T Cell-mediated Lethal Shock Triggered in Mice by the Superantigen Staphylococcal Enterotoxin B: Critical Role of Tumor Necrosis Factor

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Summary

Because mice are more resistant than humans to the pathogenic effects of bacterial toxins, we used D-Galactosamine- (D-Gal) sensitized mice as a model system to evaluate potential toxic shock symptoms triggered by the superantigen staphylococcal enterotoxin B (SEB). We show that similar to endotoxin (lipopolysaccharide) [LPS], the exotoxin SEB causes lethal shock within 8 h in D-Gal–sensitized mice, inducing 100% and about 50% lethality with 20 and 2 μg SEB, respectively. The lethal shock triggered by the superantigen SEB is mediated by T cells, a conclusion based on the observation that T cell repopulation of SCID mice conferred sensitivity to SEB. Since CSA also conferred protection, the role of T cell–derived lymphokines in mediating lethal shock was evaluated. Within 30–60 min after SEB injection, serum tumor necrosis factor (TNF) levels peaked, followed immediately by interleukin-2 (IL-2). Serum-borne lymphokines were detected well in advance of signs of T cell activation, as assessed by IL-2 receptor expression of SEB-reactive Vβ8$^+$ T cells. Passive immunization with anti-TNF-α/β–neutralizing monoclonal antibody also conferred protection, indicating that it is TNF which is critical for initiating toxic shock symptoms. Taken together, this study defines basic differences between endotoxin (LPS)- and exotoxin (SEB)-mediated lethal shock, in that the former is mediated by macrophages and the latter by T cells. Yet the pathogenesis distal to the lymphokine/cytokine–producing cells appears surprisingly similar in that TNF represents a key mediator in inducing shock.

Bacterial endotoxins are recognized as a major factor in the pathogenesis of Gram-negative septic shock, a disease with significant morbidity and mortality in humans (1, 2). Macrophages appear to be essential in mediating endotoxin reactions, since adoptive transfer of endotoxin-sensitive macrophages renders previous endotoxin-resistant mice sensitive to endotoxin (3). Further, it has been established that the macrophage product TNF-α/cachectin is central in causing endotoxin lethality, a cytokine produced by macrophages in response to endotoxins (4).

Not only endotoxins, but also bacterial exotoxins from certain Gram-positive bacteria cause toxic shock in humans (5–7). Whereas the mechanisms of endotoxin-induced shock became increasingly defined (3, 4), the mode of action of bacterial exotoxins is unclear as yet. A clue might represent the recent demonstration that some of these exotoxins exhibit the unusual properties of superantigens thereby stimulating in vitro and in vivo massive T cell proliferation (8, 9). Superantigens have been operationally defined as bifunctional molecules

binding to MHC class II structures and activating T cells expressing appropriate Vβ segments of the TCR (9–12).

The ability of exotoxins to act as superantigens for T cells represents a logical starting point to hypothesize that exotoxin-mediated rapid T cell activation leading to systemic lymphokine secretion may be causally linked to the pathogenesis of shock. Indeed T cell–derived lymphokines appear to cause shock-like syndromes as deduced from observations in human after therapeutic anti-CD3 mAb application in allograft recipients (13, 14).

Here, we have studied the pathogenic effects of one staphylococcal toxin, enterotoxin B (SEB)[1] in mice. To bypass the known natural resistance of mice to the effects of bacterial toxins (8), we increased their sensitivity by pretreat-

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[1] Abbreviations used in this paper: CSA, Cyclosporin A; D-Gal, D-Galactosamine; SEB, staphylococcal enterotoxin B; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin 1.

ment with D-Galactosamine (D-Gal) (15, 16). We now report that in D-Gal–sensitized mice the superantigen SEB triggers a T cell–mediated lethal shock syndrome.

