

TUMOR NECROSIS FACTOR/CACHECTIN

Induction of Hemorrhagic Necrosis in Normal Tissue Requires the Fifth Component of Complement (C5)

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Tumor necrosis factor (TNF) originally described to cause hemorrhagic necrosis (HN)¹ of tumors (1, 2), has also been associated with several other important effects (3, 4). For example, cachexia, which can develop in chronically infected animals, has been shown to be dependent upon cachectin (5, 6), a substance that was later found to be identical to TNF (7). Furthermore, shock occurring in animals with septicemia or in animals exposed to endotoxin has been shown to require TNF (8, 9). While these findings demonstrate that TNF can have detrimental effects under certain conditions, a primary, beneficial role of TNF may be in the defense of the host against infections. For example, the damage of vessels by TNF observed at the site of bacterial infection may represent a defense mechanism capable of stopping local blood circulation and, thereby, the dissemination of toxins and bacteria in the host (10). Thus, systemic toxicity and lethal shock would only occur when this first line of defense breaks down.

The induction of HN in the skin of mice injected with bacteria and TNF provides a model for studying this TNF-mediated vessel damage (10). Using this model, we have demonstrated that the interaction of several different products is required for HN to occur (10). In addition to TNF, two other factors were identified; one, a bacterial component, and the other, an x-ray-sensitive host cell (10). In this paper we provide several lines of evidence that the complement component C5 also plays a critical role in the induction of HN by TNF and bacteria.

Materials and Methods

Agents. Heat-killed *Corynebacterium parvum* purchased from Behring Diagnostics (San Diego, CA) was diluted in nonpyrogenic saline (Invenex Pharmaceuticals, Inc., Melrose Park, IL) to 10 mg/ml before use. Human TNF (lot L9085AX; Genentech, Inc., South San Francisco, CA) had a specific activity of 5.03×10^7 U/mg, as determined in a microcytotoxicity assay using actinomycin D-treated L929 cells as targets with 1 u defined as equal to the reciprocal of the dilution of TNF required to kill 50% of the target cells, as previously described (11).

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¹ *Abbreviation used in this paper:* HN, hemorrhagic necrosis.

TNF had an endotoxin level of 0.06 endotoxin units (EU)/mg as measured in the *Limulus* amoebocyte lysate assay, in which 1 EU is equal to 0.1 ng/ml of USP standard *Escherichia coli* endotoxin (lot EC5). Zymosan A from *Saccharomyces cerevisiae* (Sigma Chemical Co., St. Louis, MO) was boiled for 30 min, washed three times with nonpyrogenic saline, and resuspended to 50 mg/ml before use. Purified cobra venom factor from *Naja naja kaouthia* (0.5 mg/ml; Sigma Chemical Co.) was stored at -20°C and diluted in nonpyrogenic saline for use. LPS from *E. coli* 0111:B4 (lot 755012; Difco Laboratories, Inc., Detroit, MI) was diluted in nonpyrogenic saline to 10 mg/ml before use. *Mycoplasma orale* was passaged by intracellular growth in vitro and expanded with infected cells for the generation of lysate as previously described (10).

Mice. 6–10-wk-old, pathogen-free, female mice of the C3H/HeN and AKR strains were purchased from a germ-free-derived, defined flora colony at the Frederick Cancer Research Institute (Frederick, MD). DBA/2, A/J, B10.D2/o, B10.D2/n, CBA/J, and C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in pathogen-free conditions in laminar flow hoods.

Assay for HN. Mice were challenged with *C. parvum*, mycoplasma-infected cell lysates, or LPS and TNF as described previously (10). Briefly, the lower backs of mice were shaved and depilated with a chemical depilator (Nair; Carter-Wallace, New York, NY). *C. parvum* (400 μg) or mycoplasma-infected cell lysates (10^7 cells) were co-injected with TNF (10 μg s. c.). LPS (100 μg s. c.) was injected, followed 24 h later by TNF (10 μg) at the same site. HN was assessed 24 h later either macroscopically, seen as a dark red to black discoloration of the skin, microscopically, seen as infiltrating leukocytes and extravasation of blood, or by measuring the retention of ^{51}Cr -labeled blood cells (see below).

Quantification of HN. In some cases, HN was quantified using a modification of a method described previously (12). In principle, this assay measures the radioactivity retained in skin after intravenous injection of ^{51}Cr blood cells. Blood for ^{51}Cr labeling was collected into citrate-treated tubes (Becton Dickinson & Co., Rutherford, NJ) from the retroorbital sinus of syngeneic mice. Erythrocytes were washed three times in nonpyrogenic saline before labeling. To each 0.5 ml of packed blood cells we added 0.5 ml of nonpyrogenic saline containing 200 μCi of $\text{Na}_2^{51}\text{CrO}_4$ having a specific activity of 300–400 $\mu\text{Ci}/\text{mg}$ (Amersham Corp., Arlington Heights, IL). The blood cell suspension was incubated for 60 min at 37°C , washed three times in nonpyrogenic saline, and resuspended to a final volume of 2.0 ml. Mice were each injected with 0.25 ml of the ^{51}Cr -labeled blood cell suspension 60 min after injection of TNF and/or other agents. Mice were killed by cervical dislocation 24 h later and 2×2 cm square segments of full thickness skin containing the site of injection in the center were removed. The pieces of skin were placed into 12×75 mm glass tubes (Fisher Scientific, Pittsburgh, PA) and the amount of labeled blood cells retained in lesions was determined using a Micromedic ME-plus gamma counter (Micromedic Systems, Horsham, PA). Previous studies (10) have shown that HN reaches a maximum at 24 h after co-injection of *C. parvum* and TNF and, therefore, all mice were killed for analysis at that time. Results were reported as counts per minute per skin segment.

