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Development of Multiple Necrotizing Enteritis Induced by a Tumor Necrosis Factor-like Cytokine from Lipopolysaccharide-stimulated Peritoneal Macrophages in Rats

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We report the development of an animal model of multiple necrotizing enteritis (MNE) in rats. When rats were injected directly with a culture supernatant of lipopolysaccharide (LPS)-stimulated rat peritoneal macrophages into the abdominal aorta, the overt pathologic lesions of MNE developed within 30 minutes after injection. The rats showed an elevated level of blood fibrinogen degradation product content even 30 minutes after injection. Furthermore the rats that were pretreated intravenously with heparin sulfate did not develop MNE, indicating the acute disturbances of blood microcirculation in the intestine. Multiple necrotizing enteritis was developed also by the injection with recombinant tumor necrosis factor (rTNF) but rarely was observed with even a high dose of recombinant interleukin-1 (rIL-1) or platelet-activating factor (PAF). The supernatant was cytotoxic in vitro ***to TNF-susceptible LM and many other cells but was less cytotoxic to the TNF-resistant LR line. Partial purification of the supernatant suggested that the supernatant contained a cytokine that has biochemical features of TNF. Furthermore polyclonal anti-TNF antibody could inhibit not only the cytotoxicity*** in vitro ***but also MNE development*** in vivo ***by this factor. These data strongly indicate that MNE possibly could be caused by a TNF-like cytokine produced by macrophages that are stimulated by the endotoxin. (Am J Pathol 1990, 137:1103–1111)***

Multiple necrotizing enteritis (MNE) is a disease with a poor prognosis.[1] The lesion of this disease usually is found to be both multiple and segmental in the small intestine. The etiologic agent or pathogenesis is unknown but the disease seems to be caused by the acute ischemic change. It is generally considered that this lesion appears in patients with severe infection, such as those having septic shock. The possible relevance of MNE patients with the infection, especially by gram-negative bacterias, simply suggests that one of the disease processes of MNE has, at the least, a correlation with the bacterial endotoxin lipopolysaccharide (LPS).[2,3] Lipopolysaccharide could strongly induce many humoral factors such as interleukin 1 (IL-1), Tumor necrosis factor (TNF), procoagulants, growth factors, chemotactic factors, platelet-activating factor (PAF), and prostaglandins,[4–9] and it is considered that these factors are mainly produced from macrophages.

In this paper, we demonstrated the establishment of the experimental MNE in rats and analyzed the most responsible factor that is required for the development of this disease. The data has strongly suggested that MNE resulted from the acute disturbance of the blood microcirculation after the injection into the abdominal aorta with a supernatant of cultured LPS-stimulated macrophages. The development of MNE was associated closely with the production of a TNF-like molecule from macrophages, and this cytokine was supposed to be the most responsible factor of this disease.

Materials and Methods

Animals

Inbred Wister-King-Aptekman (WKA)-H and Sprague-Dawley (SD) rats were obtained from CLEA Japan Inc., Shizuoka, Japan. In the experiment, 10- to 15-week-old male rats were used.

Production of a Supernatant from Macrophages Cultured with Lipopolysaccharide

The peritoneal macrophages were obtained by intraperitoneal washing with PBS of WKA rats. Cells were cultured

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for 24 hours in fetal calf serum (FCS)-free RPMI 1640 medium. The fluorescein-activated cell sorter analysis using R2-1A6 monoclonal antibody (MAb)[10] that reacts with the cell-surface antigen expressed on rat macrophages showed that nearly all of the adherent cells expressed this antigen. In addition these cells have phagocytic activity and are positive for the nonspecific esterase. Approximately 3×10^7 of these adherent macrophages were cocultured for 24 hours at 37°C in 5% CO_2 with 1 μg LPS (*Escherichia coli*, 0127:B8, Difco, Detroit, MI) per milliliter in 200 ml, and the culture supernatant was concentrated up to 10-fold by YM-10 membrane (Amicon Corp., Lexington, MA), and this concentrate was used as a working supernatant (WS) in the *in vivo* experiment.

Development of Multiple Necrotizing Enteritis

Approximately 250 μl of the above WS was injected directly into the abdominal aorta just above the celiac artery of SD rats with confirmation of the anatomic location by laparotomy. We also injected in a similar manner the medium and/or LPS alone, human recombinant IL-1 (rIL-1) (10^8 unit of specific activity/mg; Genzyme, Boston, MA.), human recombinant TNF (rTNF) (2×10^7 unit of specific activity/mg; Genzyme), and PAF (Ono Pharmaceutical Co., Osaka, Japan) into the aorta. The mixture of a WS or rTNF with rabbit anti-human TNF (Endogen, Boston, MA) was injected to study the effect of TNF in MNE development. In some experiments, SD rats were injected with 15 U/rat of heparin sulfate (Novo Industry A/S, Bagsvaerd, Denmark) into the tail vein, and were given subsequently with a WS.