Materials and Methods

Mice and Culture Medium. Mice of the strain BALB/c (H-2^d), C3H/He (H-2^k), and C57BL/6 (H-2^b) were purchased from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. SCID mice were obtained from the Gesellschaft für Strahlen- und Umweltforschung (Munich, Germany). The medium used was Click/RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% (vol/vol) FCS, 10 mM Hepes buffer, 5 × 10^{-5} 2-ME, 1 μg/ml indomethacin, and antibiotics, respectively.

mAb and Reagents. Hybridoma F23.1 (producing anti-Vβ8 mAb) was kindly provided by Dr. M. Bevan (Scripps Institute, La Jolla, CA). The supernatant of this hybridoma was purified using a protein G column (Pharmacia, Freiburg, Germany). Hybridoma 7D4 producing a mAb reactive with the murine IL-2R was obtained from American Tissue Culture Collection (Rockville, MD). The mAb was purified from ascites fluid and FITC labeled according to the method of Goding (17). Anti-Vβ8 mAb was labeled with biotin (17). The mAb V1q neutralizing murine TNF-α and TNF-β has been described recently (18).

The exotoxin SEB (S4881), the endotoxin LPS (L3129), and MTT (M2128) were purchased from Sigma, Munich, Germany. D-Gal hydrochloride (7411) was from Roth, Karlsruhe, Germany. Recombinant human IL-2 (batch no. LP-3170B) was a kind gift from Eurocetus, Amsterdam, The Netherlands. Cyclosporin A (CSA) was generously provided by Dr. Borel, Sandoz AG, Basel, Switzerland.

FACS® Analyses. The draining, popliteal lymphnodes were removed and a single cell suspension was prepared. Thereafter, cells were stained on ice with FITC labeled anti–IL-2R and PE-conjugated anti-Vβ8 mAb, respectively, washed twice with PBS containing 2% FCS, and subsequently fixed with 1% paraformaldehyde. The labeled cells were analyzed on a Elite flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Preparation of Serum. Immunized and control animals were killed, and blood was drawn from the heart. Subsequently, the samples were centrifuged at 10,000 rpm, the supernatant collected and stored at −70°C until used in the IL-2 and TNF assay, respectively.

TNF Assay. TNF levels in mouse serum were determined using TNF ELISA kits (Genzyme Corp., Cambridge, MA). The assay was performed exactly as described by the manufacturer. Serum samples were used at a dilution of 1:5. Each sample was determined in triplicate.

Assay for IL-2 Activity. To measure IL-2 levels, the CTLL bioassay was performed as described (19). Briefly, 1.5 × 10^4 cells/well were incubated with serum samples, diluted 1:100 for 24 h. MTT, which was metabolized by living cells in 2 h, was then added. After solubilization of MTT crystals with isopropanol, the OD was determined at 570 nm. To express the IL-2 activity in serum samples in U/ml, a standard curve was established by incubating CTLL cells with different amounts of recombinant human (rhu) IL-2. Each serum sample was measured in triplicate.

Results

Lethal Effect of SEB in D-Galactosamine–treated Mice. The lethal toxicity of SEB was evaluated in D-Gal–sensitized mice, and compared with that of LPS. Groups of mice received a

Table 1. *Lethal Effect of SEB (or LPS) in D-GAL–sensitized Mice*

	Treatment		Lethality within 8 h (dead/total)
D-Gal	SEB	LPS	
mg	μg/mouse	μg	
20	–	–	0/3*
40	–	–	0/3*
–	300	–	0/3*
–	200	–	0/3*
–	20	–	0/3*
20	200	–	4/4
20	20	–	4/4
20	2	–	1/4
20	0.2	–	0/4
–	–	100	0/3
–	–	50	0/3
–	–	5	0/3
20	–	10	3/3
20	–	1	3/3
20	–	0.1	1/3