Zymosan-activated Mouse Plasma. Citrate-treated blood was obtained from the retroorbital sinus of either “C5-sufficient” (C3H/HeN) or “C5-deficient” (AKR and B10.D2/o) mice. Pooled C5-sufficient or -deficient blood was centrifuged 500 g for 15 min. Plasma was obtained and immediately frozen at -20°C until use. Freshly thawed plasma was activated with zymosan (5 mg/ml) as modified from a method described previously (13). Briefly, plasma was incubated with zymosan for 2.5 h at 37°C with frequent agitation. At the end of the incubation period, zymosan was removed by centrifugation and the supernatant (activated plasma) was used to reconstitute C5-deficient mice. In some experiments, HCl was added to the activated plasma to a final concentration of 1 N and the precipitated proteins were removed by centrifugation at 2000 g for 40 min. This acidification step removes a majority of the plasma proteins (13). Acidified zymosan-activated plasma was dialyzed in small-pore tubing (MWCO 1000; Spectrum Medical, Los Angeles, CA) against 0.1 M ammonium formate (pH 7.0) before reconstitution of mice or gel filtration chromatography.

Plasma Reconstitution of C5-deficient Mice. C5-deficient mice co-injected with *C. parvum* (400 μg s. c.) and TNF (10 μg) were injected 3 h later slowly with 0.3 ml i.v. of C5-sufficient mouse (C3H/HeN) plasma. In some cases, mice were injected intravenously with C5-deficient plasma,

C5-sufficient mouse plasma heat inactivated at 56°C for 30 min, zymosan-activated plasma, or column fractions obtained from zymosan-activated plasma after gel filtration chromatography. Preliminary experiments showed that injection of plasma 3 h after co-injection of *C. parvum* and TNF was optimal for reconstituting C5-deficient mice so that they could develop HN.

Hemolytic Complement Assays. Classical pathway activity of mouse plasma was determined by hemolysis of antibody-sensitized rabbit erythrocytes as previously described (14). Guinea pig anti-rabbit antibody for sensitization of rabbit erythrocyte targets was prepared as previously described (15). Briefly, samples of plasma were serially diluted in nonsterile 12 × 75 mm culture tubes in 0.1 ml of Veronal-buffered saline (pH 7.5) containing 5% glucose (wt/vol), 0.1% gelatin (wt/vol), 1 mM MgCl₂, 0.15 mM CaCl₂, and 1 mM NaN₃. An equal volume of washed sensitized rabbit red cells was added (10⁷ cells/ml) and the mixture was incubated at room temperature for 30 min. At the end of incubation, 1.0 ml of Veronal-buffered saline containing 0.1% gelatin (wt/vol) and 1 mM EDTA was added and the red cells remaining unlysed were pelleted by centrifugation. Supernatants were quantified for released hemoglobin by absorbance at 412 nm in a spectrophotometer (No. DU-50; Beckman Instruments, Fullerton, CA). Hemolytic activity was expressed as the reciprocal of the dilution of plasma required to lyse 50% of the added red cells per milliliter (CH₅₀). The reduction of C5 activity in zymosan-activated plasma was determined by comparing the hemolytic activity of C5-deficient plasma to which serial dilutions of either zymosan-activated or nonactivated C5-sufficient plasma had been added. Sensitized red cells were added, incubated for 60 min at room temperature, and the remaining red cells were pelleted by centrifugation to determine the amount of hemolysis as described above. The percent reduction of C5 activity was calculated by the formula: $100 \times [1 - (\text{CH}_{50} \text{ of zymosan-activated plasma} / \text{CH}_{50} \text{ of C5-sufficient plasma})]$.

Gel Filtration Chromatography. Bio-gel P60 (Bio-Rad Laboratories, Richmond, CA) was used to fractionate acidified zymosan-activated C5-sufficient or -deficient mouse plasma. The fractionation range of Bio-gel P60 is 3,000 to 60,000 *M_r* and, therefore, ideal for the isolation of anaphylatoxins (13). Plasma samples were applied to a 1.5 × 75 cm Bio-gel P60 column equilibrated in 0.1 M ammonium formate, pH 7.0. A majority of the high molecular weight plasma proteins were eluted in the void volume. Single column fractions or pooled fractions were tested for their capability to reconstitute B10.D2/o mice to respond with HN after co-injection of *C. parvum* and TNF.