Cytotoxicity and Fractionation on Chromatographic Procedures of the Culture Supernatant

The cytotoxicity of a WS was assessed by the dye-uptake method with only a slight modification.[11] Various cultured cells were used as the targets. These included TNF-sensitive LM and TNF-resistent LR mouse cells, which were gifts from Drs. Y. Niitsu and N. Watanabe at the 4th Section of Internal Medicine, Sapporo Medical College, Sapporo, Japan. D10 and D14 are malignant endothelial cells of BALB/c mice.[12] C-C26 and C-C36 are mouse colon cancer and fibrosarcoma lines, respectively.[13] WFB is a WKA rat fetal fibroblast line, and W14 and W31 are EJras oncogene-transformed cells of WFB.[14] T24 is a human transitional carcinoma line, and U937 is a monocytic leukemia line. These cells were cultured overnight with approximately 100 μl of 10% FCS-RPMI 1640 medium (complete medium) in 96 flat wells of a plate (Falcon 3072, Oxnard, CA). Approximately 10 μl of the culture supernatant was added to the wells and the cells were cultured for 18 hours at 37°C in 5% CO_2 incubator. Similarly we cultured cells with LPS (1 μg/ml), rTNF (5 and 200 μ/ml), and rIL-1 (5 and 200 μ/ml). Platelet-activating factor was dissolved in ethanol and stored at −70°C. Working solution (1 μg/ml) was made before use. These cells were washed by PBS, fixed by 0.25% glutaraldehyde for 10 minutes at room temperature, washed once again, and then dried. The solution containing 0.05% methlene blue was added into each well for 20 minutes, aspirated, and dried. Then 0.2 ml of 0.33 N HCl solution was added. The absorbance at 630 nm of each well was examined by an enzyme-linked immunoassay analyzer (Microplate reader MR 600, Dynatech Product, Chantilly, VA). The percentage cytotoxicity was calculated as (absorbance of control sample − absorbance of test sample/absorbance of control sample) × 100.

To compare the *in vivo* effect for developing MNE of recombinant cytokines, we studied the effect of cytotoxicity of rTNF and rIL-1. We also assessed PAF and LPS alone on various cells. In some experiments, we used rabbit anti-human TNF antibody to study the involvement of TNK-like cytokine in our experimental system.

We further studied the partial purification of the cytotoxic molecule in the culture supernatant. Approximately 10,000 ml (approximately 0.6 to 0.7 μg/ml of protein content) of the supernatant of macrophages cultured for 24 hours with FCS-free RPMI in the presence of 1 μg LPS/ml was concentrated to 10 ml using YM-10 membrane (Amicon Corp., Lexington, MA). Approximately 6 ml of the nondenatured concentrate (1 mg/ml in protein content) was dialyzed against 50 mmol/l (millimolar) TRIS-HCl, pH 8.0, and applied on Sephadex G75 gel filtration (2.6 × 40.0 cm) at 32 ml/hour of the flow rate. Approximately 4.5 ml of fraction was collected and examined for the cytotoxicity against LM cells. As indicated in the result section, the fractions with approximately 60 kd of molecular weight were run on Mono Q column chromatography (Pharmacia, Uppsala, Sweden), as previously described.[13] Briefly the concentrate of the molecules with approximately 60 kd of molecular weight was dialyzed against 20 mmol/l TRIS-HCl, pH 8.0. The Mono Q FPLC column was equilibrated with the same buffer, and 2 ml (0.1 to 0.5 mg/ml in protein content) of the sample was applied on the column. Then a linear NaCl gradient buffer from 0 to 0.4 and 0.4 to 1.0 mol/l (molar) of ionic strength was applied to this column. Depending on the protein peak at an absorbance of 280 nm, we sampled 4 fractions from 0 to 0.1, 0.1 to 0.2, 0.2 to 0.3, and 0.3 to 0.4 mol/l in NaCl of the ionic strength, and each fraction was assessed for the cytotoxicity against LM cells.

