

Distribution and Kinetics of Superantigen-induced Cytokine Gene Expression in Mouse Spleen

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Summary

The polyclonal stimulation of T cells by bacterial superantigens is involved in the pathogenesis of the toxic shock syndrome in certain staphylococcal and streptococcal infections. Here we describe the onset and kinetics of superantigen-induced cytokine production in situ in spleens of normal BALB/c mice monitored at the level of cytokine mRNA expression by in situ hybridization. Messenger RNAs for interleukin 2 (IL-2), interferon γ , and tumor necrosis factors (TNF) α and β were not expressed at detectable levels in spleens of unstimulated animals but became visible already 30 min after intraperitoneal application of 50 μ g staphylococcal enterotoxin B. All mRNA levels showed peak expression approximately 3 h after injection and a slow decrease up to 24 h after injection. Expression of the mRNAs was restricted to the T cell-dependent area of the periarteriolar lymphatic sheets of the spleen. Interestingly, TNF- α mRNA showed a biphasic response, the early appearing mRNA had the same localization as the other mRNAs, whereas after 3 h TNF- α mRNA showed a broader distribution indicating a second cell population producing TNF- α . The expression of IL-2 and TNF proteins in the serum increased in parallel to the observed mRNA changes with a slight delay. The presence of macrophages was not required for the expression of the cytokine mRNAs in the spleen as the expression was unchanged in macrophage-depleted mice. Only the second phase of TNF- α mRNA expression was abrogated in such animals. The expression of all mRNAs was completely suppressed by prior administration of cyclosporin A. These data show that nonphagocytic cells are the essential superantigen-presenting cells in vivo and indicate that at least part of the pathogenetic TNF- α is T cell derived.

An in situ analysis of a T cell response should provide information on the distribution of induced events in the affected organs and on the kinetics of the response. In contrast, in vitro experiments allow only a vague approximation of the kinetics of an immune response and do not give much information on the significance of cellular interactions for the events in vivo. It is therefore surprising that such analyses in vivo have only rarely been performed so far, possibly because of the usually low frequency of responding cells in antigen-specific immune reactions.

The study of a superantigen response has the advantage that a superantigen activates a large fraction of T cells by a mechanism closely resembling antigen recognition. Superantigens, such as the staphylococcal enterotoxins, cross-link variable parts of the TCR with MHC class II antigens on APCs. Consequences of this massive T cell activation are the transient expansion of the stimulated T cell subset followed by death and development of anergy in these cells, and even-

tually shocklike symptoms due to a burstlike expression of different cytokines (1, 2).

TNF has been reported to play a critical role in the development of superantigen-induced toxic shock because an antibody against TNF has a protective effect (3). The cellular source of TNF and the pattern of cytokine secretion in vivo have not yet been characterized. In vitro studies have demonstrated that staphylococcal enterotoxins can induce TNF- α from purified macrophages, probably by binding to their MHC class II molecules (4, 5). Studies with mutant superantigens have indicated that class II binding alone was insufficient to cause toxicity in mice (6, 7). A current model therefore postulates that TNF is produced by macrophages but that T cells are required to induce an enhanced TNF production by macrophages (7), most likely via released IFN- γ (7). Although T cells are able to produce TNF- α after stimulation with superantigens (8), they have not been implicated as a major source of this cytokine.

In the present report we have analyzed the cytokine response in the spleen to a single injection of the staphylococcal enterotoxin B (SEB)¹, a prototypic superantigen activating in mice all T cells bearing V β 8 TCR (9), by *in situ* hybridization for mRNAs. We demonstrate an extremely rapid accumulation of cytokine mRNA *in situ* and describe evidence that at least part of the TNF is T cell derived.

Materials and Methods

Reagents. SEB was produced as a recombinant protein in *Staphylococcus aureus* SK291 (10), and was purified to apparent homogeneity as described previously (11). LPS from *Salmonella typhimurium* was purchased from Sigma Chemical Co. (Munich, FRG). Cyclosporin A (CyA), a gift of Sandoz Inc. (Basel, Switzerland), was dissolved in olive oil. The anti-V β 8 mAb F23.1 and the antimacrophage mAb F4/80 (12) were obtained from the American Type Culture Collection (Rockville, MD). They were purified on protein G-Sepharose columns and conjugated with FITC according to standard procedures. Unless indicated, all chemicals were from Sigma, and all molecular biology reagents from Boehringer Mannheim (Mannheim, FRG).

DNA Probes and *In Vitro* Transcription. Four cytokine cDNA probes were used as RNA probes. The IL-2 probe was a 504-bp HindIII-EcoRI fragment of exon 4 cloned into pGEM (gift of Dr. A. Schimpl, University of Würzburg, FRG); the IFN- γ probe was a 643-bp cDNA fragment cloned into pSP65 (gift of Dr. E. Rude, University of Mainz, FRG); the TNF- α probe was a 470-bp cDNA fragment cloned into pGEM3(-) (gift of Dr. D. Männel, University of Regensburg, FRG); and the TNF- β probe was a 700-bp BstXI-XbaI fragment of murine TNF- β cDNA (gift of Dr. N. Ruddle, Yale University Medical School, New Haven, CT) cloned into pGEM7Zf(+). The plasmids were linearized with the appropriate restriction enzymes and transcribed to antisense or sense cRNA using SP6 or T7 polymerases as appropriate in the presence of ³⁵S-UTP (New England Nuclear, Dreieich, FRG). With the exception of IFN- γ , all experiments were performed with both antisense and sense RNA probes.