Results

C5-Deficient Mice Do Not Develop HN when Injected with TNF and a Bacterial Component. When comparing various strains of mice for induction of HN by TNF and *C. parvum*, LPS, or lysates of cells infected with *M. orale*, we found that certain strains of mice consistently failed to develop HN (Table I, Fig. 1). In search of a genetic trait shared by these mice, we found that all of these nonresponsive strains were those known to have no detectable levels of C5 in the plasma (18, 19; for review see reference 20). In contrast, the C5-sufficient CBA, B10.D2/n, and C3H mouse strains regularly developed HN characterized by a round, dark red to black, flat lesion (Fig. 1) involving the full thickness of skin, 0.5–2.5 cm in diameter (mean diameter, 1.4 cm ± 0.1 SEM). These HN lesions could also be quantified in a ⁵¹Cr-labeled blood cell assay that had been used previously to quantify HN induced in tumors by TNF (12). Thus in Fig. 2., C5-sufficient mice show significant extravasation of blood cells as quantified by the retention of ⁵¹Cr-labeled blood cells in skin with HN. Just like the induction of macroscopic HN, Fig. 2 also shows that this effect required injection of *C. parvum* as well as TNF. C5-deficient mice that show no macroscopic HN also show no significant differences in retention of ⁵¹Cr-labeled blood cells between skin injected with *C. parvum* alone and skin injected with *C. parvum* and TNF. These

TABLE I
*C5-deficient Mouse Strains Fail to Develop HN
 when Injected with a Bacterial Agent and TNF*

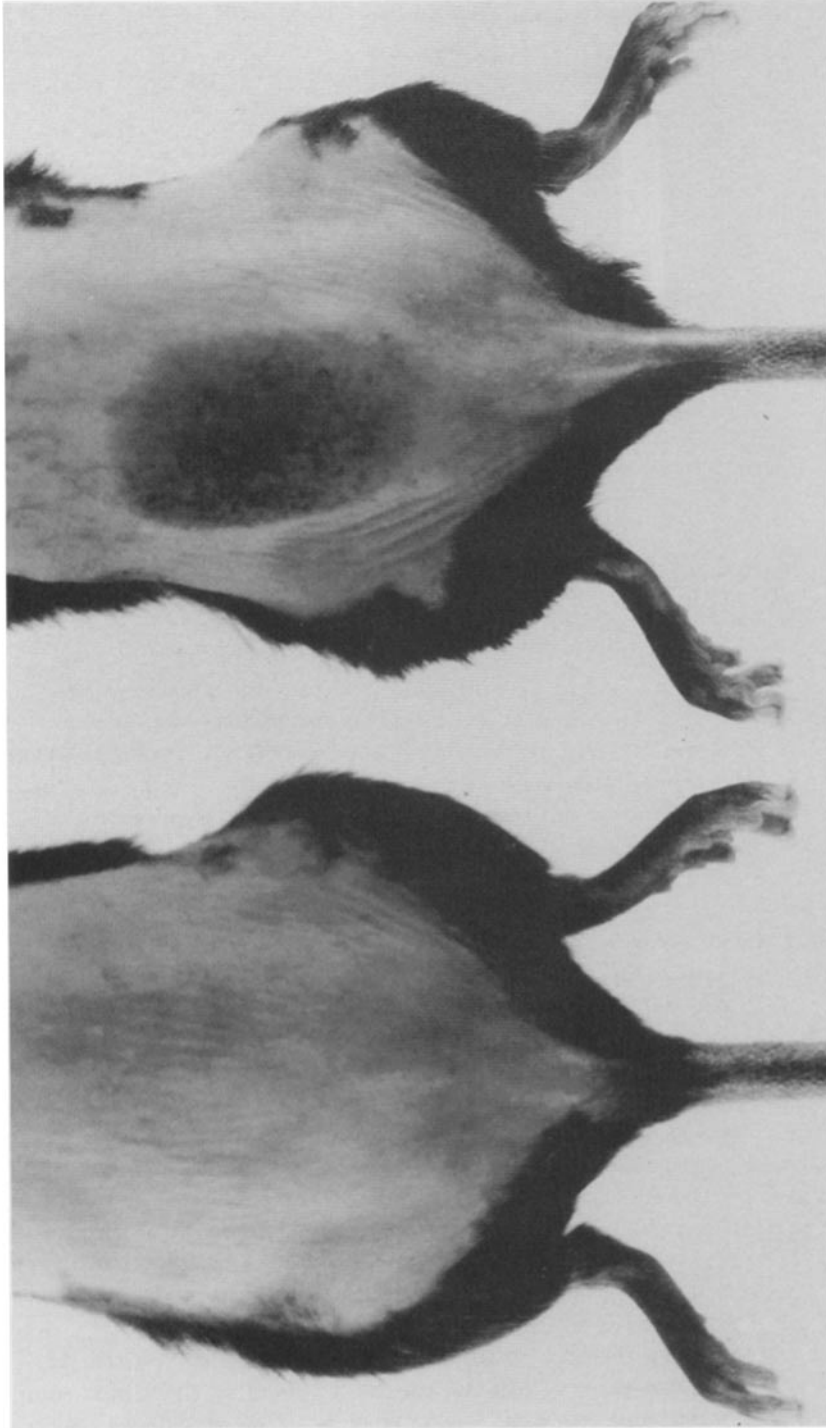
Bacterial agent*	Mouse strain	Complement deficiency [†]	Incidence of HN (%) [§]
<i>C. parvum</i>	C3H/HeN	None	13/14 (93)
	C3H/HeJ	None	4/4 (100)
	CBA/J	None	9/10 (90)
	B10.D2/n	C3	14/22 (64)
	DBA/2	C3, C5	0/10 (0)
	B10.D2/o	C3, C5	0/13 (0)
	A/J	C5	0/12 (0)
	AKR	C5	0/7 (0)
LPS	C3H/HeN	None	11/11 (100)
	B10.D2/o	C3, C5	0/10 (0)
<i>M. orale</i> -infected cell lysates	C3H/HeN	None	10/10 (100)
	DBA/2	C3, C5	0/10 (0)

* HN was induced by subcutaneous co-injection of *C. parvum* and TNF or *M. orale*-infected cell lysates and TNF. Alternatively, HN was induced by subcutaneous injection of LPS followed 24 h later by TNF.