Measurement of Blood Content of Rat Fibrinogen and Fibrinogen Degradation Product

The blood content of fibrinogen and the FDP of rats that were injected with various factors was assessed at various

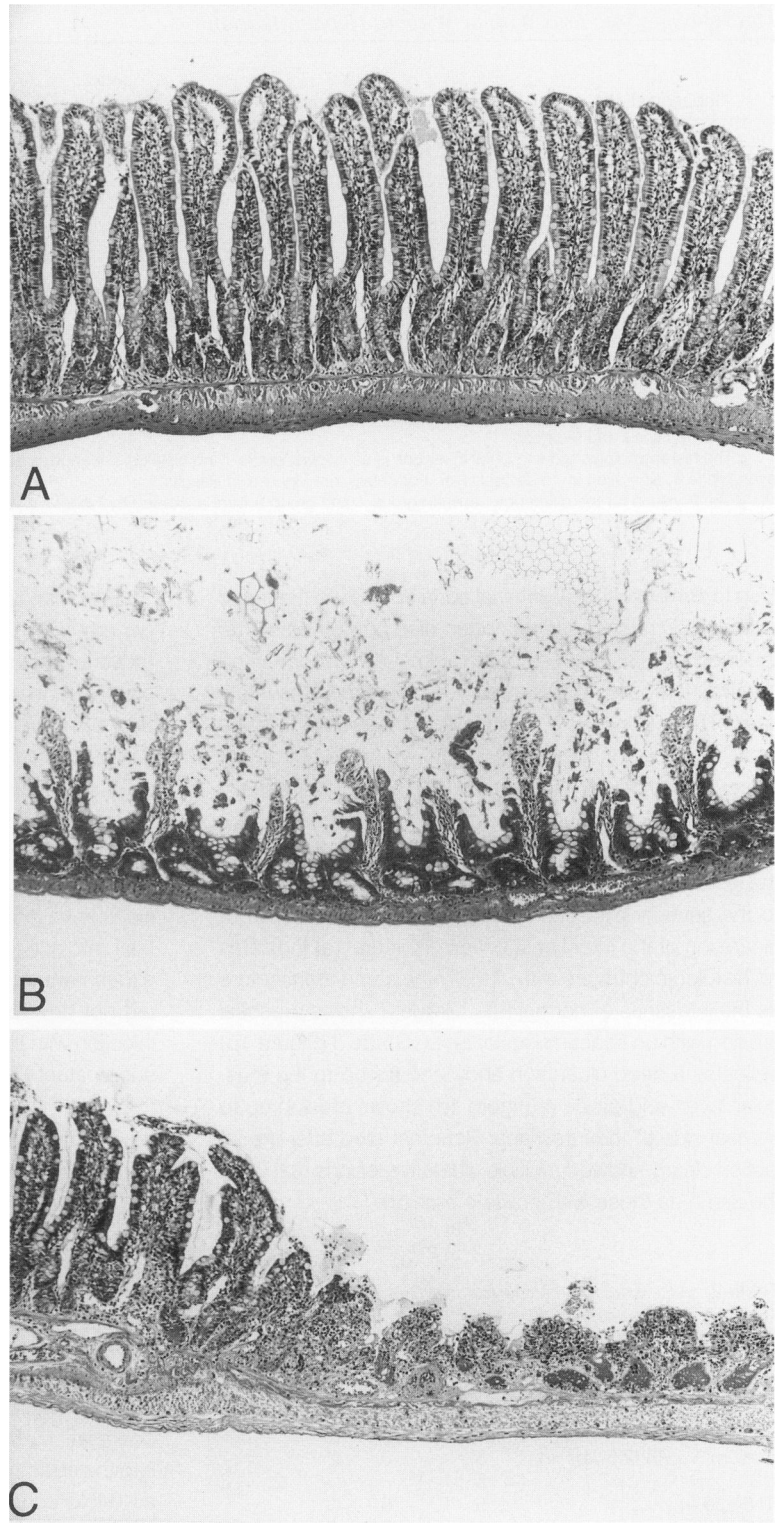

Figure 1. *Histopathologic photographs of rat MNE lesions induced by WS.* **a**: *This figure indicates normal rat intestines and we designate this grade 1. Grade 3* **(b)** *indicates the deep ulceration and necrosis up to the muscular layer, and grade 4* **(c)** *up to the serosal side of the intestines. All figures are H&E stained and* ×*100 in magnification.*

times. Fibrinogen was measured by the fibrinogen assay kit Coagmaster (Sankyo, Tokyo, Japan). The content of FDP was assayed using a latex-FDP agglutination method (Nihon Roche, Tokyo, Japan). The data was expressed as a relative ratio: blood level of fibrinogen of FDP content of MNE rats/that of normal rats.