All labeled cRNAs were purified over Nick columns (Pharmacia, Freiburg, FRG) and diluted in hybridization buffer (100 mM Tris, pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.5 mg/ml t-RNA, 0.1 mg/ml sonicated salmon sperm DNA, 1 \times Denhardt's solution, 10% dextran sulfate, 50% formamide) to 50,000 cpm/ μ l. cRNA was stored for not longer than 2 wk at -75°C.

Treatment of Mice. BALB/c mice were injected intraperitoneally with 50 μ g of SEB or 5 μ g of LPS. Higher and lower doses of SEB have been shown to lead to strong T cell stimulation *in vivo* (3, 13). This dose has been used and spleens were removed after 0.5, 1, 2, 3, 4, 9, 24, or 48 h. Organs were immediately embedded in Tissue Tek (Miles Laboratories, Elkhart, IN) and frozen in -30°C cold isopentane. Tissues were stored at -75°C until 16- μ m cryostat sections were cut and stored at -75°C. CyA was injected intraperitoneally at 50 mg/kg 4 h before the enterotoxin. For depletion of macrophages from spleens, mice were injected intravenously with 200 μ l of liposomes containing dichloromethylene diphosphonate (Cl₂MDP) as described (14). This treatment leads to a complete disappearance of phagocytic cells in spleen and liver after 48 h (14).

Bioassays for IL-2 and TNF- α . IL-2 in the serum was measured

by a CTLL test. CTLL2 cells were cultured in a 96-well plate with 2,500 cells per well in Iscove's medium supplemented with 5% FCS, 0.1% gentamicin, 1% glutamine, and 0.1% 2-ME. Serum samples were used at a dilution of 1:100 and measured in triplicates. After 24 h, cells were pulsed with [³H]thymidine overnight, harvested, and incorporation was measured by liquid scintillation counting. TNF- α was measured using its cytotoxic effect on actinomycin D-pretreated Wehi 164 cells. The cells were incubated with dilutions of serum, and after overnight incubation, viable cells were determined by incubation with methylthiazol-tetrazolium (Sigma Chemical Co.).

***In Situ* Hybridization.** *In situ* hybridization was performed with small modifications as described by Schäfer et al. (15). Serial 16- μ m cryostat sections were fixed in 4% paraformaldehyde in PBS at 4°C for 1 h, washed three times in PBS, pretreated by 0.4% Triton X-100 in PBS for 5 min, and acetylated for 10 min in 0.1 M triethanolamine, pH 8.0, with 0.25% acetic anhydride. Tissues were washed in 2 \times SSC, dehydrated in ethanol, and stored at -20°C until hybridization. Hybridization with cRNA, prepared by *in vitro* transcription, was performed by incubation of 35 μ l of cRNA on tissue sections for 16-18 h at 52°C in a moist chamber. Sections were washed in 2 \times SSC and 1 \times SSC for 10 min each and single-stranded RNA was digested by 10 μ g/ml RNase and 1 U/ml T1 RNase (Boehringer Mannheim) in Tris-EDTA, pH 8.0, and 150 mM NaCl at 37°C for 1 h. Afterwards, sections were desalted by passing them through 1 \times SSC, 0.5 \times SSC, 0.2 \times SSC for 10 min each, and washed in 0.2 \times SSC at 60°C for 1 h. The tissue sections were then washed in H₂O for 10 min, dehydrated by ethanol, and air-dried. Autoradiograms were taken by exposing the sections to an autoradiography film (Hyperfilm-MP; Amersham, Dreieich, FRG) for 1-3 d. Autoradiograms were digitized and evaluated with an M4 MCID image analysis system (Imaging Res. Inc., St. Catherines, ON, Canada). Sections were then coated with NBT film emulsion (Eastman Kodak, Rochester, NY). Coated tissues were developed and fixed after 15-25 d (D19 developer, Eastman Kodak) and counterstained with hematoxylin-eosin as described. Although not shown in the figures, sense probes were used in each experiment and never gave any signal above background.

Results

Kinetics and Distribution of Cytokine mRNA in the Spleens of SEB-treated Mice. In spleens of untreated or mock-injected mice, the expression of mRNA coding for IL-2, IFN- γ , TNF- α , and TNF- β was below detection level (Figs. 1, A and F and 2, A and F). Intraperitoneal injection of SEB generated a rapid appearance of mRNAs for these cytokines. IL-2 mRNA was detectable as early as 30 min after injection by x-ray film and also in emulsion-coated autoradiograms (not shown). Levels of IL-2 mRNA were maximal 3-4 h after injection, decreased thereafter, and completely disappeared 48 h after injection (Fig. 1, G and K). Similarly, mRNA for IFN- γ could be detected 30 min after injection and thereafter increased to a peak period lasting from 3 to 9 h (Fig. 1, B and C). This mRNA had a slower decay than IL-2 mRNA, but had also disappeared within 48 h (Fig. 1 E).

The localization of both mRNAs was mainly restricted to the T cell areas. Both IFN- γ and IL-2 mRNA showed a region-specific distribution pattern. Under dark-field illumination, numerous positively labeled cells with medium to very high density of grains were concentrated around nonla-

¹ Abbreviations used in this paper: Cl₂MDP, chloromethylenediphosphonate; CyA, cyclosporin A; PALS, periarteriolar lymphatic sheet; SEB, staphylococcal enterotoxin B.