[†] Mice deficient in C3 have low but detectable levels of C3 (16, 17). Mice deficient in C5 have no detectable C5 (18, 19; for review see reference 20).

[§] Number of mice responding with HN per total number of mice tested. HN was assessed 24 h after injection of TNF. The mean diameter of the hemorrhagic lesions of different C5-sufficient mouse strains was similar (1.8 cm \pm 0.2 SEM). Data are pooled from two or more independent experiments, each containing at least one C5-sufficient and one C5-deficient group of mice.

data can be corroborated microscopically by demonstrating that the increased retention of ⁵¹Cr-labeled blood cells in skin showing HN is indeed associated with the extravasation of red blood cells (Fig. 3, *D* and *E*). C5-deficient mice show inflammatory infiltrates but no evidence of extravasation of red cells (Fig. 3, *F* and *G*). Therefore, these inflammatory white blood cell infiltrates may be responsible for the slightly increased retention of ⁵¹Cr-labeled blood cells in the skin of these C5-deficient mice (Fig. 2). Although this level is increased significantly above the level observed in noninjected skin, it is much lower than in mice developing HN. Since C5-deficient mice lack C5a, a potent leukocyte chemoattractant derived from the activation of C5 (21), one might argue that the failure of these mice to develop HN was due to insufficient leukocyte infiltration. However, C5-deficient mice show equal or even greater inflammatory infiltrates when compared with C5-sufficient mice (Fig. 3). Therefore, the failure of C5-deficient mice to develop HN was not due to the absence of a leukocyte infiltrate. Interestingly, B10.D2/n mice did not show the same high incidence of HN as the other C5-sufficient strains, which responded 90–100% of the time. One known difference is that B10.D2/n mice have a lower level of C3 than the three other C5-sufficient mouse strains (17). Together, these data suggest that complement components at or beyond C5 in the complement pathway are necessary, whereas early acting components of the classical (C1, C2, C4, and C3) and



mice (*left*) co-injected with *C. parvum* (400 μ g) and TNF (10 μ g) fail to respond with a macroscopic hemorrhagic lesion.

FIGURE 1. C5-deficient mice fail to develop HN when infected with *C. parvum* and TNF. C5-sufficient (BI0.D2/n) mice (*right*) respond with HN 24 h after co-injection of *C. parvum* (400 μ g) and TNF (10 μ g). C5-deficient (BI0.D2/o)

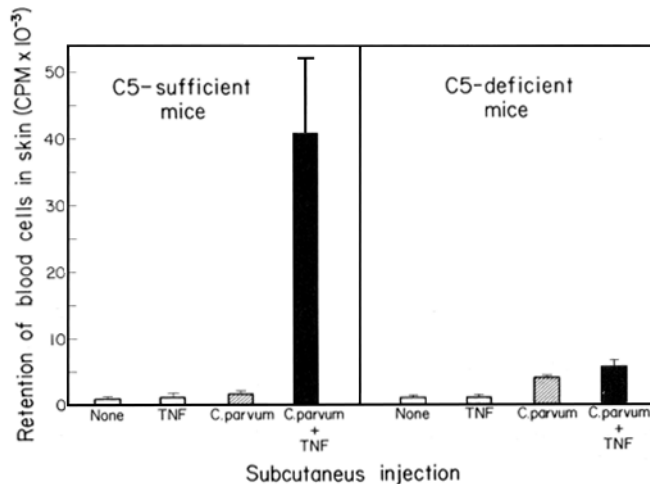


FIGURE 2. Quantification of HN developing in C5-sufficient, but not C5-deficient, mice after injection of *C. parvum* and TNF. C3H/HeN (C5-sufficient) or B10.D2/o (C5-deficient) mice were injected with either TNF (10 μ g) alone, heat-killed *C. parvum* (400 μ g) alone, or heat-killed *C. parvum* (400 μ g) and TNF (10 μ g). 24 h later, retention of ⁵¹Cr blood cells in the skin was quantified by measuring the radioactivity of a 2 \times 2 cm square piece of skin containing the site of injection in the center. For details see Materials and Methods. Data are pooled from three independent experiments.

the alternative (B, D, P, C3) pathway may contribute, but are not sufficient alone to cause HN in animals injected with *C. parvum* and TNF.

Depletion of Complement in C5-sufficient Mice by Cobra Venom Factor (CVF) Prevents the Induction of HN. If C5 was truly needed for the formation of HN in C5-sufficient mice, then the depletion of complement should abrogate the capacity of such mice to respond with HN. CVF isolated from *Naja naja kaouthia* venom, by generating a C3/C5-convertase, causes the depletion of C3 and factor B as well as the depletion of the terminal (C5-9) components of the complement pathway (22, 23). Indeed, C5-sufficient C3H/HeN mice pretreated with two injections of purified CVF (50 μ g i.v. injections 4 h before and, again, at the time of co-injection of *C. parvum* and TNF) failed to develop HN 24 h later (Exp. 1, 0/4, Exp. 2, 0/4). In contrast, nontreated control mice responded with HN (Exp. 1, 4/4, Exp. 2, 4/4). CVF, however, did not prevent leukocyte attraction since CVF-pretreated mice had as great an influx of leukocytes as the nontreated mice (data not shown). These data confirm that the depletion of complement components in C5-sufficient mice can prevent the development of HN.