Results

Development of Multiple Necrotizing Enteritis

We first injected 250 μl of 10-fold concentrate (WS) of the supernatant of macrophages cultured for 24 hours by

Table 1. *The Kinetics of Development of MNE After the Injection with Supernatant of LPS-stimulated Peritoneal Macrophages into Rats With or Without Heparinization*

Heparinization*	Minutes after injection†	No. of rats	Grade of lesions‡			
			1	2	3	4
Group 1						
	0	5	5			
	10	5		5		
	30	10				10
	60	5				5
No	180	5				5
Group 2						
	10	5	5§			
	60	5	5§			
Yes	180	5	5§			

* Rats in group 2 were injected with 15 U/rat of heparin sulfate into the tail vein, and subsequently received WS.
† Approximately 250 μl of WS was injected directly into the abdominal aorta of SD rats after confirming the anatomical location by laparotomy. For details see Materials and Methods.
‡ The histopathologic grading of MNE lesions is as follows: grade 1, no histologic change; grade 2, mucosal erosion; grade 3, ulceration of muscular layer; grade 4, ulceration up to serosal layer. For details see Figure 1 in Results.
§ The *P* values for the difference between group 1 and group 2 were assessed by Fisher's exact probability test, indicating $P < 0.004$.

1 μg LPS/ml into the abdominal aorta just above the celiac artery of SD rats. These rats often died 3 to 6 hours after the injection. The autopsy of the animals showed marked edema, necrosis, and eventually perforation in the intestines. The lesions were multiple and more severe in the small intestines. In the other organs, we could not detect any overt pathologic changes, except for the focal hepatic necrosis, pancreatic edema, and the visceral congestion.

To study the details of the developmental process of the lesion, we classified the lesions into grade 1 to 4 due to the severity of tissue degeneration and the depth of ulceration of the intestines. Grade 1 (Figure 1a) indicates no histologic changes with virtually the same architecture as the intestines of normal rats. Grade 2 shows mucosal erosion with an intact muscular layer. Grade 3 (Figure 1b) indicates a deep ulceration and necrosis up to the muscular layer, and grade 4 (Figure 1c) shows erosion up to serosal side of the intestines. Rats that died after the injection clearly showed multiple ulcerative lesions that were the same as those with grade 4 erosion.

Table 2. *Assessment of Various Factors for MNE Development*

Factors*	No. of rats	Grade of lesions†			
		1	2	3	4
Medium alone	5	4	1		
Medium + LPS (2.5 μg)	5	3	2		
WS	5			2	3
rTNF (50 μ)	5	5			
rTNF (500 μ)	5	3	2		
rTNF (20,000 μ)	5		2	3	
rIL-1 (50 μ)	5	5			
rIL-1 (20,000 μ)	5	5			
PAF (2.5 μg)	5	4	1		

* These factors were resolved to units shown in parentheses in a total 250 μl of solution and were injected into the rat abdominal aorta.
† MNE lesion was assessed histopathologically 3 hours after injection of factors.

We analyzed the kinetics of MNE development, especially within the first 3 hours after injection, because rats showed immediate deterioration after injection of the supernatant. As shown in Table 1, rats injected with 250 μl of WS developed pathologic lesions of MNE with grade 2 erosion after 10 minutes of the injection. Within 30 minutes, rats showed intestinal bleeding and the multiple ulcerative lesions with grade 4 erosion. Although the overt intravascular thrombus formation was not microscopically detected, these facts may indicate strongly that MNE lesions were induced by an acute disturbance of the intestinal microcirculation. This notion was supported by the experiment in which rats pretreated with heparin sulfate did not develop an MNE lesion even 180 minutes after injection with the same quantity of the macrophage-culture supernatant (Table 1).

Factors Leading to the Develop of Multiple Necrotizing Enteritis

It is known that macrophages can produce biologically active humoral factors in response to various stimuli. Among these factors, TNF, IL-1, and PAF were assessed for the capability to develop MNE because it is suggested that these factors may participate in the intravascular blood coagulation. Three or five rats per group were injected into the abdominal aorta with 250 μl of RPMI medium only, a 10-fold concentrate of medium plus LPS, WS, human rTNF (50, 500, and 20,000 μ), human rIL-1 (50 and 20,000 μ), and PAF (2.5 μg). Working supernatant contained approximately 10 μ in rat TNF/ml as assessed by the method using Actinomycin D described by Flick et al.[15] As shown in Table 2, rats injected with medium alone or medium plus LPS showed the histologic finding with grade 1 or 2 erosion. Platelet-activating factor and rIL-1 in very high