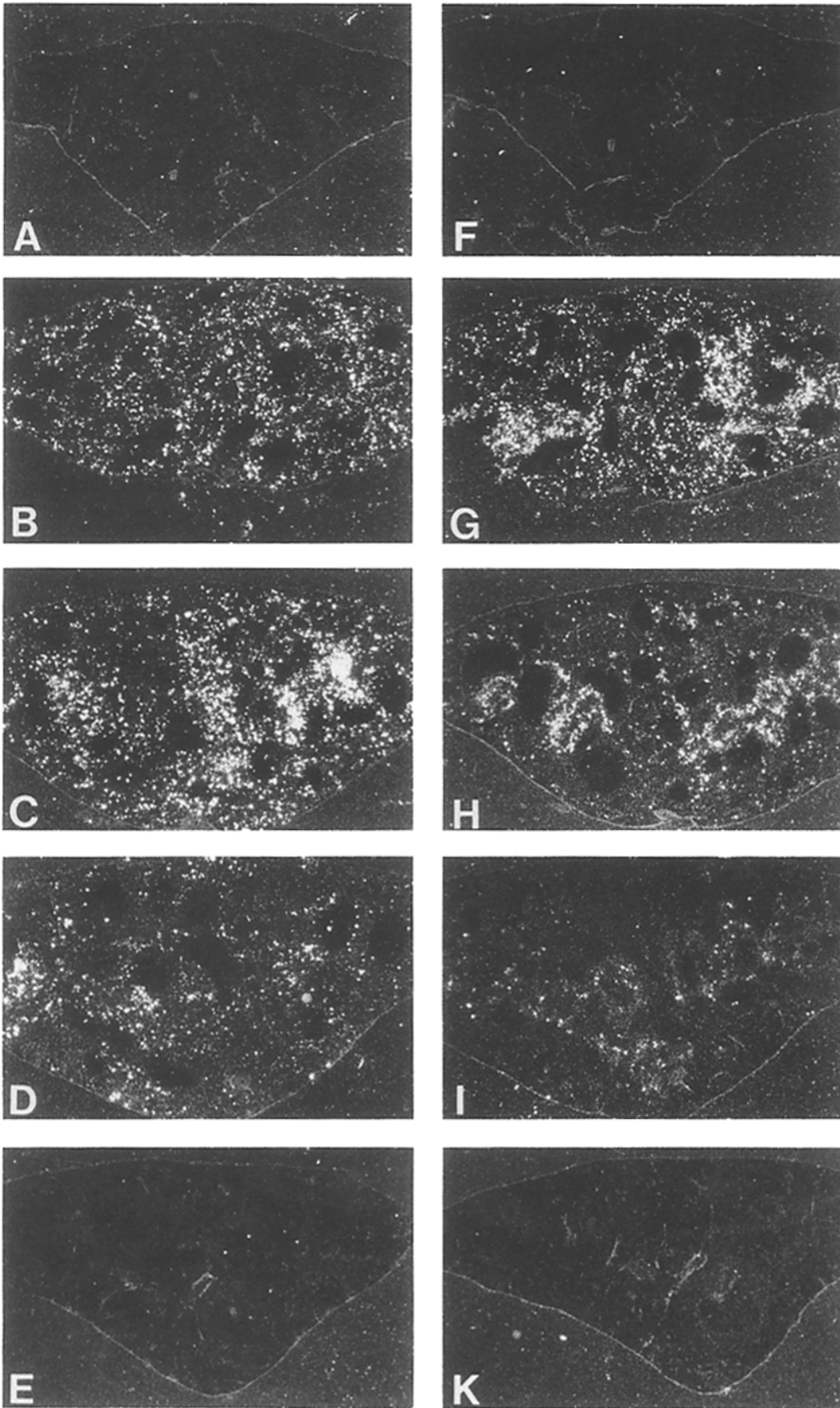


Figure 1. Kinetics and distribution of SEB-induced IL-2 mRNA and IFN- γ mRNA in the spleen. Serial sections (16 μ m) of spleens were hybridized with 35 S-labeled cRNA probes for IL-2 (A-E) and IFN- γ (F-K) and emulsion-coated autoradiograms were evaluated. (A and F) Spleens of untreated mice; (B and G) 3 h after injection; (C and H) 9 h; (D and I) 24 h; (E and K) 48 h. $\times 15$.