C5-deficient Mice Can Be Reconstituted with C5-sufficient Plasma to Develop HN. To obtain further evidence that the C5 defect in C5-deficient mice was responsible for their failure to develop HN, C5-deficient mice were reconstituted with plasma from C5-sufficient mice. Table II shows that such plasma was fully capable of reconstituting two different C5-deficient mouse strains, B10.D2/o and AKR. Heat inactivation of this plasma at 56°C for 30 min abrogated its capacity to reconstitute C5-deficient mice, a finding consistent with the notion that complement was indeed the active component. As expected, plasma from C5-deficient mice could not reconstitute B10.D2/o or AKR mice to respond with HN to co-injection of *C. parvum* and TNF. (Table II). These data support the notion that C5 can completely correct the defect in C5-deficient mice to respond with HN to coinjection of *C. parvum* and TNF. These reconstitution experiments also provided us with an assay to identify the specific complement component required for the induction of HN.

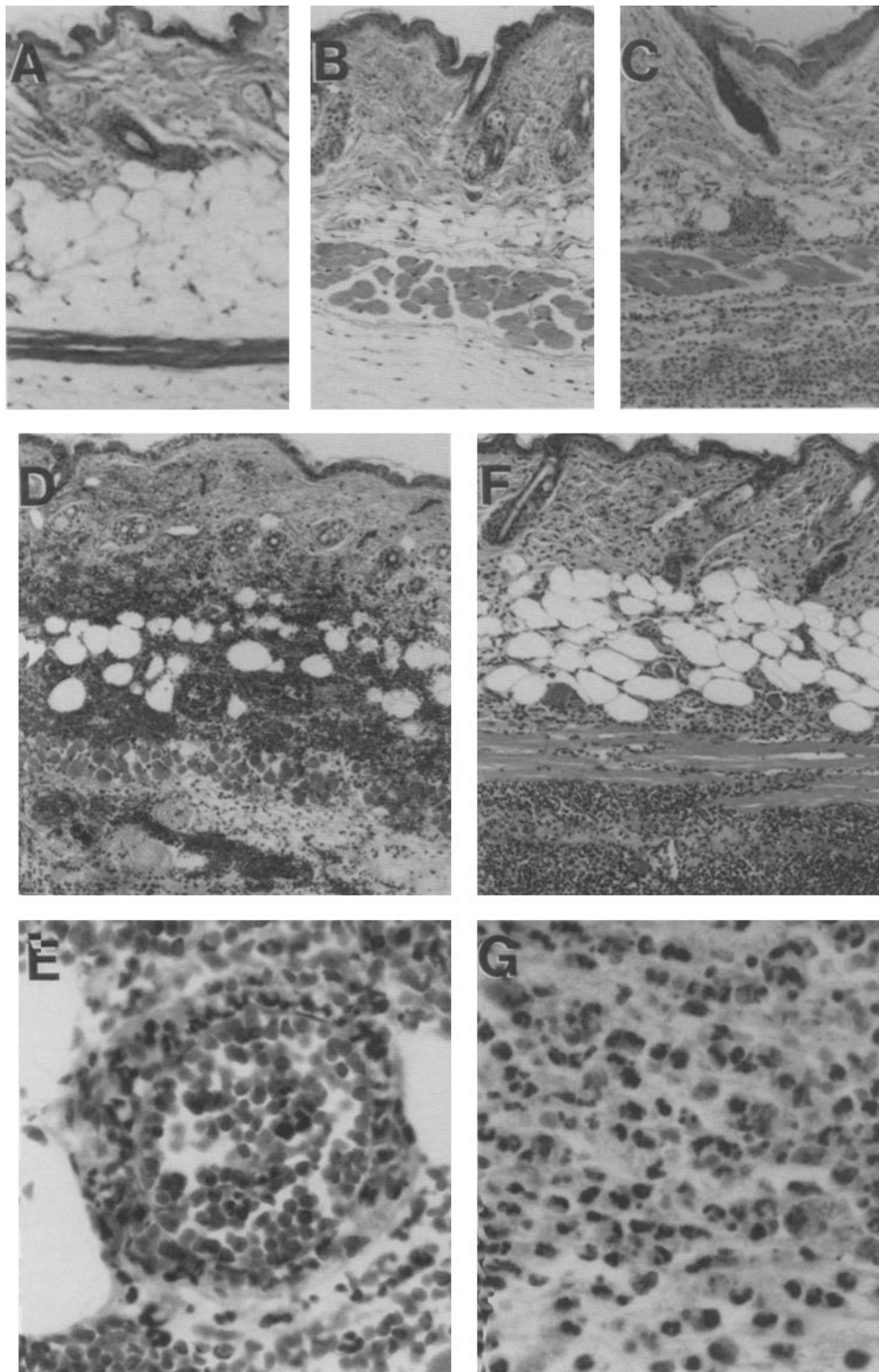


FIGURE 3. Inflammatory infiltrate without extravasation of red blood cells in skin of C5-deficient mice injected with TNF and *C. parvum*. (A-E) Skin sections of C5-sufficient C3H/HeN mice. (A) Noninjected. (B) TNF (10 µg); no infiltrate. (C) *C. parvum* (400 µg); extensive leukocyte infiltrate. (D and E) *C. parvum* (400 µg) and TNF (10 µg); extensive leukocyte infiltrate with extravasation of red blood cells. (F and G) skin sections of C5-deficient A/J mice injected with *C. parvum* (400 µg) and TNF (10 µg); extensive leukocyte infiltrate but no red cell extravasation (A-D, F: × 99; E and G × 600).