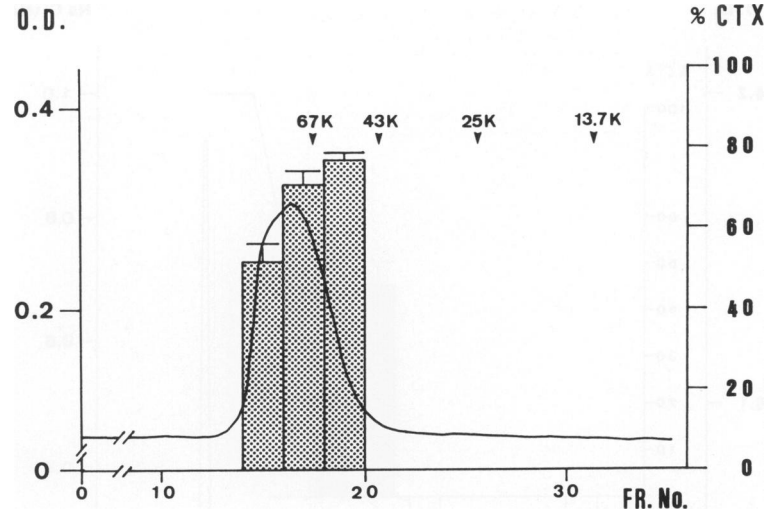

Figure 2. *The gel filtration on Sephadex G75 of LPS-stimulated macrophage supernatant. Approximately 6 ml of the nondenatured concentrate (1 mg/ml in protein content) was dialyzed against 50 mmol/l TRIS-HCl, pH 8.0, and was applied on Sephadex G75 gel column (2.6× 40.0 cm) at 32 ml/hour of the flow rate. Approximately 4.5 ml of fraction was collected and examined for the cytotoxicity against LM cells.*

amounts (2.5 μg and 20,000 μ, respectively) did not induce foci of more than grade 2. In contrast, the high content of human rTNF (20,000 μ) could lead to the development of MNE with grade 3 histologic foci in the rat small intestines, although these rats rarely showed grade 4 foci. These data suggested that while other cofactors may be involved, the main cofactor that led to the development of MNE seems to be TNF. Furthermore rTNF was derived from the human TNF-α gene so that the effect was weaker than that of the rat origin.[16–18]

Characterization of the Supernatant

We determined whether supernatants contain ample TNF, which was produced from peritoneal macrophages, because the above-mentioned data suggest that TNF could induce MNE lesions. As described above, the WS injected into rats contained approximately 10 μ of rat TNF. We also studied the cytotoxicity of WS against various cells, including TNF-sensitive LM and TNF-resistent LR cells. We found that the spectrum of cytotoxicity of supernatant was compatible with human rTNF, that is, supernatant was cytotoxic to LM cells but not LR cells. It was also cytotoxic to many cell lines tested, such as D10, D14, C-C26, C-C36, WFB, W14, W31, T24, and U937. Lipopolysaccharide, rIL-1, and PAF did not have the cytotoxic potential for any of these cells tested. Furthermore we studied the stability for heat treatment of the activity of supernatant. When the supernatant was treated with 1) 56°C, 30 minutes; 2) 70°C, 30 minutes; and 3) 100°C, 10 minutes, the cytotoxic activity against LM was retained only with 56°C at 30 minutes but not at more than 70°C (data not shown).

The supernatant was further characterized by the gel filtration on Sephadex G75 and ion-exchange column chromatography on Mono Q FPLC. The data showed that in the gel filtration the cytotoxic molecule against LM cells was eluted from high to low molecular weight fractions with nondenatured condition of the molecules (Figure 2). But it appeared that approximately 60 kd of the molecular weight was most cytotoxic. This correlates with the fact that the native TNF could form trimer of subunits with approximate molecular weights of 20,000.[19] Furthermore it was eluted also by 0.3 to 0.4 mol/l in NaCl of the ionic strength (Figure 3). These data may indicate that the active molecules in the supernatant was TNF-like molecules, although previous reports[20] showed that TNF has a weaker ionic strength, such as 0.2 to 0.3 mol/l in NaCl. To test this possibility, we have studied the blocking experiment using polyclonal rabbit anti-TNF serum. When the supernatant was preincubated for more than 18 hours at 37°C with polyclonal anti-TNF serum, the cytotoxicity against LM cells was greatly reduced (Figure 4a). Furthermore the same material containing the supernatant and antiserum could not develop MNE lesions when injected into rats (Figure 4b). However the mixture of the supernatant and normal rabbit serum could induce the development of MNE lesions of grade 4. Together these data suggest that the factor most likely to cause MNE is a TNF-like cytokine.

