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Effect of Anti-Tumor Necrosis Factor Alpha Antibodies on Histopathology of Primary *Salmonella* Infections

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We reported that administration of anti-tumor necrosis factor alpha (anti-TNF-α) antibodies exacerbates the course of a *Salmonella* infection in both susceptible and resistant mice by preventing the suppression of bacterial growth in the reticuloendothelial system. In the present study, we evaluated the effect of in vivo neutralization of TNF-α on the histopathology of primary *Salmonella* infections. We show that in primary infections, the suppression of bacterial growth in the reticuloendothelial system coincides with granuloma formation in the spleen and liver. Administration of anti-TNF-α globulins on day −1 of salmonellosis affected neither the histological picture nor the course of the infection in the early stages of the disease (days 1 to 3), with splenic and hepatic lesions consisting mainly of polymorphonuclear leukocytes (PMNs); conversely, later in infection (days 3 to 7), the treatment inhibited the formation of granulomas. When the anti-TNF-α treatment was started well after the suppression of bacterial growth in the reticuloendothelial system and the formation of granulomatous lesions in the spleen and liver, a prompt relapse of the infection and regression of already established granulomas were seen. In anti-TNF-α-treated mice, salmonellae were found inside macrophages and PMNs and extracellularly in the necrotic tissue of the spleen, while in the liver the organisms were seen mainly in inflammatory mononuclear cells, resident Kupffer cells, and hepatocytes and occasionally in the extracellular compartment within necrotic lesions. The bacteria appeared most often in clusters, being morphologically intact when in the extracellular space or within hepatocytes, while undergoing various degrees of degeneration when inside phagocytes. The results suggest that TNF-α is required for granuloma formation in salmonellosis and that its neutralization does not completely abrogate the bactericidal activity of macrophages and PMNs. Salmonellae were observed to grow within both hepatocytes and phagocytes but were killed only in the latter.

Salmonellae are intracellular parasites which can grow in mononuclear cells as well as parenchymal nonphagocytic cells and can persist in polymorphonuclear phagocytes (PMNs) (2–5, 11).

The mechanisms of natural resistance and acquired immunity to *Salmonella* spp. are studied mainly with a mouse model using host-adapted strains able to cause systemic infections. In mice, lethal *Salmonella* infections progress rapidly, and the animals succumb when high bacterial numbers (ca. 10^8 CFU) are reached in the reticuloendothelial system (RES). In sublethal infections, bacterial growth is suppressed towards the end of the first week, resulting in a plateau phase. This phase is mediated by an input of radiation-sensitive bone-marrow-derived cells (10), is dependent on the presence of both tumor necrosis factor alpha (TNF-α) (16, 18, 23, 24) and gamma interferon (21, 23), and does not require T cells (10).

TNF-dependent granuloma formation is associated with resistance to *Mycobacterium bovis* BCG, *Listeria*, and *Schistosoma mansoni* infections in the mouse model (1, 7, 13). We showed previously that TNF-α is crucial to host resistance to *Salmonella* spp., being constantly required for the suppression of bacterial growth in the RES (plateau phase) (16, 19). Although some evidence for the presence of granulomas in the organs of *Salmonella*-infected mice has been provided (22), information regarding the immunological mechanisms involved in the formation of granulomatous lesions in sublethal salmonellosis is still lacking.

The aim of the present study was to investigate the effect of early and late administration of anti-TNF-α antibodies on the histopathology of primary *Salmonella* infections in the mouse model and the location (intra- or extracellular) and viability of the bacteria in the organs of the anti-TNF-α-treated mice.

MATERIALS AND METHODS

Mice. Female, innately *Salmonella*-resistant A/J $Ity^{r/r}$ and susceptible BALB/c $Ity^{s/s}$ mice were purchased from Harlan OLAC Ltd. (Blackthorn, Bicester, United Kingdom) and used when more than 8 weeks old.

Bacteria. The virulent strain *Salmonella typhimurium* C5 and *S. typhimurium* M525, a strain with intermediate virulence, have been described elsewhere (9). Aliquots of 37°C stationary-phase overnight cultures in tryptic soy broth (Oxoid) were snap frozen and stored in liquid nitrogen. For intravenous inoculation, a vial was thawed rapidly and diluted appropriately in phosphate-buffered saline (PBS). Animals were inoculated with 0.2 ml of bacterial suspensions in a lateral tail vein, and the inoculum was checked by tryptic soy agar pour plates.

