By intrasplenic immunization we raised a rat mAb (mAb V1q; IgG2a,κ) with a potent neutralizing activity against natural mouse TNF (1 μg/ml mAb V1q/100 U/ml TNF). mAb V1q was used to study the role of endogenous TNF in experimental peritonitis induced by sublethal cecal ligation and puncture. mAb V1q persisted for over 5 days in the serum of mice injected with 100 μg of the antibody and, therefore, proved useful for in vivo experiments. As little as 20 μg mAb V1q/mouse prevented lethal shock of the animals by 400 μg LPS/mouse. In sublethal cecal ligation and puncture i.p. injection of mAb V1q directly and up to 8 h after induction of experimental peritonitis lead to death of the animals within 1 to 3 days. The lethal effect of mAb V1q was compensated by injection of recombinant mouse TNF. Similar mAb V1q effects as in immunocompetent mice were shown in severe combined immune deficiency mice deficient of mature functional B and T cells. Taken together, these data suggest that during the early phase of peritonitis endogenous TNF may stimulate nonlymphoid cells such as granulocytes, macrophages, platelets, and fibroblasts to ingest bacteria and to localize inflammation, respectively. These beneficial effects of TNF may determine survival. Thus, our data may have implications for the therapeutic management of a beginning peritonitis.

Many different in vitro and in vivo effects of TNF have previously been described (1–3). TNF has beneficial as well as deleterious effects. In experimental infections it was shown that locally, TNF accelerates elimination of bacteria and to localize inflammation, respectively. These beneficial effects of TNF may determine survival. Thus, our data may have implications for the therapeutic management of a beginning peritonitis.

In the experimental system of CLP (12), a model of bacterial sepsis that resembles the clinical situation of an infection with a mixed bacterial flora of intestinal origin, pretreatment with LPS or TNF was shown to increase the survival rates of the injected animals (13, 14). There are two possible explanations for this effect: LPS or TNF pretreatment may induce 1) LPS or TNF tolerance or 2) activation of macrophages and infiltration of granulocytes that help to fight the infection, e.g., by ingestion of bacteria. To distinguish between these possibilities and to investigate the contribution of endogenous TNF to the recovery from sepsis, we tested the effect of a neutralizing anti-mouse TNF mAb in sublethal CLP (15). A neutralizing rat mAb against mouse T cell cytotoxin was raised by intrasplenic immunization of rats with partially purified cytotoxin from T cells. Injection of this mAb i.p. during the first 8 h after CLP lead to death of all mAb-treated mice. These experiments show the beneficial effect of endogenous TNF and its requirement for recovery from sublethal CLP.

MATERIALS AND METHODS

Animals. DBA/2, C3H/HeN, and C57Bl/6 mice were obtained from the Institute für Versuchstierforschung, Hannover, F.R.G., and female Sprague-Dawley rats from Mus-Rattus, Brunntal, F.R.G. The animals were housed in the barrier conditions of the University of Heidelberg Ludwigshafen, F.R.G. SCID mice, a generous gift of W. Schuler and E. Wagner, Basel Institute of Immunology, Basel, Switzerland, were kept in filter top cages. All SCID mice were tested for leakiness by ELISA for mouse IgG and IgM. Only animals with serum levels less than 5 μg/ml IgG were used in the experiments.

Media. Cells were cultured in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with l-glutamine (2 mM), 50 μg/ml gentamycin, HEPES (5 mM), mercaptoethanol (30 μM), and 10% heat-inactivated FCS. For culture of T cell clone 29 medium supplemented with 2% C5/36/2RIL-2 containing conditioned medium (16) was used.

Cells. The IL-2 dependent long term mouse T cell clone 29 was established by limiting dilution cloning from activated T cells (17). L929 is a fibrosarcoma line from C3H mice. C57/6 is a L929 clone transfected with the human IL-2 cDNA secreting IL-2 into the supernatant (16).

Reagents. rHuTNF-α (sp. act. 9×10^6 U/mg) and rmTNF-α, sp. act. 8×10^6 U/mg were a generous gift from BASF/Bioll AG, Ludwigshafen. F.R.G. rHuTNF-β (TNFβ lymphotoxin), sp. act. 3×10^7 U/mg was kindly provided by Genentech, South San Francisco, CA. The sp. act. were tested in the L929 TNF bioassay as described below.