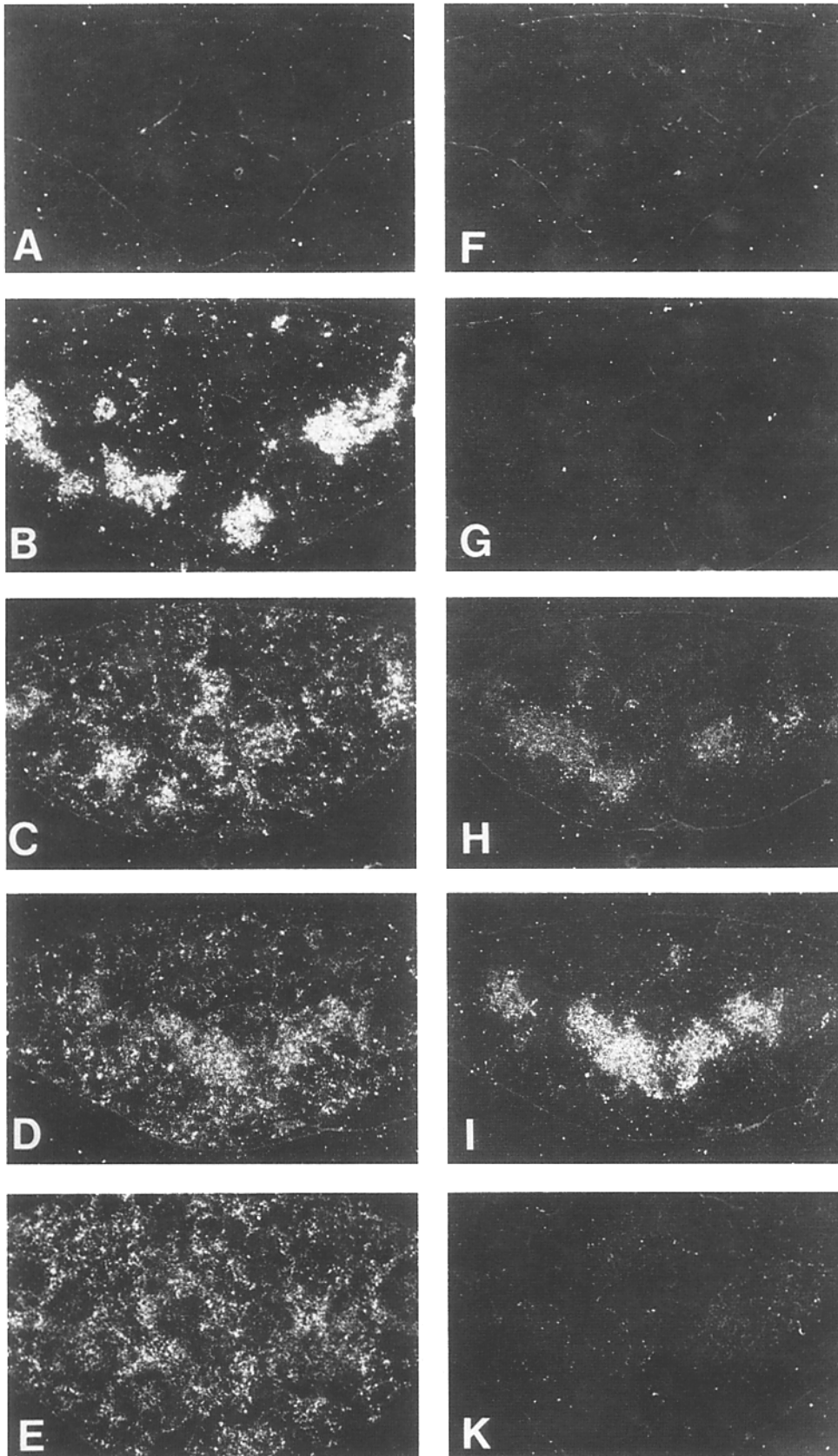
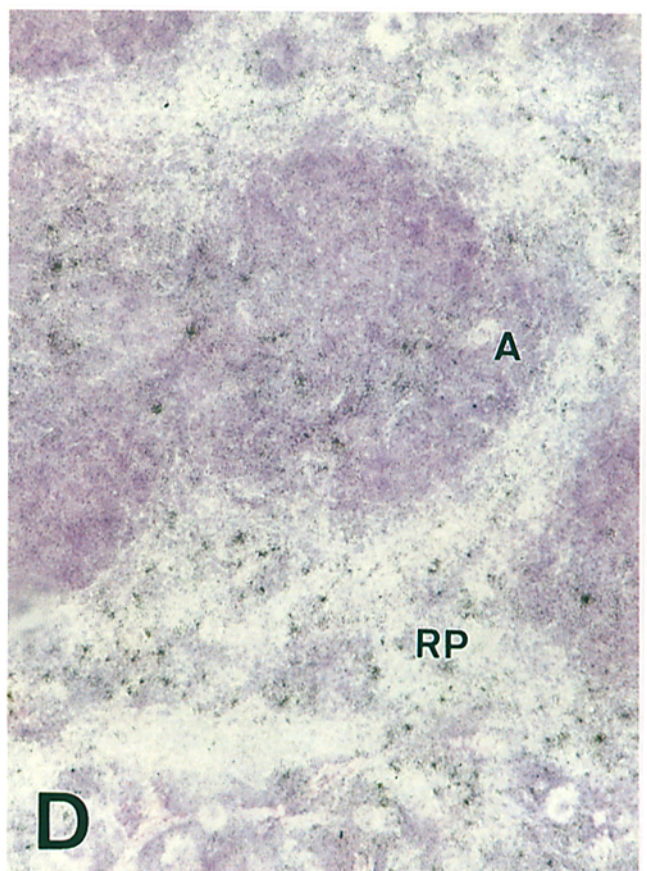
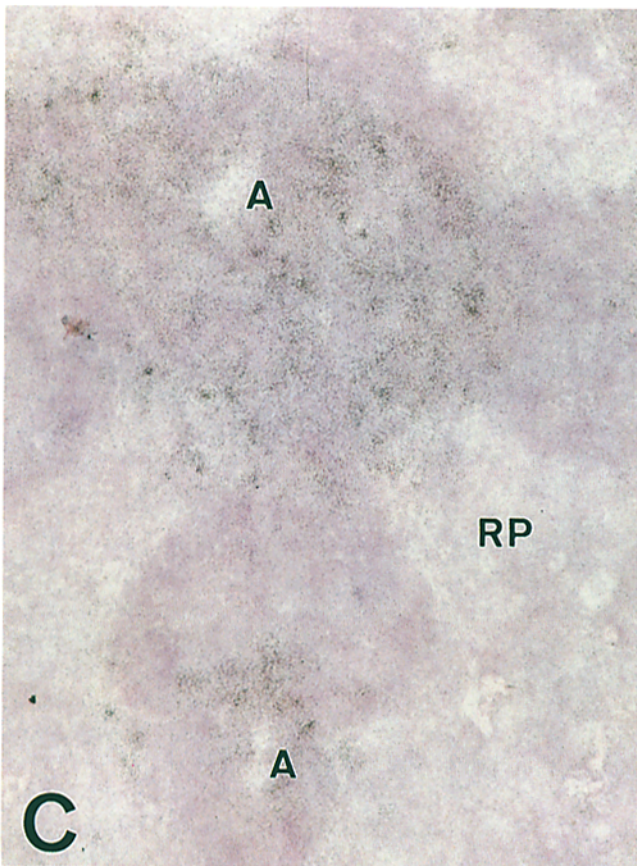