TABLE II
*C5-deficient Mice Can Be Reconstituted with Plasma
 from C5-sufficient Mice to Respond with HN*

C5-deficient responder*	Plasma used for reconstitution†	Retention of blood in skin‡ <i>cpm ± SEM</i>	Incidence of HN (%)§
B10.D2/o	Sufficient	22,385 ± 8,552	16/17 (94)
	Deficient	—	0/4 (0)
	Sufficient (HI)	5,474 ± 1,375	0/5 (0)
	None	4,105 ± 110	0/5 (0)
AKR	Sufficient	—	12/14 (86)
	Deficient	—	0/4 (0)
	Sufficient (HI)	—	0/3 (0)
	None	—	0/9 (0)

* Mice were co-injected with *C. parvum* and TNF followed by plasma reconstitution 3 h later (for details see Materials and Methods).

† C5-sufficient plasma was collected from C3H/HeN mice. C5-deficient plasma was collected from either AKR, DBA/2, or B10.D2/o mice. C5-sufficient plasma was heat inactivated (HI) by incubation at 56°C for 30 min.

‡ Retention of ⁵¹Cr-labeled blood cells in skin of mice isolated 24 h after injection of *C. parvum* and TNF. For details see Fig. 2.

§ Number of mice responding with HN per total number of mice tested. HN was observed 24 h after reconstitution. The mean diameter (± SEM) of the hemorrhagic lesions was similar between strains (1.8 cm ± 0.3 cm for B10.D2/o; and 1.7 cm ± 0.3 cm for AKR). Data are pooled from two or more independent experiments, each containing a positive control group (C3H/HeN mice injected with *C. parvum* and TNF).

Hemolytic Activity of Plasma Is Not Needed to Reconstitute C5-deficient Mice to Respond with HN. Since C5-deficient mice have a selective genetic defect in their ability to secrete C5 (19, 24, 25), while having normal levels of all other complement components, two possible pathways involving C5 in the pathological process of HN could be proposed: (a) C5 could be activated locally and the active cleavage product C5b could trigger the assembly of the membrane attack complex (C5b-9) which, in the presence of leukocytes and TNF, could cause direct vessel damage leading to HN; or (b) C5 could be activated locally and the C5-derived fragment C5a, together with TNF, could interact with infiltrating leukocytes to induce the secretion of hydrolytic enzymes, reactive oxygen intermediates, and/or vasoactive substances which ultimately cause vessel damage and HN.

Zymosan can deplete plasma of many of the hemolytic components of complement while leaving the anaphylatoxins, C3a and C5a, behind (26). Table III shows that plasma depleted of detectable hemolytic activity was still completely capable of reconstituting C5-deficient B10.D2/o mice for the induction of HN by *C. parvum* and TNF. In fact, C5 activity of zymosan-activated plasma was depleted by 96% as measured in vitro by a hemolytic assay specific for C5 (Table III). These data suggest that the terminal complement components (membrane attack complex) were not needed for HN to occur. The data are consistent, however, with the notion that C5a may be the missing component required for C5-deficient mice to develop HN.

Partially Purified C5a and TNF Can Cause HN in C. parvum-treated C5-deficient

TABLE III
*C5-sufficient Plasma Depleted of Hemolytic Activity Can Reconstitute
 C5-deficient Mice to Respond with HN*

Treatment of plasma in vitro*	Plasma hemolytic activity [†]	Reduction of plasma C5 activity [‡]	Incidence of HN (%) [§]
	U/ml	%	
4°C	165 ± 15	0 ± 0	5/6 (83)
37°C	103 ± 23	51 ± 6	7/8 (88)
37°C + Zymosan	<50 ± 0	96 ± 2	9/11 (82)

* C5-sufficient plasma was either kept at 4°C, incubated for 2.5 h at 37°C, or incubated at 37°C with 5 mg/ml zymosan for 2.5 h followed by removal of zymosan by centrifugation.

† Hemolytic activity of plasma was determined using a standard rabbit erythrocyte hemolysis assay. CH₅₀ values of each sample are reported as U/ml ± SEM.

‡ Represents the percent reduction in C5 activity as measured by the capacity of treated plasma to reconstitute hemolytic activity in C5-deficient plasma. Data are reported as percent reduction in CH₅₀ from control ± SEM. For details see Materials and Methods.

§ B10.D2/o mice were co-injected with *C. parvum* (400 µg) and TNF (10 µg) 3 h before the start of the experiment and then reconstituted with plasma as described in the footnote of Table II. HN was assessed 24 h later and reported as the number of mice responding with HN per total number of mice tested. The mean diameter (± SEM) of the hemorrhagic lesions was similar between the groups (1.6 cm ± 0.1 cm). Data are pooled from three independent experiments.