Measurement of Blood Fibrinogen and Fibinogen Degradation Product

To assess whether the intravascular blood coagulation was virtually induced by the TNF-like cytokine, we also measured the blood content of fibrinogen and FDP at various times after the injection of WS. As shown in Figure 5, both of these levels in the blood showed a peak at about 6 hours after injection. Furthermore we assessed

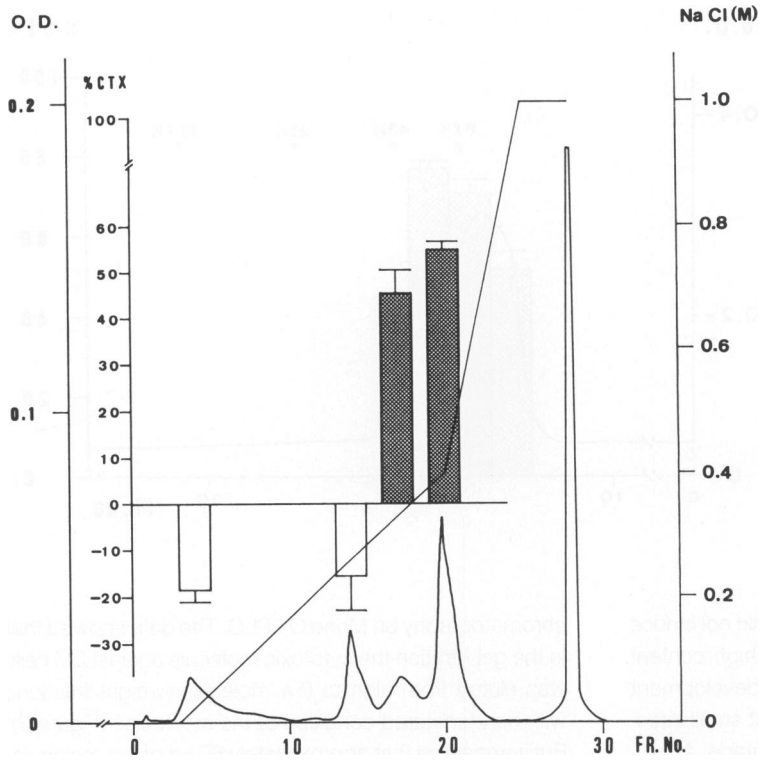

Figure 3. *FPLC using Mono Q column chromatography of the concentrate of molecules with approximately 60 kd of molecular weight from Sephadex G75 gel filtration. The concentrate was dialyzed against 20 mmol/l TRIS-HCl, pH 8.0. Mono Q FPLC column was equilibrated with the same buffer, and 2 ml (0.1 to 0.5 mg/ml in protein content) of the sample was applied on the column. A linear NaCl gradient buffer from 0 to 0.4 mol/l and then from 0.4 to 1.0 mol/l was applied to the column. Four fractions from 0 to 0.1, 0.1 to 0.2, 0.2 to 0.3, and 0.3 to 0.4 mol/l in NaCl of the ionic strength were obtained and dialyzed overnight with PBS. Then these fractions were assessed for the cytotoxic potentials against LM cell.*

the content of fibrinogen and FDP when rats were injected with the mixture of WS plus anti-TNF antibody. These rats did not show any obvious changes of the content of fibrinogen and FDP. This was true for rats injected by rIL-1 (data not shown).

Discussion

The pathogenesis of MNE in human patients still remains unknown.[1] However MNE usually develop in patients who suffer from severe bacterial infection, especially by gram-negative bacterias. It is well known that endotoxemia could induce serious illness, and the bacterial endotoxin LPS is a powerful stimulator for the production of various humoral factors including TNF, IL-1, and PAF by macrophages.[4-9,21-25]

In this paper we described the development of MNE by the supernatant of LPS-stimulated peritoneal macrophages in rats and analyzed the humoral factor that is most responsible for the development of MNE. Our data indicate that the lesion of MNE was caused by acute ischemia possibly due to the disturbed microcirculation in the small intestines. Multiple necrotizing enteritis was induced 10 to 30 minutes after injection when the supernatant was administered into the abdominal aorta. Furthermore the study by using not only a gel filtration and ion exchange chromatography but also anti-TNF antibody of the LPS-stimulated macrophage supernatant suggested that it contained a certain amount of the TNF-like molecule and that MNE is caused by this TNF-like molecule, which is secreted by LPS-stimulated macrophages.