Groups of BALB/c mice simultaneously received D-Gal intraperitoneal and SEB (hind footpads), or D-Gal and LPS (intraperitoneal), respectively. Controls received D-Gal, SEB, or LPS alone.
* No apparent signs of illness.
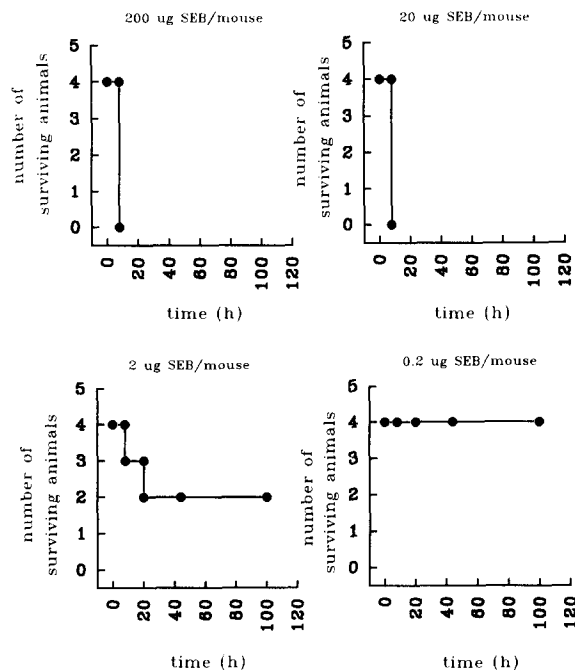
Figure 1. Groups of BALB/c mice (four/group) were simultaneously treated with 20 mg D-Gal (i.p.) and titrated amounts of SEB (hind footpads) as indicated.

dose of 20 mg D-Gal i.p., a protocol previously shown to enhance sensitivity to LPS (15), and in addition, SEB or LPS. Animals receiving D-Gal, SEB, or LPS alone served as controls. In accordance with published data (15) Table 1 shows that D-Gal treatment confers high sensitivity to LPS. For example, 1 μg LPS was lethal in D-Gal–sensitized BALB/c mice, while in unsensitized mice 100 μg caused only marginal signs of illness such as immobility and rough fur, but no lethality. As in the case of LPS, none of the unsensitized BALB/c mice died upon injection of up to 300 μg SEB. At these high doses the animals showed marginal signs of illness (immobility, rough fur), but recovered within 24–48 h. However, upon D-Gal sensitization 20 μg SEB sufficed to cause death in all injected mice (Table 1). There was no significant difference in toxicity between SEB, given intraperitoneal, intravenous, or subcutaneously (data not shown). In addition, D-Gal–sensitized C3H and C57BL/6 mice behaved almost identically to BALB/c mice in their shock response to SEB (data not shown).

The time course of lethality induced by LPS or SEB in D-Gal–sensitized mice was ~8 h. The first signs of illness (immobility, rough fur) became apparent ~3–4 h before death. Only when sublethal doses of SEB were administered did D-Gal–sensitized mice survive up to 48 h. Thereafter, some of the animals recovered again (Fig. 1). As reported for endotoxin lethality in D-Gal–sensitized animals (15), D-Gal–mediated sensitization to SEB was most effective when D-Gal and SEB were injected with a time interval of less than 2 h. When D-Gal was administered 4 h before SEB, or 4–6 h after SEB, lethality was not observed (data not given). The lethal toxicity of SEB in D-Gal–sensitized mice was seen in many duplicate experiments with some variations in the effects of a particular dose of toxin. We conclude that similar to endotoxin (15), D-Gal renders mice susceptible to lethal shock triggered by the exotoxin SEB.

T Cell Requirement for the Lethal Toxicity of SEB. It has been established that SEB acts as a superantigen for T cells expressing appropriate Vβ chains of the TCR (10). Therefore we evaluated the requirement for T cells to cause lethal toxicity. In the first approach, we functionally depleted mice for T cells by administering CSA, a reagent known to suppress lymphokine secretion by T cells (20). All CSA-treated and D-Gal–sensitized mice survived without signs of illness when challenged with an otherwise lethal concentration of SEB (Table 2). These data indicated that the release of lymphokines from SEB-reactive T cells may be central for the lethal shock.