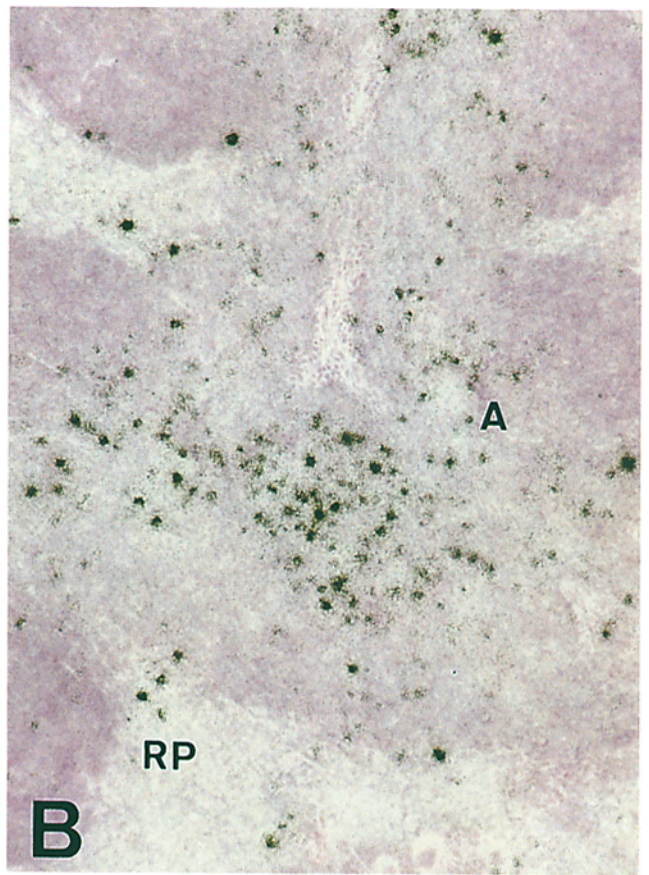
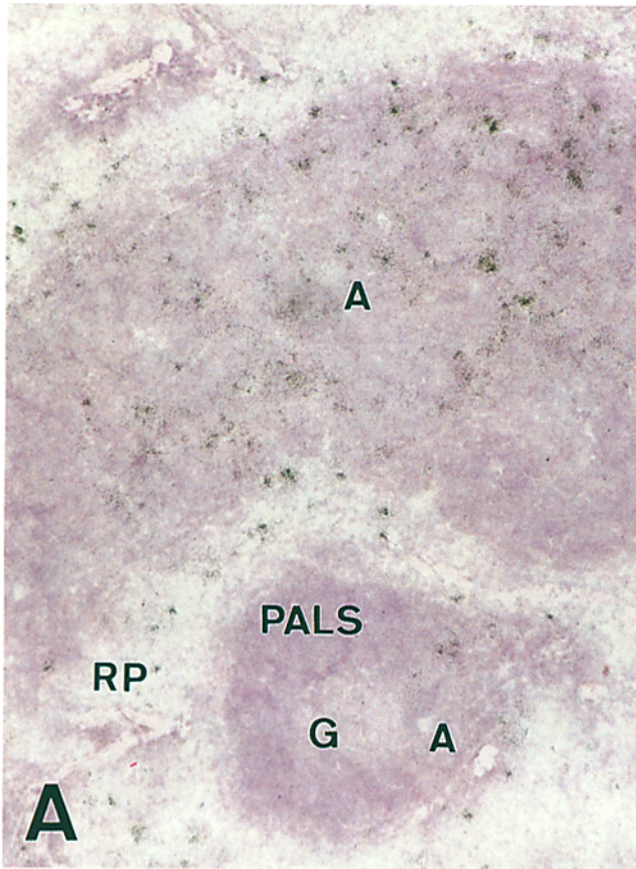