In our present model of MNE, the small intestines are more susceptible to MNE development than the large intestines. But this fact is simply derived from the anatomic differences among intestines because the blood supply in the small intestines is distributed by only one route of the superior mesenteric artery. However the large intestines are supplied doubly by the superior and inferior mesenteric artery. Hence it appears that the TNF-like molecule from the aorta was directly distributed in the small intestines. However this molecule was diluted in its concentration by the blood flow from the inferior mesenteric artery in the large intestines.

TNFα is considered to be the putative mediator of endotoxemia.[3] Our present data suggest that the TNF-like molecule is most responsible for the development of MNE lesion because the biochemical characteristics of the culture supernatant of LPS-stimulated macrophages is compatible with that of TNF.[3,21] This lesion also could be clearly developed by human TNF, but IL-1 and PAF could rarely develop the overt lesions of MNE. However a high amount of human TNF could develop the MNE lesion with grade 3. This fact may be due to the species difference of the effectiveness of human TNF against rat cells, as suggested by several investigators.[16-18] Rats are

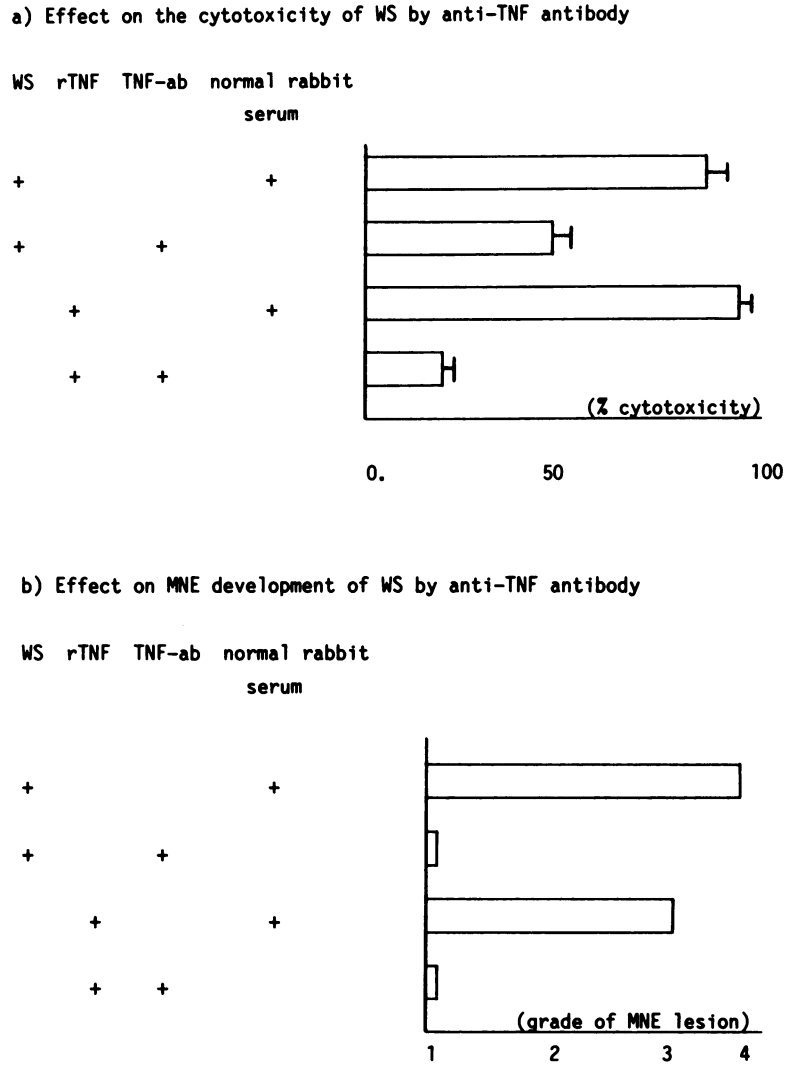

Figure 4. a: *The* in vitro *effect of rabbit anti-TNF antibody (TNF-ab) to WS. Twenty-five microliters of WS or 25 μl of rTNF (5 μ/ml) were preincubated for more than 18 hours at 37 °C with 25 μl of rabbit anti-TNF antibody (5 μ/ml) and with 25 μl of ×1000 diluted normal rabbit serum, and these mixtures amounting to 50 μl, was added to the wells in which LM cells were cultured in 100 μl complete medium. The cytotoxicity was assessed by culturing for 18 hours at 37 °C in 5% CO_2 incubator. This rabbit anti-human TNF antibody has a specific activity of more than 10,000 neutralizing units/ml. This activity was titered as TNF units by using standard cytotoxicity assay using LM cells treated with actinomycin-D, in which one unit equal to the amount of TNF is needed to induce 50% lysis of LM cells* in vitro. b: *The* in vivo *effect of rabbit anti-TNF antibody to WS. One hundred twenty-five microliters of WS or 125 μl of rTNF (total 20,000 μ) were similarly pretreated with 125 μl of rabbit anti-TNF antibody (total 20,000 μ) and with 125 μl of ×1000 diluted normal rabbit serum, and these mixtures, amounting to 250 μl were injected into the animals. Five rats for each group were assessed pathohistologically for the development of MNE lesion 6 hours after injection.*

relatively resistant to human TNF, and the LD50 for human TNF in rats is 700 μg/kg.[17]