In the second approach, SCID mice known to lack T and B cells, were used. As shown in Table 2, D-Gal–sensitized SCID mice were sensitive to LPS, indicating that their macrophage reactivity to LPS was sufficient to cause endotoxin-mediated lethal shock. Table 2 also shows that SCID mice treated with D-Gal were resistant to the lethal toxicity of SEB, indicating that T cells are required for the manifestation of toxicity. This was confirmed by the observation that upon reconstitution of SCID mice with T cells (15 × 10⁶ cells/mouse), administration of SEB caused lethal shock. We

Table 2. *T Cell–mediated Lethal Shock Syndrome*

D-Gal (20 mg i.p.)	CSA (0.5 mg i.p.)	SEB (20 μg/ mouse)	LPS (10 μg i.p.)	Lethality (dead/total)	
				BALB/c	SCID
−	−	+	−	0/3	ND
+	−	+	−	3/3	0/9
+	+	+	−	0/5	ND
+	−	+	−	3/3	5/5*
+	−	−	+	3/3	3/3

Treatment of mice with more than one agent was done at the same time with doses indicated in the table.
* SCID mice were reconstituted with 15 × 10⁶ BALB/c T cells 6 d before injection.

concluded that T cells are mediating the lethal toxicity triggered by SEB.

Kinetics of Lymphokine Release and T Cell Activation in D-Gal–treated Mice. The acute (within 8 h) T cell–mediated lethal toxicity triggered by SEB in D-Gal–sensitized mice prompted us to evaluate signs for T cell activation in LNs draining the site of SEB injection. Since Vβ8–expressing T cells are known to respond to SEB (12), we scored over time by two-color staining the appearance of IL-2R⁺ Vβ8⁺ T cells in LNs draining the side of SEB injection. Representative data as demonstrated in Fig. 2 show that 8 h after SEB administration all Vβ8⁺ T cells within the draining LN became IL-2R⁺. A timing of IL-2R expression revealed that 2–4 h after SEB application Vβ8⁺ became IL-2R⁺. After 8 h virtually all Vβ8⁺ T cells expressed the IL-2R at high densities (mean fluorescence intensity is recorded in channel 8, Figs. 2 and 3 A). In other words, IL-2R expression in vivo tags the subset of T cells reactive to SEB. Note that IL-2R expression of Vβ8⁺ T cells was equally independent whether mice were D-Gal sensitized or not (Fig. 3 A).

Because the protective effect of CSA (Table 2) suggested that release of lymphokines was central to the lethal toxicity triggered by SEB, we next evaluated IL-2 and TNF lymphokine levels in the blood of D-Gal–sensitized or unsensitized mice treated with SEB. Surprisingly, as early as 1–2 h upon SEB injection, peak levels of IL-2 were present in the blood (Fig. 3 B). Thereafter, blood-borne IL-2 concentrations sharply decreased both in D-Gal–sensitized and unsensitized mice. Peak levels of TNF were detected as early as 30–60 min upon SEB administration (Fig. 3 A). Again, TNF levels decreased sharply at later time points. We conclude that SEB-triggered release of lymphokines from T cells represents an immediate event, well in advance of SEB-mediated triggering of proliferative responses of SEB-reactive T cells, as defined by IL-2R expression.

