SEs.

We finally examined whether SE could mediate lymphocyte activation in the absence of TCR/CD3 complex expression by testing the capacity of SE to induce the proliferation of CD3⁻ NK cell clones. Table 2 shows that all four CD3⁻ MHC class II⁺ CD56⁺ NK clones tested were induced to proliferate by any of the three SEs in the presence and in the absence of accessory cells. The response of NK clones to SEs was obtained at day 6 after stimulation with feeder cells.

DISCUSSION

The data presented in this paper indicate that MHC class II molecules can transduce activation signals to MHC class II⁺ human T-cell clones. The MHC class II-mediated signal can act synergistically with an accessory-cell-derived signal or with signals derived from the TCR/CD3 complex to cause T-cell proliferation.

Synergy between MHC class II-mediated signals and TCR/CD3-mediated signals was illustrated by the restricted proliferation, in the absence of accessory cells, of four

FIG. 4. Role of accessory-cell MHC class II expression on SE-mediated T-cell clone proliferation. Prior to stimulation with a SE (1 μ g/ml; as indicated), T-cell clones were extensively depleted of residual feeder cells. MHC class II⁻ EBV-B cells were derived from an MHC class II-deficient patient (Ramia) (10, 23). MHC class II⁺ EBV-B cell lines were in each case autologous to the T-cell clones. $[^3H]$ Thymidine incorporation into DNA was measured in triplicate during the last 18 h of a 96-h proliferation assay. The SD of triplicates was always <15% of the mean. Groups of bars: A, no accessory cells; B, MHC class II⁺ accessory cells; C, MHC class II⁻ accessory cells.

Table 2. Proliferative response of human NK clones to SEs

Clones	Accessory cells	[³ H]Thymidine, cpm × 10 ⁻³			
		Medium	TSST-1	SEA	SEB
NL-1	—	0.1	5.4	5.2	11.7
	+	1.0	19.6	32.7	23.3
NL-9	—	0.1	1.4	4.9	3.5
	+	1.0	4.0	13.6	5.3
13	—	1.2	20.4	24.2	26.4
	+	2.8	42.9	30.1	33.9
4	—	6.6	33.0	32.9	31.7
	+	ND	ND	ND	ND

All four clones are CD3⁺CD56⁺. Clones NL-1, NL-9, and 4 are CD16⁺. Clone 13 is CD16⁻. At day 6 of stimulation with irradiated feeder cells in conditioned medium, cells were stimulated with SEs (1 µg/ml). [³H]Thymidine incorporation into DNA was measured in triplicate during the last 18 h of a 96-h proliferation assay. The SD of triplicates was always <15% of the mean. ND, not determined.

αβTCR clones, each to a single SE. Engagement of the TCR of these clones by SE was suggested by an increase in [Ca²⁺]_i upon stimulation with SEs. Synergy between signals delivered by TCR and MHC class II molecules was demonstrated by the capacity of crosslinked mAbs to MHC class II and to CD3 to induce proliferation of MHC class II⁺ but not MHC class II⁻ T-cell lines and T-cell clones (Table 1).

A particularly important observation in this study is the capacity of SE to polyclonally activate MHC class II⁺ T-cell clones in the presence of accessory cells. SE-mediated polyclonal activation of T-cell clones was dependent on MHC class II expression by the T-cell clones because it was not observed in the clones derived from an MHC class II-deficient patient. The MHC class II signal was not accompanied by a detectable increase in intracellular Ca²⁺ flux (Fig. 2 B and C) and was insufficient by itself to cause T-cell proliferation as shown by the failure of MHC class II⁺ T-cell clones to undergo polyclonal SE-mediated proliferation in the absence of accessory cells. The optimal concentration of SE needed for presumably non-TCR-mediated activation of MHC class II⁺ clones in the presence of accessory cells was one order of magnitude higher than optimal SE concentrations required for TCR-mediated activation of the same clones (data not shown). Because mAb to MHC class II molecules did not cause T-cell lines or clones to proliferate in the presence of accessory cells (data not shown), we cannot rule out the possibility that SE delivered an additional signal, possibly the result of unrestricted low-affinity binding of SE to the TCR. The observation that TCR/CD3⁺ NK-cell clones were stimulated by SE to proliferate polyclonally indicates that TCR/CD3-complex expression was not essential for the SE-mediated proliferation of MHC class II⁺-activated lymphocytes. We cannot exclude the possibility that, in addition to binding to MHC class II molecules, SE might bind also in an unrestricted manner to the NK-cell receptor complex (25).

MHC class II-dependent SE-mediated polyclonal proliferation of T-cell clones required a second signal derived from accessory cells. The nature of the accessory-cell signal is at present unknown. It could be replaced by intrinsic signals generated by an accessory-cell-independent T-cell clone (clone SP-B21) or by NK cells. The failure of SE to mediate polyclonal activation of MHC class II-deficient T-cell clones in the presence of MHC class II⁺ accessory cells makes it unlikely that the observed proliferation was simply the result of the action of cytokines secreted by MHC class II⁺ EBV-B-cell lines in response to SE. Because the accessory-cell signal could be delivered, albeit less efficiently, by EBV-B cell lines derived from MHC class II-deficient patients, engagement of MHC class II molecules on accessory cells by SE is not essential for these accessory cells to support the SE-

mediated proliferation of T-cell clones. Interaction of SE with MHC class II⁺ cells enhances LFA-1-dependent adhesiveness (16). This may facilitate cellular interactions between T-cell clones and accessory cells by LFA-1-mediated aggregation.

The capacity of SE to polyclonally activate MHC class II⁺ T-cell clones has important implications for diseases caused by bacterial toxins. Interferon γ released by T cells responding to a toxin in a Vβ-restricted fashion could result in MHC class II expression by bystander T cells. These in turn may become targets for polyclonal activation by SEs. This would result in the amplification of the T-cell response including the secretion of cytokines that play an important role in the clinical manifestations of SE-mediated diseases (5, 6, 26).

We thank Haifa Jabara for providing clones, Dr. Talal Chatila for helpful discussion, and Joe Phillips (Becton Dickinson, San Jose, CA) for his help with sorting and cloning of the NK cells. Drs. H. Yssel and M. G. Roncarolo are gratefully acknowledged for their help with clones 827 and SP-B21. This work was supported by U.S. Public Health Service Grants 5 RO1 AI20373-5 and 5 P50 AI21163-07. F.S. was supported by a fellowship from the Fondation suisse de Bourses en Médecine et Biologie. DNAX Research Institute of Molecular and Cellular Biology is supported by Shering-Plough Corporation.

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