L929 TNF bioassay. The assay was performed as previously described (18). Briefly, 30,000 L929 cells were added to serial twofold dilutions of TNF. The test was performed in the presence of 2 μg/ml Actinomycin D (Sigma Gmbh, München. F.R.G.). After 20 h at 37°C and 5% CO_2 in air at 80% relative humidity TNF-mediated cytopathic effects on L929 cells were evaluated microscopically or by crystal violet (Sigma Chemical Co., St. Louis, MO) staining.

Cytotoxin purification. T cell clone 29 was induced with 10 μg Con A/ml in 10 ml phenol red free medium (Biochrom KG, Berlin.}

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2 Address correspondence and reprint requests to Dr. Peter H. Krammer, Institute for Immunology and Genetics, German Cancer Research Center, Heidelberg, F.R.G.
that mAb V1q neutralizes cytotoxin contained in the Con A-induced supernatant of mouse T cell clone 29 and natural TNF-α contained in serum from LPS injected mice with equal efficiency. One μg/ml mAb V1q was required to neutralize 100 U/ml mTNF-α or cytotoxin. Considerably higher quantities of mAb V1q (16 μg/ml V1q per 100 U/ml rmTNF-α) were required to neutralize rmTNF. mAb V1q did not neutralize rhuTNF-α or rhuTNF-β. These data show that mAb V1q neutralized both mouse TNF and mouse T cell cytotoxin activity and that its neutralizing titer allowed its use in in vivo experiments.

Persistence of mAb V1q in mouse serum. To determine mAb V1q serum clearance rates mice were injected i.p. with 100 μg purified mAb V1q. The mice were bled 2 h and 1 to 5 days after injection. The neutralizing titer of mAb V1q persisting in mouse serum was then determined by testing the neutralizing capacity on 128 U/ml of cytotoxin from mouse T cell clone 29. Figure 1 shows that the titer of mAb V1q in the serum of the injected mice declined with time after injection as expected. A considerable neutralizing titer (1:40), however, was still found 5 days after injection. These results suggested that mAb V1q is suitable for in vivo experiments even in situations in which a prolonged TNF neutralizing capacity is required.

Protection of mice from lethal LPS effects by mAb V1q. It was shown previously that polyclonal and mAb neutralizing TNF-α protected the animals from the lethal effects observed after injection of LPS into mice (11). To assess the potency of mAb V1q in vivo groups of four mice were injected i.p. with 100 pg purified mouse anti-mTNF mAb V1q at various concentrations 2 h before the injection of a lethal dose of LPS (400 μg). Amounts of mAb V1q as low as 20 μg per mouse neutralized the lethal TNF-mediated LPS effects completely. Control mice injected with PBS were dead at day 3 or 4 after LPS injection whereas all mAb V1q injected mice survived even though they showed symptoms of endotoxin shock such as ruffled fur and diarrhea. These data supported our assumption that mAb V1q was suitable for in vivo experiments because it had a potent TNF neutralizing capacity.

Requirement of TNF for recovery from CLP. TNF-mediated endotoxin shock is a model for the deleterious

**RESULTS**

**Specificity of rat anti-mouse TNF mAb V1q.** We found five hybridomas that produced neutralizing anti-cytotoxin mAb. The mAb V1q (lgG2a,α) was used for further investigation. As an isotype-matched control antibody we used mAb V10.12 from the same fusion. Table I shows