Anti-TNF-α/β mAb Confer Protection. There is compelling evidence that the lethal effects of endotoxin during septicemia can be prevented by passive immunization of mice
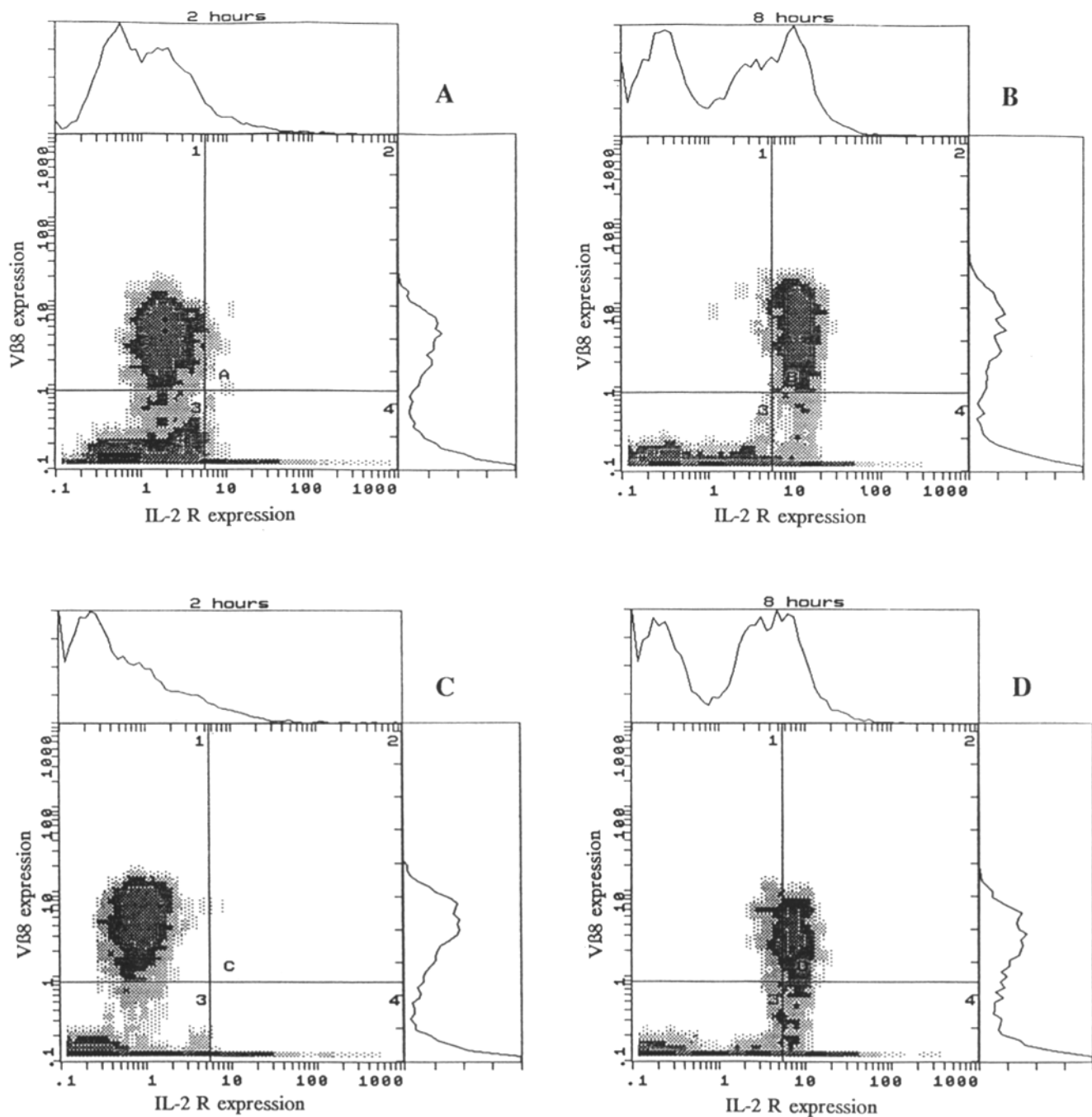
Figure 2. T cells of the draining popliteal lymph node were stained with FITC-labeled anti-IL-2R mAb and biotin/PE-labeled anti-Vβ8 mAb. In *A* and *B*, Balb/c mice were treated with SEB (20 μg/footpad) alone, while in *C* and *D*, mice received D-Gal (20 mg i.p.) and SEB (20 μg/footpad). *A* and *C* depict cells 2 h, and *B* and *D* 8 h after treatment.

against TNF-α/cachectin (4). Because of the apparent similarities between LPS- and SEB-triggered lethal shock in D-Gal–sensitized mice (Tables 1 and 2), we speculated whether T cell–dependent lymphokines such as TNF-α/cachectin and/or TNF-β/lymphotoxin might represent key mediators for the T-cell–mediated lethal shock induced by SEB. We therefore used the neutralizing anti–mouse TNF mAb V1q, shown

