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REQUIREMENT OF ENDOGENOUS TUMOR NECROSIS FACTOR/CACHECTIN FOR RECOVERY FROM EXPERIMENTAL PERITONITIS[1]

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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 pathogens, enhances leukocyte infiltration, induces angiogenesis, and improves wound healing. Thus, beneficial in vivo effects were shown in the cases of BCG (*Mycobacterium bovis*) (4), listeria (5, 6), and leishmania infections (7). The deleterious role of TNF was shown in many in vivo models. TNF is involved in cachexia (1) and exacerbates murine cerebral malaria (8) and graft vs host disease (9). Furthermore, it mediates shock induced by bacteria and LPS (10, 11).

In the experimental system of CLP[3] (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 Institut für Versuchstierforschung, Hannover, F.R.G., and female Sprague-Dawley rats from Mus-Rattus, Brunntal, F.R.G. The animals were kept under the barrier conditions of the German Cancer Research Center, Heidelberg, 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 Ig 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% C63/26 rIL-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. C63/26 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. 8×10^7 U/mg) were a generous gift from BASF/Knoll AG, Ludwigshafen, F.R.G. rhuTNF-β (TNF-β(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 90% relative humidity TNF-mediated cytopathic effects on L929 cells were evaluated microscopically or by cristal 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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[3] Abbreviations used in this paper: CLP, cecal ligation and puncture; hu, human; m, mouse; SCID, severe combined immune deficiency; V1q, IgG2a, κ monoclonal rat anti-mouse TNF-α/T cell cytotoxin antibody.

F.R.G.) in cell culture flasks (no. 3375, Costar, Cambridge, MA) under serumfree conditions for 20 h at 37°C and 5% CO_2 in air at 90% relative humidity. The supernatant was centrifuged at 15,000 × *g*, sterile filtered and precipitated at 80% ammonium sulfate saturation. The precipitated material was centrifuged again at 15,000 × *g*, dissolved in 10 mM NaCl/50 mM Tris/HCl, pH 8, and dialysed against this buffer. This buffer was also used as the starting buffer for anion exchange HPLC (Spherogel TSK DEAE-5PW, Beckman, München, F.R.G.). The material was applied to the HPLC column in a volume of 10 ml at a flow rate of 1 ml/min. The column was washed with loading buffer until baseline absorption at 280 nm was reached. The adsorbed material was eluted using a linear gradient from 10 to 300 mM NaCl in 50 mM Tris/HCl pH8. The 1-ml fractions were collected. The cytotoxin activity in individual fractions was measured using the L929 TNF bioassay. Peak cytotoxin activity was found at 150 mM NaCl. The most active fractions were pooled, dialysed against 10 mM NaCl/50 mM Tris/HCl, pH 8, and concentrated 100-fold in a vacuum concentrator.

Intrasplenic immunization, fusion, and mAb purification. A total of 130,000 U of partially purified cytotoxin was applied to 15 mm^2 of nitrocellulose (Millipore S.A., Molsheim, France). Cytotoxin-coated pieces of nitrocellulose were deposited in the spleen of a Sprague-Dawley rat as described by Nilsson et al. (19). After four intrasplenic immunizations rat spleen cells were fused with the mouse myeloma X63.Ag8.653 (20). Anti-cytotoxin hybridomas were selected by testing hybridoma supernatants for neutralization of the cytopathic effect of cytotoxin in the L929 TNF bioassay. mAb were purified from serumfree hybridoma supernatant using the same column and conditions as described in *Cytotoxin purification.*

Determination of mAb persistence in mice. DBA/2 mice were injected i.p. with 100 μg purified rat anti-mTNF mAb V1q. The injected mice were bled daily from the tail and the serum dilutions were used to neutralize 128 U/ml cytotoxin in the L929 TNF assay.

Effect of rat anti-mTNF mAb V1q on LPS-induced shock. To determine the lethal dose of LPS 10- to 14-wk-old female DBA/2 mice were injected i.p. with 50 to 1000 μg LPS (LPS W *Salmonella minnesota* 9700, Difco Laboratories, Detroit, MI). A total of 400 μg LPS was determined as the lethal dose of LPS for this strain which killed 100% of the mice within 3 to 4 days. Subsequently, groups of four mice were injected i.p. with different quantities of mAb V1q or with PBS 2 h before i.p. injection of 400 μg LPS. The mice were observed for at least 2 wk.