<table>
<thead>
<tr>
<th>Source of TNF/Cytotoxin</th>
<th>mAb V1q Required to Neutralize 100 U/ml of TNF/Cytotoxin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A induced C129 cytotoxin</td>
<td>1</td>
</tr>
<tr>
<td>LPS-Induced mouse TNF serum</td>
<td>1</td>
</tr>
<tr>
<td>Mouse rTNFp</td>
<td>16</td>
</tr>
<tr>
<td>Human rTNFm</td>
<td>≥40</td>
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<tr>
<td>Human rTNF</td>
<td>≥40</td>
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</tbody>
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* Neutralization was tested in the L929 TNF bioassay.
* C129 is a mouse T cell clone in long term culture.
* 100 U/ml LPS 2:25 ng/ml.
* 20 U/ml.
* No neutralization was seen with ≥40 μl mAb V1q.
effects of a massive septicemia at later stages of a bacterial infection. To assess the effect of TNF in a more localized protracted infection with a mixed bacterial flora, however, we used the system of cecal ligation and puncture as published by Wichterman et al. (12). To generate a peritonitis the cecum of mice was ligated and punctured. Most strains of mice including C3H/HeN mice used in our experiments survive the ensuing peritonitis that develops after CLP. Previous experiments by Urbas-chek et al. (13) and Sheppard et al. (14) had shown that the injection of small quantities of LPS or TNF into mice that had undergone an otherwise lethal CLP saved a significant percentage of the animals from death. This suggested that TNF had a beneficial effect in CLP. Alternatively, this protection may be the consequence of induction of LPS/TNF tolerance. To test whether endogenous TNF was required for survival from peritonitis C3H/HeN mice were injected with 100 μg purified mAb Vlq at 0 h or 8 h after CLP and mAb Vlq injection. We concluded from these results that endogenous TNF was required to survive the peritonitis after CLP. This was further supported by experiments displayed in Figure 3 which show that the deleterious effect of mAb Vlq was compensated by i.p. administration of as little as 200 ng rmTNF-α 1 h after injection of 100 μg mAb Vlq. To further determine the time span during which TNF is required for survival from CLP we injected C3H/HeN mice which had undergone sublethal CLP with 100 μg mAb Vlq at 0 to 16 h after CLP. Figure 4 shows that all mice injected with mAb Vlq at 6 h or 8 h after CLP died at approximately 1 to 3 days after the surgical procedure. In contrast, three of six mice that had received mAb Vlq 16 h after CLP survived. These results show that the beneficial effect of TNF for survival from CLP-induced peritonitis was exerted during the first 8 h after onset of the experimental disease.

**TNF-mediated recovery from CLP induced peritonitis is independent of functional mature lymphocytes.** The fact that TNF was required for survival during the first 8 h of peritonitis suggested that rather than inducing a response by lymphoid cells TNF may affect nonlymphoid cells in the peritoneal cavity. To support this assumption SCID mice devoid of mature functional T and B lymphocytes were used (21). Figure 5 shows that injection of 100 μg mAb Vlq after CLP of SCID mice was lethal in the same way as in the experiments depicted in Figure 2, in which normal C3H/HeN mice with an intact immune system were used. These data strongly suggest that the beneficial effect of TNF in recovery from CLP-induced peritonitis is an early effect on nonlymphoid cells, possibly granulocytes and macrophages, platelets, and fibroblasts.

**DISCUSSION**

We investigated whether endogenous TNF supports the recovery from sublethal peritonitis caused by CLP. To this end we raised a neutralizing rat anti-mouse TNF
mAb (V1q) by intrasplenic immunization. We showed the in vivo efficiency of mAb V1q by demonstrating that it protected mice from LPS-induced shock. Injected with mAb V1q after sublethal CLP, however, mice died within 1 to 3 days after injection. Kinetic experiments showed that endogenous TNF was required for survival of the mice during the first 8 h of peritonitis since even 8 h after CLP mAb V1q injection was lethal. In addition, the effect of mAb V1q was obtained in SCID mice suggesting that TNF acted directly on nonlymphoid cells.

It is difficult to obtain sufficient quantities of pure natural cytokines for standard immunization. Therefore, we used the small quantities of a cytotoxic that we isolated from the supernatant of a long term mouse T cell clone (C129) for intrasplenic immunization—a method that requires only small quantities of Ag (19). We obtained five mAb, among them V1q, that neutralized both TNF-α/cachectin and T cell cytotoxicity from C129. C129 cytotoxicity may be identical to TNF-β/lymphotoxin because in Northern blots clone 29 mRNA gives a signal with a mouse TNF-β cDNA probe (a generous gift of N. H. Ruddle, Yale University Medical School, New Haven, CT). This result agrees with the data of Sheehan et al. (22) who also found a hamster mAb that neutralized both TNF-α and TNF-β after immunization with rmTNF-α (22).