Mice. Since many molecules other than C5a are present in zymosan-activated, C5-sufficient plasma, we attempted further purification of the active component in zymosan-activated plasma which could reconstitute C5-deficient mice. Thus, zymosan-activated, C5-sufficient and -deficient plasma were acid precipitated to remove unwanted plasma proteins without affecting the acid soluble C5a (13). The supernatant was dialyzed against 0.1 M ammonium formate and fractionated through a Bio-Gel P60 column (Fig. 4). Acid-soluble plasma proteins >60,000 *M_r* eluting in the void volume had no activity. Pooled smaller sized fractions were tested for their ability to reconstitute C5-deficient mice to develop HN after injection of *C. parvum* and TNF. Fig. 4 and Table IV show that only a pool of fractions corresponding to proteins within the molecular weight range of 8,000–15,000 could reconstitute C5-deficient mice for the induction of HN. Further analysis of single fractions within this size range revealed that the activity corresponded to a protein with a *M_r* of 9,000 (Fig. 4, Table IV). Similar fractions tested from the zymosan-activated C5-deficient plasma did not reconstitute treated mice. The molecular weight of this acid-stable protein is consistent with published reports of the molecular weight of rat, guinea pig, porcine, and human C5a, which ranges from 8,000 to 15,000 (27, 28). These data further implicate C5a in the induction of HN by *C. parvum* and TNF.

Discussion

This paper shows that TNF requires the fifth component of complement in order to induce hemorrhagic necrosis in skin exposed to bacterial agents. This is supported

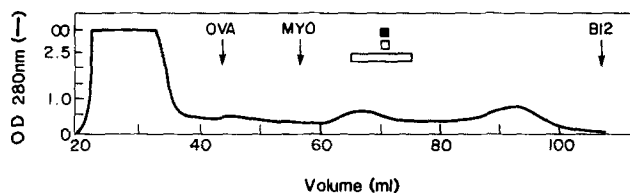


FIGURE 4. Gel filtration chromatography of acidified zymosan-activated plasma from C5-sufficient mice. Column fractions were collected and monitored for total protein at 280 nm. Elution volumes of low molecular weight standards are shown. The molecular weights of ovalbumin (*OVA*), myoglobin (*MYO*) and vitamin B12 (*B12*)

are 44,000, 17,000, and 1,350, respectively. Protein that eluted at a volume corresponding to a M_r of 9,000 reconstituted C5-deficient mice to develop HN when injected with *C. parvum* and TNF (filled bar). The open bars represent the relative location of the active single and pooled fractions tested from two independent gel filtrations, thus confirming this M_r .

by several lines of evidence: First, mouse strains that do not have C5 do not develop HN after injection of TNF and the bacterial agent into skin (Table I, Figs. 1 and 2). Second, plasma from C5-sufficient mice corrected the defect in C5-deficient mice (Table II). Third, heating plasma at 56°C for 30 min inactivated its capacity to reconstitute the C5-deficient mice, suggesting that complement is the critical component in the plasma (Table II). Fourth, C5V-treatment of mice, known to deplete complement in vivo, abrogated the ability of C5-sufficient mice to respond.

C5 is known to mediate several biological functions associated with complement activation: anaphylactic, chemotactic, and leukocyte-activating functions, which require C5a (M_r of 8,000–15,000) and hemolysis, which requires C5b (M_r of 160,000). We found that plasma activated with zymosan to enrich for C5a and acidified to deplete C5 was still fully capable of reconstituting C5-deficient mice. As expected, such plasma did not cause hemolysis in the presence of appropriate antibody in an in vitro assay. Finally, only the plasma fraction corresponding to the size range of C5a reconstituted C5-deficient mice. These findings indicate that C5a and not the membrane attack complex is required for HN to develop when TNF is injected into

TABLE IV
*C5-deficient Mice Reconstituted with a Partially Purified
9,000 M_r Protein Respond with HN*

Elution volume <i>ml</i>	M_r	Incidence of HN (%)*
65–75	11,500–8,000	4/5 (80)
68	10,000	0/4 (0)
71	9,000	2/4 (50)
75	8,000	0/4 (0)
78	7,000	0/4 (0)
78–100	7,000–4,000	0/3 (0)

* B10.D2/o mice were reconstituted with column fractions as described in the footnote of Table II. Data are pooled from two independent experiments, each containing as positive control C5-deficient B10.D2/o mice co-injected with *C. parvum* and TNF followed by reconstitution with unfractionated C5-sufficient plasma.

skin exposed to bacteria. Our studies do not exclude the fact that earlier complement components participate in the development of TNF-mediated HN. In fact, C3 is normally required for the generation of C5a and the lower responsiveness of mouse strains that have reduced levels of C3 (but are sufficient in C5) is consistent with this. Interestingly, the serum level of C3 is linked to a gene within the MHC complex (16), which is also where the TNF gene is located (29, 30).

Although C5a is required for the formation of TNF-mediated HN in skin exposed to bacterial agents, it is likely that the much more stable derivative of C5a, C5a-des-arg, is the actual molecule involved *in vivo* since the terminal arginine residue of C5a is quickly cleaved off in the serum by carboxypeptidase N (31). In humans, this derivative is greatly reduced in its biological activity both *in vivo* and *in vitro* and it has completely lost spasmogenic activity (32). In contrast, rodent C5a-des-arg retains much of the original activity of the parental C5a molecule (27). It is possible, therefore, that C5a-des-arg behaves the same way as C5a in the HN response described here. At present, we do not know whether this apparently unique difference between mouse and human C5a may explain our inability to reconstitute C5-deficient mice with human plasma (Rothstein, J. L., unpublished observation).