Cecal ligation and puncture. The 10- to 14-wk-old C3H/HeN mice were anesthetized by i.p. injection of 75 mg/kg Ketanest (Parke, Davis & Company, München, F.R.G.) and 16 mg/kg Rompun (Bayer AG, Leverkusen, F.R.G.) in 0.2 ml sterile, pyrogen-free saline (Fresenius AG, Bad Homburg, F.R.G.) (12, 15). The abdominal skin of the mice was shaved and a 0.7-cm midline incision was made. The cecum was exteriorized and filled with feces by milking stool back from the ascending colon. The cecum was ligated below the ileocecal valve and punctured once with a 0.9 × 40-mm needle. Gentle pressure was applied on the ligated cecum to exteriorize a small amount of feces. The cecum was then returned to the peritoneal cavity and the incision closed with clamps and Histoacryl blau tissue glue (B. Braun Melsungen AG, Melsungen, F.R.G.). Mice were observed for at least 2 wk.

RESULTS

Specificity of rat anti-mouse TNF mAb V1q. We found five hybridomas that produced neutralizing anti-cytotoxin mAb. The mAb V1q (IgG2a,κ) was used for further investigation. As an isotype-matched control antibody we used mAb V10.12 from the same fusion. Table I shows 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.

TABLE I

Specificity of rat anti-mouse TNF mAb V1q

Source of TNF/Cytotoxin	mAb V1q Required to Neutralize 100 U/ml of TNF/Cytotoxin (μg/ml)[a]
Con A induced C129[b] cytotoxin	1
LPS-induced mouse TNF serum	1
Mouse rTNF[c]	16
Human rTNF[d]	≥40[e]
Human rLT[d]	≥40[e]

[a] Neutralization was tested in the L929 TNF bioassay.
[b] C129 is a mouse T cell clone in long term culture.
[c] 100 U/ml ≙ 1.25 ng/ml.
[d] 20 U/ml.
[e] No neutralization was seen with ≥40 /ml mAb V1q.

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 with 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

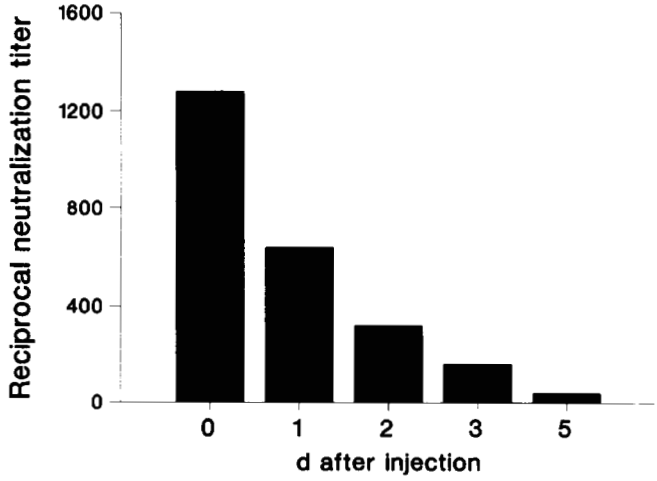

Figure 1. Persistence of mAb V1q in mouse serum. Mice were injected i.p. with 100 μg mAb V1q and bled from the tail vein 2 h, 1, 2, 3, and 5 days after injection. Serum in dilutions was used to neutralize 128 U/ml cytotoxin from mouse T cell clone 29 in the L929 TNF bioassay.

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 ensueing peritonitis that develops after CLP. Previous experiments by Urbaschek 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 V1q after sublethal CLP. Figure 2 shows that in contrast to mice injected with either PBS or isotype-matched control mAb, mice injected with mAb V1q died between 1 and 3 days after CLP and mAb V1q 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 V1q was compensated by i.p. administration of as little as 200 ng rmTNF-α 1 h after injection of 100 μg mAb V1q. 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 V1q at 0 to 16 h after CLP. Figure 4 shows that all mice injected with mAb V1q at 0 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 V1q 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 V1q 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.

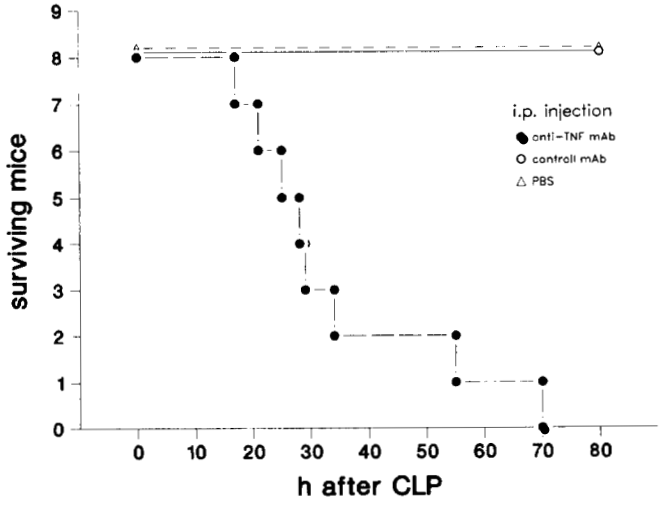

Figure 2. Requirement of TNF for recovery from CLP. Immediately after CLP eight mice/group were injected i.p. with 100 μg mAb V1q in 0.2 ml PBS, 100 μg isotype-matched control mAb in 0.2 ml PBS, or 0.2 ml PBS. These injections were repeated 12 h after CLP.