Bacterial enumeration in organ homogenates. The mice were killed by cervical dislocation. The spleens and livers were removed aseptically and homogenized in a Colworth Stomacher (Seward) in distilled water (9). Viable counts were performed with pour plates of tryptic soy agar or by the droplet technique with a Colworth Droplette (Seward) as described previously (9).

Anti-TNF-α antibodies. Anti-TNF-α antiserum was raised in rabbits by immunization with recombinant murine TNF-α kindly provided by G. R. Adolf, Boehringer Ingelheim, Vienna, Austria, as described previously (24). Whole globulins were used (2 mg per dose) after 40% ammonium sulfate precipitation and dialysis against PBS. Control animals received a similar amount of normal rabbit globulins (Sigma, Poole, Dorset, United Kingdom). Two milligrams of globulins neutralized the biological activity of 3×10^4 U of recombinant TNF-α in the L929 cytotoxicity assay as described previously (19).

Histological sections. Five-micrometer-thick sections stained by hematoxylin-

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eosin were prepared from tissues fixed by immersion in 10% (vol/vol) formal saline and embedded in paraffin wax.

Electron microscopy and thin sections. Mice were anesthetized deeply with sodium pentobarbitone. A midline thoracotomy was performed, and a 20-gauge needle was inserted into the left ventricle. An incision was made in the left auricular appendage, and the mice were exsanguinated by perfusion with physiological saline containing final concentrations of 10 mM PIPES buffer [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 139 mM NaCl, 2.7 mM KCl, 19.4 mM glucose, 0.0075% mM polyvinylpyrrolidone with a molecular weight of 40,000 as a molecular weight colloid to minimize edema (8), and 1% procaine at pH 7.2. The perfusion was continued until the heart ceased beating, typically 1 to 2 min. Exsanguination was followed by perfusion with 50 ml of fixative containing 3% glutaraldehyde, 0.5% formaldehyde, and 2.5% polyvinylpyrrolidone with a molecular weight of 40,000 in 0.1 M PIPES buffer at pH 7.2. The spleens and livers were removed and cut into 100-μm slices with an Oxford Vibratome, and fixation was continued by immersion in the same fixative for 4 h at 4°C. Five slices from each organ were selected at random for further processing.

The fixed tissues were rinsed in 0.1 M PIPES buffer, osmicated, bulk stained, dehydrated in an ascending series of ethanol solutions, and embedded in Spurr's epoxy resin. Sections were cut at a nominal thickness of 1 μm with a Reichert Ultracut E, stained with methylene blue, and viewed with a Leitz Labourlux S. The same block faces were sectioned for transmission electron microscopy, and 50-nm sections were mounted on 300-mesh grids and stained with uranyl acetate and lead citrate.

RESULTS

Effect of early administration of anti-TNF-α antibodies on the histopathology of a primary *Salmonella* infection. A/J mice were injected intravenously with a sublethal dose (3×10^3 CFU) of *S. typhimurium* C5. One group of mice received 3×10^4 neutralizing units of anti-TNF-α antibodies on days −1 and +3 of the infection, and controls received normal rabbit globulins. Viable counts in spleen and liver homogenates from groups of six mice performed at intervals thereafter showed that CFU counts were similar in both groups until day 3, increasing to ca. 10^4 to 10^5 CFU per organ at a rate of approximately 0.5 $\log_{10}$/day. Thereafter, counts in the anti-TNF-α-treated mice increased to ca. 10^7 to 10^8, compared with ca. 10^5 in the controls. Bacterial counts were higher in treated mice by day 7 (Fig. 1).

The examination of 5-μm-thick hematoxylin-eosin-stained sections prepared from livers and spleens from groups of three mice on successive days after infection revealed that early administration of anti-TNF-α antibodies (day −1) did not affect the evolution of the histological picture of the spleen and liver during the early exponential bacterial growth in the RES (days 1 to 3), dramatic differences appearing later in the infection. On days 1 to 2 of the infection, the livers of both control and anti-TNF-α-treated mice showed small lesions containing few mononuclear cells located mainly in the sinusoids and surrounded by eosinophilic hepatocytes (Fig. 2a); no lesions could be observed in the spleens at this time. By days 3 to 4, the lesions in both spleens and livers consisted mainly of PMNs and moderate necrosis (Fig. 2b and 3a). Serial daily histopathological studies revealed that in the next stages of the infection (days 5 to 7), the lesions in control mice were invaded from the periphery to the center by mononuclear cells, and granulomatous lesions were observed by day 7 in both livers and spleens (Fig. 2c and 3b). At this time, the granulomatous lesions in the liver still contained PMNs, and few lymphocytes were seen. Later in infection (day 10), the lesions in controls became almost exclusively lymphomonocytic (data not shown).