On the other hand, polyclonal anti-TNF antibody could inhibit the development of MNE *in vivo*. Furthermore the cytotoxicity *in vitro* against TNF-sensitive LM cells by the culture supernatant of LPS-stimulated rat peritoneal macrophages also was inhibited, although this inhibition was detected only partially. The fact that this antibody could almost completely inhibit the *in vivo* effect of murine TNF is very important because these data simply indicate that the cytokine most responsible for MNE development is a TNF. Although Gonzales-Crussi et al[26] and Hsueh et al[27] reported that PAF is a powerful inducer of ischemic bowel disease in rats, MNE lesion was not developed by PAF or in conjunction with LPS in our study. This was not attributed to the amounts of PAF injected because these investigators and our present study used almost the same amounts of PAF, and we could not explain this discrepancy.

Our present study seems to indicate that MNE is caused by the acute disturbance of microcirculation because 1) MNE lesion is found to be necrotic and markedly hemorrhagic and 2) MNE could be induced 10 to 30 minutes after injection. Furthermore the level of fibrinogen and FDP content in the blood was clearly increased 6 hours after the injection. Following this, it showed a relatively rapid decrease. These facts suggest that the mechanisms for developing MNE are relevant to the increased intravascular blood coagulation of the local vessels that were affected by TNF.

There are several reports demonstrating that TNF could induce the procoagulant production from the endothelial cells.[6,7] Moreover, there is the possibility that TNF itself could enhance the intravascular blood coagulation[6,24] and increase the vascular permeability, independent of neutrophiles.[18] This notion might be reasonable because MNE could develop very rapidly, as mentioned above. However the MNE lesion with grade 4 was frequently accompanied

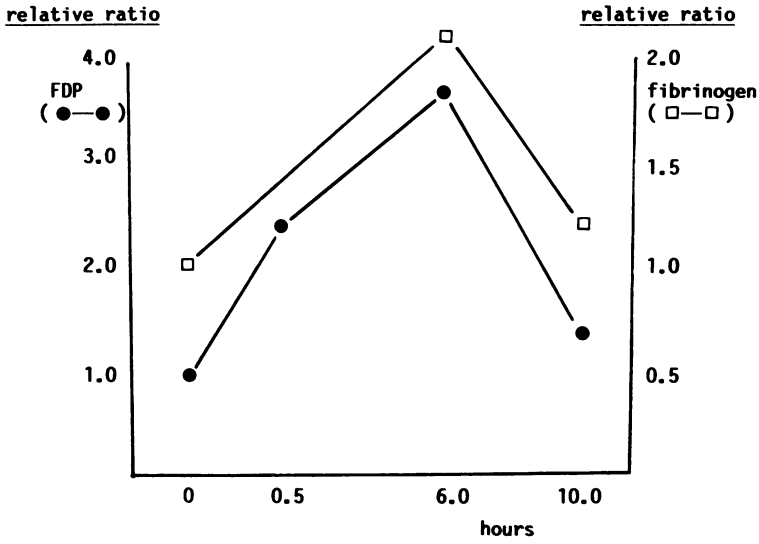

Figure 5. *The blood level of fibrinogen (□) and FDP (●) content in MNE rats. The blood content of fibrinogen and FDP of five rats that were injected with various factors was assessed at various times after WS injection. The data are expressed as a relative ratio; mean blood level of fibrinogen or mean FDP content of five MNE rats/that of five normal rats.*

by the accumulation of neutrophiles when assessed histopathologically 3 to 6 hours after injection of the LPS-culture supernatant. As Westwick et al[28] suggest, LPS-stimulated macrophages can produce the neutrophile-activating factor (NAF). Although we do not have the experimental data using purified NAF, it may be possible that NAF may enhance the pathologic lesion of MNE.

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