Figure 2. Kinetics and distribution of SEB-induced TNF- α and TNF- β mRNA in the spleen. Serial sections adjacent to those shown in Fig. 1 were hybridized with probes for TNF- α (A-E) and TNF- β (F-K). (A and F) Spleens of untreated mice; (B and G) 30 min after injection; (C and H) 1 h; (D and I) 2 h; (E and K) 4 h. $\times 15$.

Figure 3. Localization of cytokine mRNA in spleens of SEB-injected mice. Spleen sections were hybridized with probes for IL-2 mRNA (A), IFN- γ mRNA (B), and TNF- α mRNA (C and D), coated with film emulsion, and after exposure and development, counterstained with hematoxylin-eosin. The spleens were taken at different time points after SEB injection (A: 3 h, B: 9 h, C: 0.5 h, D: 3 h). Magnification: $\times 300$. (RP) Red pulp; (A) arteriole; (G) germinal center.



beled, oval-shaped areas. These regions, which appear dark in the figures, were identified as B cell follicles, because they were specifically stained with antisera against mouse Ig in adjacent serial sections (data not shown). Furthermore, bright-field analysis of counterstained spleen sections from animals expressing peak cytokine mRNA levels were analyzed and compared to the known architecture of mouse spleen (16, 17). They revealed the concentration of strongly positive labeled cells for both mRNAs in the T cell areas surrounding the B cell follicles, the periarteriolar lymphatic sheaths (PALS) (Fig. 3, A and B). A few signal-bearing cells were also present in non-PALS region.

Similar kinetics were found with TNF- α mRNA (Fig. 2, A-E). This cytokine mRNA was strongly expressed 30 min after SEB injection and completely disappeared within 48 h. Interestingly, the localization of TNF- α mRNA changed over the observation period. The early appearing TNF- α mRNA had the same periarteriolar localization as the mRNA for IL-2 and IFN- γ . After 3-4 h, however, staining of TNF- α mRNA showed a broader distribution, indicating that additional cells had been induced to express this mRNA (Fig. 2, D and E). In stained sections, the early TNF- α mRNA was restricted to the PALS (Fig. 3 C), whereas after 3 h, strong signals were found in the red pulp as well (Fig. 3 D). In contrast, TNF- β mRNA was first detectable after 1 h and had already nearly completely disappeared after 4 h without changing its distribution pattern restricted to the PALS (Fig. 2, F-K).

The time course of IL-2 and TNF- α bioactivity in the serum correlated with these findings. Both cytokines were detectable 1 h after injection, showed a maximum after 2 h (TNF- α) or 3 h (IL-2), and thereafter decreased rapidly (Fig. 4).

Effect of Macrophage Depletion on Splenic Cytokine Response to SEB. To examine if macrophages play an important role in the cytokine response to SEB, we depleted phagocytic cells by intravenous injection of liposomes containing Cl₂MDP. This regimen has been shown to deplete macrophages completely from spleen and liver (14). 2 d after liposome injection, F4/80-positive cells could neither be detected by immunofluorescence in spleen tissue sections (Fig. 5 E), nor in suspensions of spleen cells by flow cytometry (data not shown). It is noteworthy that the spatial and temporal pattern of mRNA production in the spleen for IL-2, IFN- γ , and TNF- β after SEB injection was essentially unchanged. This is shown for IL-2 mRNA in Fig. 5, B and F, but the same findings were made with IFN- γ and TNF- β mRNAs (data not shown). In contrast, the pattern of TNF- α mRNA expression was different: the shift to a broader distribution, seen in normal mice after 3-4 h, did not occur (Fig. 6). Moreover, after i.p. injection of LPS into macrophage-depleted mice, TNF- α mRNA was produced at strongly reduced levels and showed a more restricted, clustered localization (Fig. 5 H). Examination of counterstained sections by light microscopy showed that the signals were located in the T cell areas (data not shown).

Sensitivity of Cytokine Production to CyA. CyA that is a potent inhibitor of T cell activation has been shown to pro-

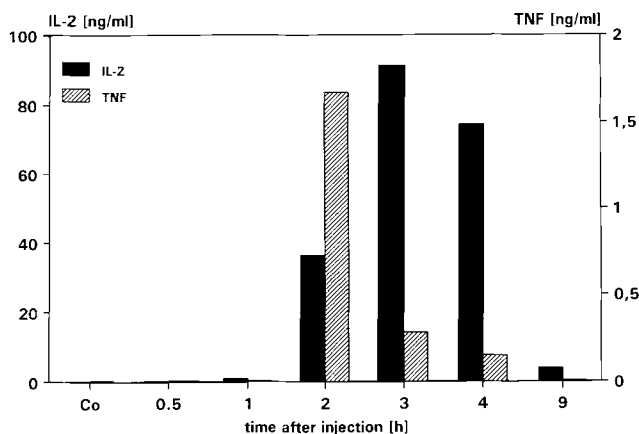


Figure 4. Detection of IL-2 and TNF bioactivity in mouse serum after SEB injection. Cytokines were assayed in serial dilutions of sera in bioassays. Data are the mean concentrations in three animals.

tect mice from staphylococcal enterotoxin-induced lethal shock (3). We therefore investigated the effect of this drug on the expression of the four cytokine mRNAs. CyA treatment completely abrogated the appearance of all four cytokine mRNAs in the spleen demonstrating their T cell-derived and activation-dependent expression (data not shown).

Discussion

The potent T cell stimulatory activity of staphylococcal and streptococcal superantigens is an important pathogenicity factor in the infections with these bacteria. In particular the stimulation of a large fraction of T cells has been implicated as responsible for the shocklike syndrome occurring during certain infections with *S. aureus* and *Streptococcus pyogenes* or after injection of a superantigen into experimental animals (3, 18). A systemic lymphokine secretion as the prime pathogenetic mechanism has been proposed. This notion is supported by the observations that experimental or therapeutic application of anti-CD3 mAb can induce a shock syndrome in mice and humans (19).