to protect mice from LPS-induced shock as well has to neutralize the T cell lymphokine TNF-β/lymphotoxin (18), to evaluate for its protective effects. As shown in Table 3, passive immunization with V1q mAb also conferred protection against the lethal effect of SEB as well as that of LPS. In fact, amounts of mAb V1q as low as 50 μg/mouse were effective. We conclude that TNF plays a critical role in inducing lethal
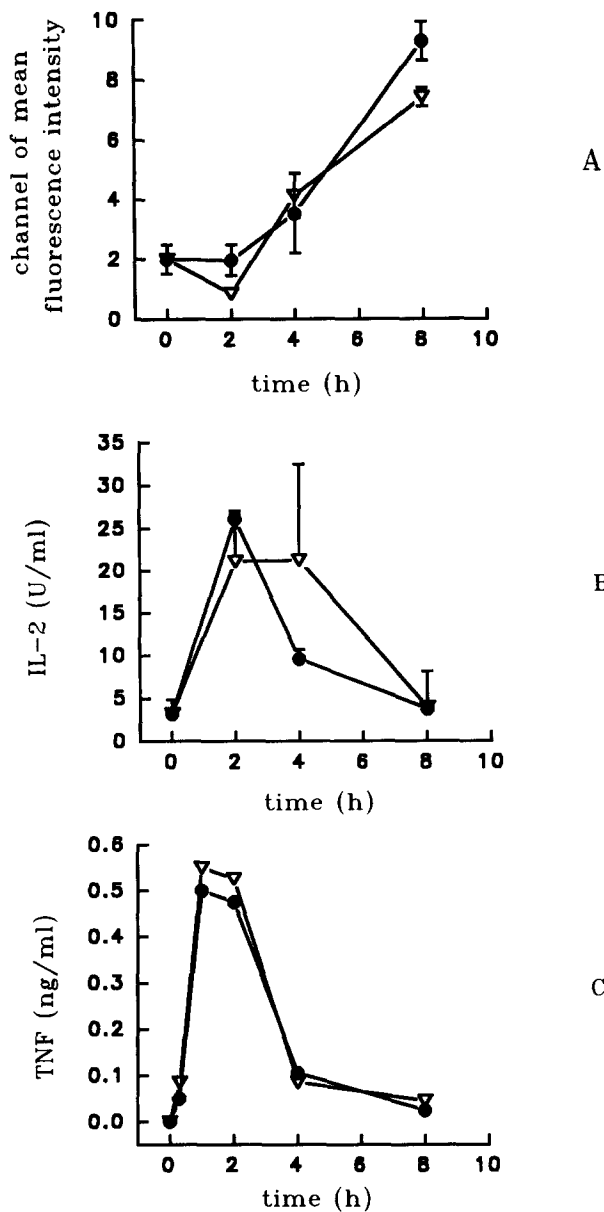
Figure 3. *A* shows the kinetic of the expression of the IL-2R on $V\beta8^+$ T cells of the draining popliteal lymph node. BALB/c mice were treated with either SEB alone (20 μg/footpad, *filled circles*), or with D-Gal (20 mg i.p.) and SEB (20 μg/footpad, *open triangles*). Each symbol represents three individual mice. *B* depicts the kinetic of serum levels of IL-2 in mice injected with SEB alone (20 μg/footpad, *filled circles*) or with D-Gal (20 mg i.p.) and SEB (20 μg/footpad, *open triangles*). Each symbol represents three individual mice. IL-2 was measured using the CTLL bioassay. In *C*, the kinetic of TNF serum levels is demonstrated. At each time point the sera of two individual mice treated with 20 μg SEB and 20 mg D-Gal (*open triangles*), or 20 μg SEB alone (*closed circles*) were analyzed. TNF was measured with commercially available ELISA kits as described in Material and Methods.

toxicity in the exotoxin shock studied here. Yet the lack of specific mAb discriminating between TNF-α and TNF-β hindered our identification of the type of TNF involved.

Table 3. *Anti-TNF mAb Confers Protection*

D-Gal (20 mg i.p.)	SEB (20 μg/mouse)	Anti-TNF mAb V1q μg i.p.	Lethality (dead/total)
+	+	250	0/2
+	+	50	0/2
+	+	10	2/3
+	+	1	3/3

Groups of BALB/c mice were injected with D-Gal and SEB at the same time. 2 h in advance titrated amounts of anti-TNF mAb V1q were administered. Isotype-matched control mAb were without effects (data not shown).