To ascertain the quality of mAb V1q for in vivo use we tested its persistence in the circulation. We showed that mAb V1q is detectable in mouse serum for at least 5 days after injection of 100 μg per animal and retains its neutralizing capacity. It has been shown previously that mAb or polyclonal anti-TNF antibodies protect animals from LPS-induced lethal shock (11). The same effect was observed with mAb V1q and 20 μg purified mAb V1q per mouse were sufficient to protect the animals from the lethal effect of 400 μg LPS. This result demonstrated that mAb V1q was useful in experiments in which large quantities of endogenous TNF had to be neutralized.

The deleterious role of TNF has been shown in various model systems of bacterial sepsis or LPS-induced shock. In these systems animals were exposed to large quantities of LPS or bacteria. This induced a rapid production of high levels of TNF that acted systemically and led to shock. In contrast, we chose sublethal CLP as a model for a slowly developing sepsis. This model that resembles the clinical situation of sepsis in intraabdominal trauma enabled us to test whether endogenous TNF also exerts supportive effects during recovery from sepsis. CLP is lethal or sublethal depending on the mouse strain, the number of cecal punctures made, and the hygiene state of the mice. The sublethal CLP model has previously been used by Moss et al. (15) to show the immunosuppressive effect of thermal injury. In our hands, C3H/HeN proved to be a suitable strain of mice for sublethal CLP. C3H/HeN mice that had received control mAb or PBS after CLP did not differ from the mAb V1q-injected mice in their clinical appearance during the first 12 h after injection. After 12 h, however, in contrast to the control mice, mAb V1q-injected mice started to die. This result suggests that endogenous TNF is the essential cytokine for recovery from CLP. Further kinetic experiments showed that for recovery from peritonitis TNF is needed during the first 8 h post CLP. It was shown that the effect was TNF specific because the lethal mAb V1q effect was overcome by subsequent injection of 200 ng rmTNF. This result suggested an important role for TNF in natural defense and prompted us to investigate the effects of TNF in CLP of SCID mice devoid of functional mature B and T lymphocytes (21). In SCID mice we obtained the same data as in mice with an intact immune system underlining the role of nonlymphoid cells in the peritoneal cavity for recovery from CLP.

Our data do not exclude that early after CLP suboptimal amounts of TNF produced act on TNF-secreting cells and induce a refractory state as found in early LPS or TNF tolerance (23, 24). However, we favor other, more direct beneficial TNF effects to explain our results. TNF secreted by peritoneal macrophages induced by bacteria may further stimulate TNF secretion in an autocrine fashion (2). Thus, TNF may stimulate chemotaxis and lead to rapid infiltration of neutrophils into the peritoneal cavity. This may be a consequence of enhanced adhesion of such cells to the vascular endothelium followed by increased diapedesis (25, 26). Complement secreted upon TNF stimulation may mediate disruption of capillaries and hemorrhagic necrosis (27). Such disruption of capillaries may prevent rapid spread of bacteria into the circulation and thus prevent general septicemia after CLP. In addition, TNF may stimulate the production of acute phase proteins such as C-reactive protein, complement factors, and fibrinogen. These proteins play an essential role in chemotaxis, activation of neutrophils, opsonization of bacteria, direct bacteriolysis, and abscess formation (28). Furthermore, TNF may activate procoagulant function on the endothelial surface. This in turn may lead to disseminated intravascular coagulation. In addition, TNF may inhibit fibrinolysis by transcriptional induction of plasminogen activator inhibitors and suppression of tissue-type plasminogen activator (29). The combination of activation of procoagulant activity, fibrinogen, and fibrin formation, and the inhibition of fibrinolysis may stabilize fibrin at the site of inflammation in the peritoneum. Stability of fibrin clots in abscess formation may further enhance from the ability of TNF to stimulate fibroblast growth and collagen production (30). Taken together these events may play a decisive role in repopulation and tissue remodeling during and after in-
flammation (2). Thus, it seems likely that TNF supports the first line of defense in peritonitis and prevents a disseminated infection with massive bacteremia (2, 3).

In conclusion, therefore, our data show a beneficial early role for TNF in recovery from peritonitis that may have implications for the therapeutic management of such a disease.

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