Although the molecular and cellular interactions during T cell stimulation in vitro by superantigens are well known, the early events after introduction of a superantigen into the organism are poorly characterized. In this study, we have analyzed the localization and the kinetics of the cytokine burst after injection of a toxic superantigen at the level of the cytokine mRNA. We found a rapid accumulation of mRNA for IL-2, IFN- γ , and TNF- α in the spleen, with significant levels of these cytokine mRNAs already after 30 min, whereas after in vitro stimulation accumulation of IL-2, mRNA has been described to require 3-6 h and to peak after 17-24 h (20, 21). Moreover, in vivo mRNAs were expressed only for a short period of time, in contrast to the in vitro findings showing that mRNAs are present for up to 72 h after stimulation (22-24). TNF- β mRNA, for example, is present at maximal levels for at least 48 h after in vitro stimulation (22),

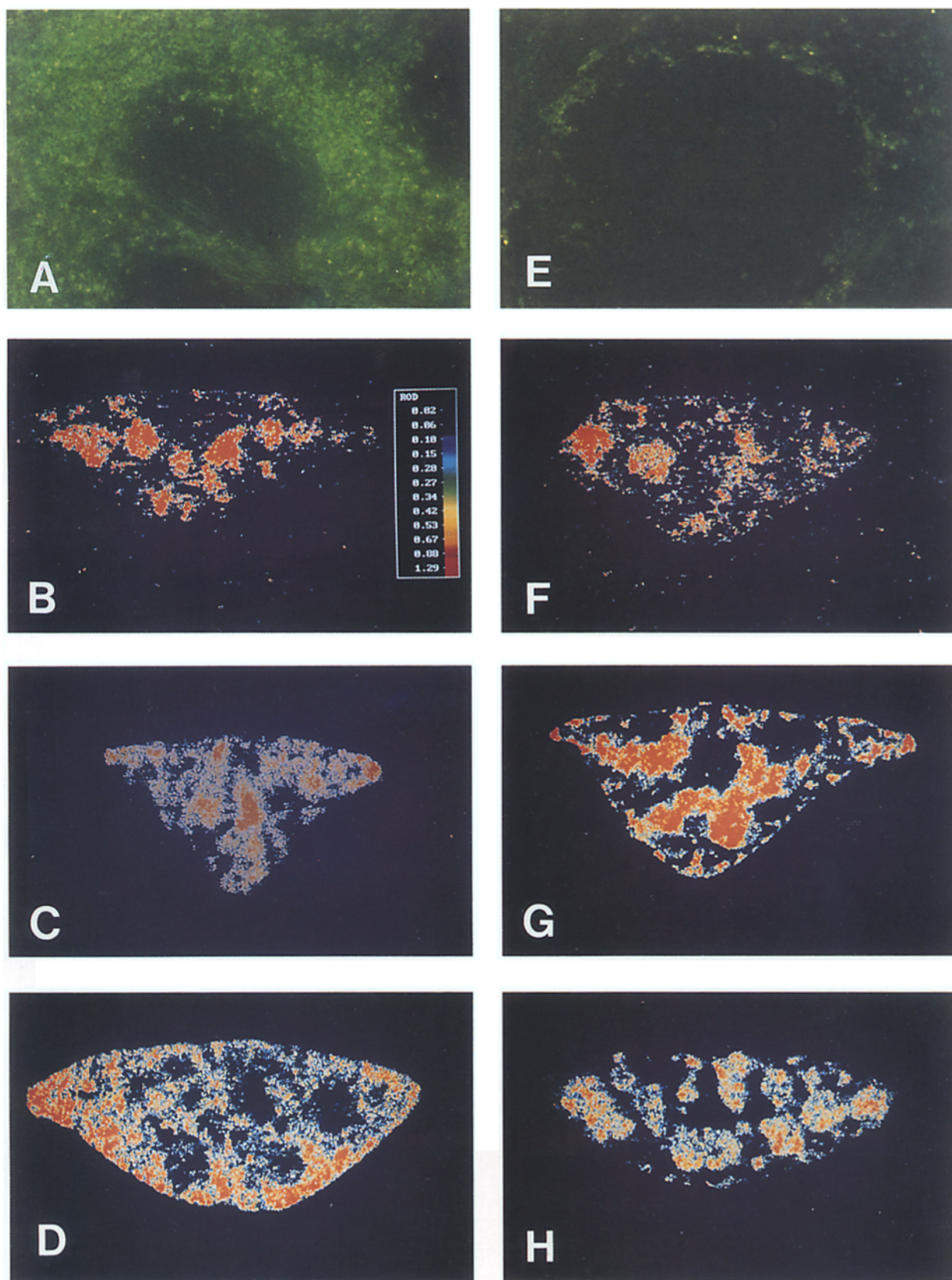


Figure 5. Effect of macrophage depletion on expression of cytokine mRNAs after SEB stimulation. Sections of spleens from normal (A–D) and macrophage depleted mice (E–H) were stained for the expression of the F4/80 antigen in immunofluorescence (A and E) and for the expression of IL-2 mRNA (B and F), and TNF- α mRNA (C and G) after injection of 50 μ g SEB or for TNF- α mRNA 3 h after injection of 5 μ g LPS (D and H). Hybridized autoradiograms were analyzed with an image analyzer, the color code indicating the intensity of labeling is introduced into panel B. $\times 280$ (A and E).