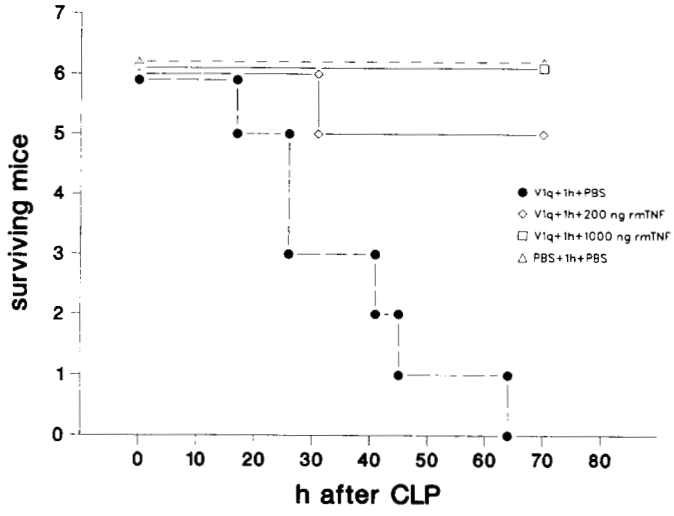

Figure 3. The deleterious effects of mAb V1q in CLP-induced peritonitis is overcome by administration of rmTNF. After CLP mice were either injected with 100 μg mAb V1q in 0.2 ml PBS or 0.2 ml PBS alone, respectively. After 1 h PBS-injected mice were re-injected with 0.2 ml PBS. mAb V1q-injected mice were injected either with 0.2 ml PBS or with 200 ng or 1000 ng rmTNF in 0.2 ml. These injections were repeated 12 h after CLP.

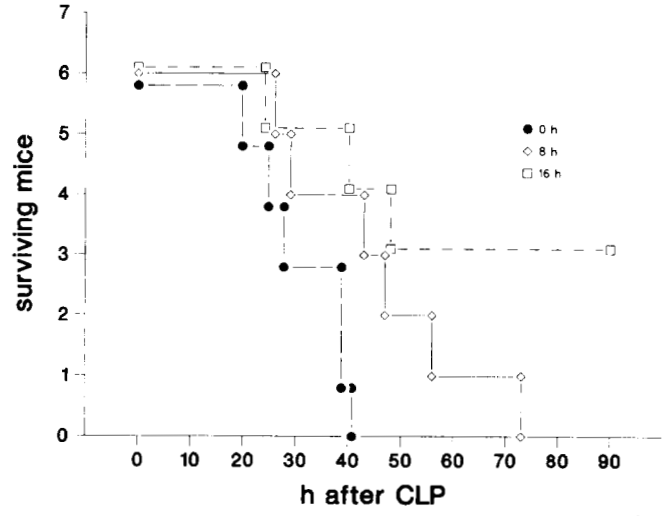

Figure 4. Requirement of TNF for survival from CLP during the first 8 h of peritonitis. 0, 8, and 16 h after CLP mice were injected with 100 μg mAb V1q in 0.2 ml PBS. These injections were repeated 24 h after CLP.

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

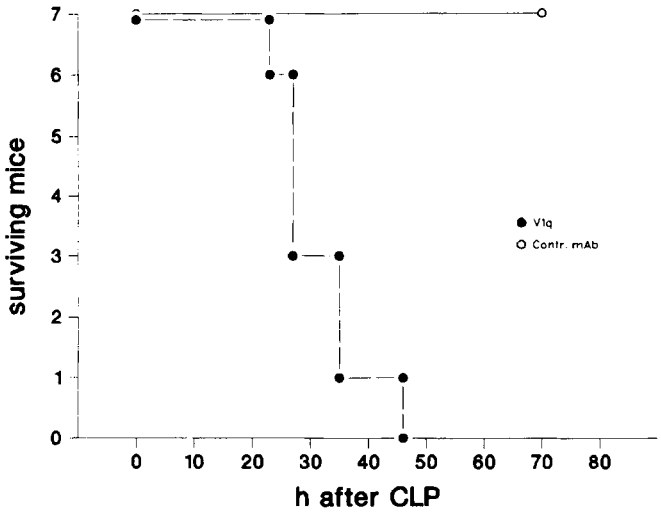

Figure 5. Requirement of TNF for recovery of SCID mice from CLP. Immediately after CLP 7 SCID mice/group were injected with 100 μg mAb V1q, or an isotype-matched control antibody in 0.2 ml PBS. These injections were repeated 12 h after CLP.

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 cytotoxin 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 cytotoxin from C129. C129 cytotoxin 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, opsonisation 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 come 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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