At present, we do not know through which mechanism C5a participates in the development of HN. Even though C5a is a potent leukocyte chemoattractant, C5-deficient and C5-sufficient mouse strains still showed very similar leukocyte infiltrates after injection of bacteria. Therefore, it is unlikely that the defect of C5-deficient mice is related to such a role for C5a. Alternatively, C5a, known to cause vessel leakage, might directly damage vessels to cause extravasation of blood. However, this is unlikely since C5a alone when injected intravenously or subcutaneously does not cause HN. Therefore, it is unlikely that C5a is the ultimate mediator of the tissue damage resulting in the development of HN. Alternatively, C5a and TNF may be two signals that are required *in vivo* for infiltrating leukocytes to release as yet undefined products that are ultimately the mediators of vessel disruption and hemorrhage. *In vitro* models are consistent with the idea that, in addition to TNF, a second signal is required for the release of mediators from neutrophils (33, 34). We do not know the precise role of the bacterial agent in the development of HN. It appears that the bacterial component is critical for attracting inflammatory leukocytes. However, this may not be the only role bacteria play since LPS-resistant mice that are complement sufficient have normal leukocyte infiltrates after injections of LPS but do not develop HN after a subsequent injection of TNF (10). In these mice, therefore, it is possible that LPS attracts but does not activate leukocytes to become responsive to mediator release by C5a and TNF.

The HN described here has many similarities with one form of the Shwartzman phenomenon, the localized Shwartzman reaction. It is characterized by the development of localized HN after a sensitizing and eliciting dose of bacterial agents (35). It is interesting, in this regard, that C6-deficient rabbits responded with HN to repeated endotoxin injections which suggests that C5b-9 assembly was also not required for the localized Shwartzman reaction (36). Other experiments show that anti-C3 antiserum or C566 prevented the development of the localized Shwartzman reaction (37, 38). Even though these early experiments did not identify C5a as a critical component in the development of HN, these findings are certainly consistent with the possibility that C5a is required for the localized Shwartzman reaction. The other

form of the Schwartzman phenomenon, which is usually referred to as the generalized or systemic Schwartzman reaction, probably can account for septic shock, but interestingly, C5a may not be needed for the pathophysiological events leading to shock. For example, we found (Rothstein, J. L., and H. Schreiber, unpublished observations) that C5-sufficient and C5-deficient mouse strains are equally susceptible to the synergistic induction of systemic shock by intravenous injection of TNF and bacterial agents. Furthermore, C6-deficient rabbits are equal or more susceptible to the induction of the generalized form of the Schwartzman reaction (39). HN is also observed in solid tumors treated with TNF (1) but, in contrast to normal skin (10), TNF alone will cause this necrosis (1). To this end, it is interesting that tumors growing in C5-deficient mice are still susceptible to HN induced by TNF (Rothstein, J. L., and H. Schreiber, unpublished observations). It is possible that the tumors in these mice supply or elicit a signal similar to that given by C5a.

The disruption of capillaries in HN in the vicinity of bacteria may prevent rapid spread of bacteria or their products into the circulation; thus, HN may be part of an important local defense mechanism. Presumably, TNF would be produced by the activated macrophages that are known to be present in such lesions and the comparatively smaller amount of secreted TNF would result in microscopic rather than macroscopic lesions. The described local response involving C5a, TNF, and bacteria may provide one explanation for why C5-deficient mouse strains have increased susceptibility to certain infections. Such mice are known to be more susceptible to pneumococcal, staphylococcal, gonococcal, and mycoplasmal infections, although they appear to have intact mechanisms of chemotaxis (40). In addition, C5-deficient mice are more susceptible to infections by cryptococcus or candida and are slightly more susceptible than C5-sufficient mice to infection by intracellular bacteria and to viral infections such as influenza (40). Thus, we propose that C5a synergizes with TNF and leukocytes to cause local vessel disruption and hemorrhage; by this defense mechanism the spread of bacteria and toxins may be prevented.

Summary

TNF induces hemorrhagic necrosis (HN) when injected into skin exposed to bacterial agents but not when injected into normal skin. In this paper, we present several lines of evidence suggesting that TNF requires the fifth component of complement (C5) to induce HN in skin exposed to bacteria. First, mouse strains that do not have C5 did not develop HN after injection of TNF and bacteria into skin. Second, plasma from C5-sufficient mice could correct the defect in these C5-deficient mice. Third, heating at 56°C for 30 min inactivated the capacity of plasma to reconstitute C5-deficient mice. Fourth, C5E, which is known to inactivate complement, abrogated the capability of C5-sufficient mice to respond. Fifth, depleting plasma of hemolytic activity while generating C5a did not affect the capacity of the activated plasma to reconstitute C5-deficient mice. Finally, only the plasma fraction containing molecules of the size range of C5a reconstituted C5-deficient mice. These findings indicate that C5a and not the membrane attack complex is required for HN. Although we do not know through which mechanism C5a participates in the development of HN, we propose that the described HN response is related to a local defense mechanism in which TNF and C5a lead to the disruption of capillaries in the direct vicinity of bacteria. By this mechanism the rapid spread of bacteria or their products

into the circulation is prevented. Such a tissue response is consistent with the known higher susceptibility of C5-deficient mice to bacterial infections and provides a model with which to search for the multiple steps involved in this important local defense mechanism.

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