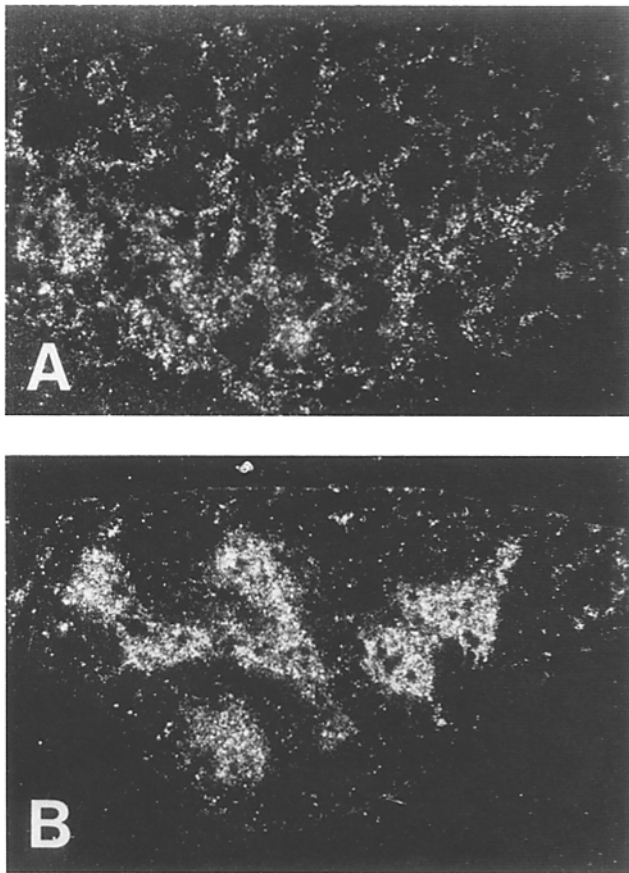


Figure 6. Differential distribution of TNF- α mRNA in normal and macrophage-depleted spleens 3 h after SEB stimulation. Normal (A) and macrophage-depleted mice (B) were injected with 50 μ g of SEB, and spleens taken after 3 h were hybridized with a TNF- α cRNA probe and analyzed in dark-field. $\times 8$.

but was present only 1–4 h after *in vivo* stimulation. A possible reason is that *in vivo* T cells are activated almost simultaneously and instantly in the intact, densely packed microenvironment by the superantigen that does not require processing and is rapidly cleared from the body (25). *In vitro*, T cell activation probably requires more time because of the need for establishing cell–cell contacts. A simultaneous activation is not possible, but the mitogen is present throughout the culture period and may activate T cells at different time points.

Within the first hour, all four cytokine mRNAs had a very similar localization: they were found in the PALS, the T cell areas of the spleen, whereas they were undetectable in the B cell-containing follicles and in the red pulp signals. This

pattern of the burstlike response to SEB of mRNA for IL-2, IFN- γ , and TNF- β was unchanged in macrophage-depleted animals. These results show that *in vivo*, the decisive superantigen-presenting cells are not macrophages but are probably dendritic cells. *In vitro*, dendritic cells have been shown to be up to 50 times more potent than other APCs as stimulators for antigens (26) and superantigens (27).

After LPS administration, the mRNA for TNF- α was still strongly induced in macrophage-depleted mice, but with a lower intensity and a different distribution pattern. Whereas in normal mice, LPS induced a strong and diffuse staining for TNF- α mRNA, in macrophage-depleted mice this mRNA was induced in a clustered expression (Fig. 5 H), with a distribution similar to the expression of the T cell-specific mRNA (Fig. 5, B and F). Thus, in the depleted mice, nonphagocytic cells, presumably dendritic cells, are the responders to LPS. The restricted localization of the TNF- α mRNA in macrophage-depleted mice is in accordance with the demonstration (17) that dendritic cells are present in the PALS of mouse spleen.

After SEB injection, the TNF- α mRNA behaved differently from the mRNAs for IL-2, IFN- γ , and TNF- β . Whereas the latter cytokine mRNAs were exclusively expressed in the T cell areas, TNF- α mRNA changed its distribution from the early clustering in the T cell areas to a broadly distributed pattern in white and red pulp after 3 h, indicating that a second cell population started to express this mRNA. This pattern was similar to the distribution of TNF- α mRNA in response to LPS in normal mice. A likely explanation is that the second phase is due to expression of TNF- α mRNA in macrophages. This notion is supported by the finding that this late phase was missing in macrophage-depleted animals. In these animals, TNF- α is produced by T cells, and, in the second phase, possibly by dendritic cells as well.

Taken together, these findings indicate that the early cytokine burst after SEB injection, including TNF- α , is T cell derived. Thus, in the first 2 h, TNF- α mRNA is expressed mainly in T cells and in the second phase, mainly in macrophages. The early peak of TNF activity in the serum and the delayed expression of TNF- β mRNA could indicate that most of the TNF protein is TNF- α derived from the first phase, *i.e.*, is produced by T cells. The complete sensitivity to CyA of all mRNAs suggests that the production of TNF- α in the second phase by macrophages is T cell dependent. This would be in agreement with *in vitro* studies showing that a strong TNF- α production is only seen when both T cells and monocytes are incubated together (7).

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