Discussion

Exotoxins of certain Gram-positive bacteria are known to contribute to toxic shock pathogenesis and to function as superantigens as well (10, 21). Whether both facets are causally linked is at present unknown. Probably because mice are relatively resistant to bacterial toxins, use of murine animal models in studying pathogenesis of exotoxin-induced toxic shock has been limited (22). The sensitivity of mice to endotoxin can be greatly enhanced by impairing liver metabolism with D-Gal (15). Here we adopted this approach to evaluate the pathogenesis of exotoxin-mediated toxicity. We show that upon D-Gal sensitization of mice the superantigen SEB causes lethal toxicity within 8 h, while D-Gal–sensitized SCID mice become sensitive to SEB only upon repopulation with T cells. Manifestation of lethal toxicity requires SEB-reactive $V\beta8^+$ T cells, and is inhibited by CSA, a reagent known to block production of lymphokines by T cells. Within 30–60 min upon SEB administration, high concentrations of TNF and, within 1–2 h, high concentrations of IL-2 are noted in the blood. In contrast to the immediate lymphokine release into the blood, activation of $V\beta8^+$ T cells by SEB in terms of IL-2R expression, an early marker for T cell activation, requires 4–8 h. Passive immunization of mice with TNF-α/β neutralizing mAb V1q effectively conferred resistance to T cell–mediated shock. We conclude that D-gal–induced sensitization of mice represents a useful model system in unraveling not only the central role of T cells, but also that of T cell–dependent TNF in the pathogenesis of the lethal shock triggered by the exotoxin SEB.

The TSS-toxin 1 produced by toxigenic strains of *Staphylococcus aureus* (21, 23) is probably the best documented example of an exotoxin causing TSS in humans. While TSST-1 has no direct toxic effect on a variety of tissues tested (24), it represents a powerful in vitro activator of lymphocytes and monocytes thereby inducing the production of many T cell lymphokines including IL-2 (25), IFN-γ, TNF-β-lymphotoxin (26), and colony stimulating factors (27), as well as cytokines such as IL-1 and TNF-α/cachectin (28, 29). Since the symptoms observed in patients suffering from TSS are similar in

many respects to those observed in humans upon administration of large quantities of IL-2 during human cancer therapy (30) or anti–CD3 mAb administration to transplant recipients (13, 14), we searched for a useful in vivo model for dissecting the pathogenesis of shock symptoms as triggered by superantigenic bacterial exotoxins.

Because mice are more resistant to bacterial toxins than are humans, high doses of the toxin SEB, for example, are needed to observe T cell–mediated stress symptoms such as weight loss and thymic involution (22, own observations). Since in humans acute toxic shock reactions dominate the response of mice to superantigenic exotoxins (21, 23), we searched for means to enhance the sensitivity of mice to superantigenic exotoxins. To this we evaluated the effect of D-Gal (2-amino-2-deoxy-D-galactose), a substance known to increase the sensitivity of mice to the lethal effects of endotoxin (15, 16) severalfold. D-Gal is a hepatotoxic agent, and the development of sensitization to endotoxin requires depletion of UTP and changes in uracyl nucleotides thereby impairing biosynthesis of macromolecular cell constituents (3).

In both D-Gal–treated and untreated mice there was no obvious difference in the kinetics of appearance of serum-borne TNF and IL-2, and the kinetics of IL-2R expression within SEB-reactive V$\beta 8^+$ T cells (Fig. 3, B and C). However, only the former succumbed to lethal shock (Tables 1 and 2, see below). We take this as argument that the effect of D-Gal is only to augment sensitivity to endogeneous mediators (15), rather than to affect their rate of production.

As in the case of LPS (reference 15, Table 1), we show here (Table 1) that SEB is highly toxic in D-Gal–sensitized mice, inducing 100 and 50% lethality with 20 and 2 μg SEB, respectively. In the absence of D-Gal, up to 300 μg SEB caused no lethality (Table 1). As in the case of LPS (15), the state of D-Gal–induced hypersensitivity to SEB lasts only about 4–6 h, and mice sensitized with D-Gal and a lethal dose of SEB developed the first signs of illness about 4 h before death, which took place 6–8 h upon injection.

In contrast to endotoxin (LPS), the exotoxin SEB caused no lethal toxicity in D-Gal–sensitized SCID mice known to lack B and T cells. Yet adoptive transfer of T cells into SCID mice conferred sensitivity to the lethal effect of SEB (Table 2). In addition, the compound CSA, known to inhibit lymphokine production by T cells (20), effectively protected D-Gal–sensitized mice towards SEB-induced toxicity. It follows that upon D-Gal application mice developed sensitivity to lethal shock induced not only by LPS but also by SEB. However, the basic difference between both toxins is that macrophages are mediating the endotoxin reactions (3) while the lethal shock triggered by the superantigen, i.e., exotoxin SEB, are mediated by SEB-reactive T cells.

In Gram-negative septicemia, the macrophage product TNF-α/cachectin appears early in circulation as a brief peak upon infusion of bacterial LPS (31), but is sufficient to induce secondary cytokines including IL-1 and IL-6 (32). Further, upon infusion of high doses of TNF-α/cachectin in experimental animals, toxic shock symptoms such as cardiovascular collapse, lactic acidosis, and extravascular fluid sequestration ensue (31). Because T cells are mediating SEB-induced lethal shock, we considered T cell–dependent TNF-α/ cachectin (33) or TNF-β/lymphotoxin (33) as prime candidates in effecting lethal shock. This conclusion is supported by our finding that passive immunization with anti-TNF mAb neutralizing both TNF-α/cachectin and TNF-β/lymphotoxin (18) effectively conferred resistance to SEB-triggered T cell–mediated shock (Table 3). These results define TNF as a key mediator not only in endotoxin/macrophage-mediated lethal shock (34) but also in T cell–dependent shock. TNF-α and TNF-β can be produced by T cells (33), and are lymphokines with limited-sequence homology, (35) but bind the same receptors (31, 36) and have common effects on a large number of target cells (31, 36). Excessive production of TNF-α or TNF-β might therefore yield in similar toxic symptoms, a conclusion supported by our observation that endotoxin/TNF-α–mediated lethal shock symptoms are phenotypically indistinguishable from the lethal shock induced by the exotoxin SEB.

The kinetics of production and elimination of serum-borne TNF and IL-2 during SEB-induced shock and its relation to IL-2R expression in SEB-reactive V$\beta 8^+$ T cells were unexpected. Within 30–60 min upon SEB injection, serum-borne TNF peaked, followed 60 min later with a peak of IL-2. Both mediators declined thereafter, as if an SEB-induced immediate burst of T cell–dependent mediators were followed by a phase of elimination from circulation at a constant rate. On the other hand, there was a clear delay in time until SEB-reactive T cells became IL-2R$^+$, an early marker for T cell proliferation. It thus appears that T cell–dependent mediators such as TNF trigger toxic shock symptoms well in advance of the signal events yielding in cellular activation of the lymphokine-secreting T cells (Fig. 2).

In contrast to the marked lethality associated with TNF-α in endotoxin-mediated septic shock (37), TNF-α is tolerated when administered to humans in clinical trials (38). Since in mice the lethal effect of TNF-α is potentiated by Il-1 (39) and fatal outcome in human septic shock is associated with high levels of not only TNF, but also IL-6 and IL-1, (32) the role of additional cytokines such as IL-1 and IL-6 in SEB-mediated shock needs now to be evaluated.

In conclusion, our study demonstrates the applicability of D-Gal–sensitized mice in studying the pathogenesis of bacterial exotoxin-mediated lethal shock. In this system, exotoxin-reactive T cells cause shock via systemic and immediate release of lymphokines. Since passive immunization with neutralizing anti-TNF-α/β mAb confers protection, T cell–dependent TNF appears to play a principal role. Thus, exotoxin and endotoxin shock seem to differ in the sense that T cells are mediating the former and macrophages the latter, but the events distal to the mediator-producing cells might be very similar in that TNF is central in causing toxicity.

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