Conversely, by day 5, the spleens of anti-TNF-α-treated mice showed macroscopically visible irregular pale lesions that increased in size and number until the death of the animals (days 7 to 8). Histology revealed wide patches of necrosis together with cellular depletion in the red and white pulp of the spleen. In the red pulp, the marked depletion of mononuclear cells resulted in a (proportional) manifest abundance of PMNs. Intravascular thrombosis was clearly visible in both small and large vessels (Fig. 3c). Granulomas were absent. The histological picture of the livers of the anti-TNF-α-treated mice revealed a few lesions consisting of a few mononuclear cells and PMNs that were not organized in granulomatous lesions, as they were in the livers of control mice, and which were surrounded by damaged or eosinophilic hepatocytes (Fig. 2d). Granulomas were absent, and necrosis was evident.

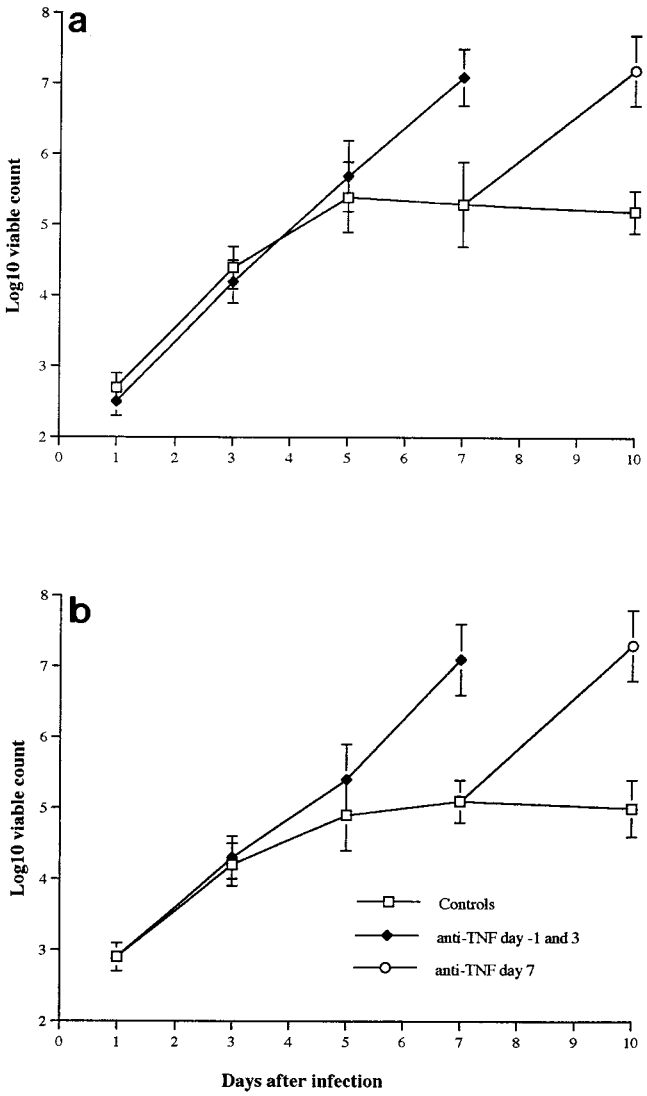


FIG. 1. Effect of early or late administration of anti-TNF-α antibodies on the course of a *Salmonella* infection in the spleens (a) and livers (b) of mice. A/J mice were injected intravenously with 3×10^3 CFU of *S. typhimurium* C5. One group of mice received 3×10^4 neutralizing units of anti-TNF-α antibodies on days −1 and +3 of the infection (closed diamonds). A second group received a similar amount of anti-TNF-α antibodies on day 7 of the infection (open circles). Controls received normal rabbit globulins (open squares). Results are expressed as $\log_{10}$ viable counts ± standard deviations of groups of six mice per point.

We have reported (19) that the anti-TNF-α treatment has a similar effect on the evolution of the infection in innately susceptible (*Ity*^{s}) BALB/c mice infected sublethally with moderately virulent salmonellae. In the present study, BALB/c mice were infected with ca. 500 CFU of *S. typhimurium* M525. The overall course of the infection and the evolution of the histological picture (results not shown) were similar to those observed in control and anti-TNF-α-treated A/J mice infected

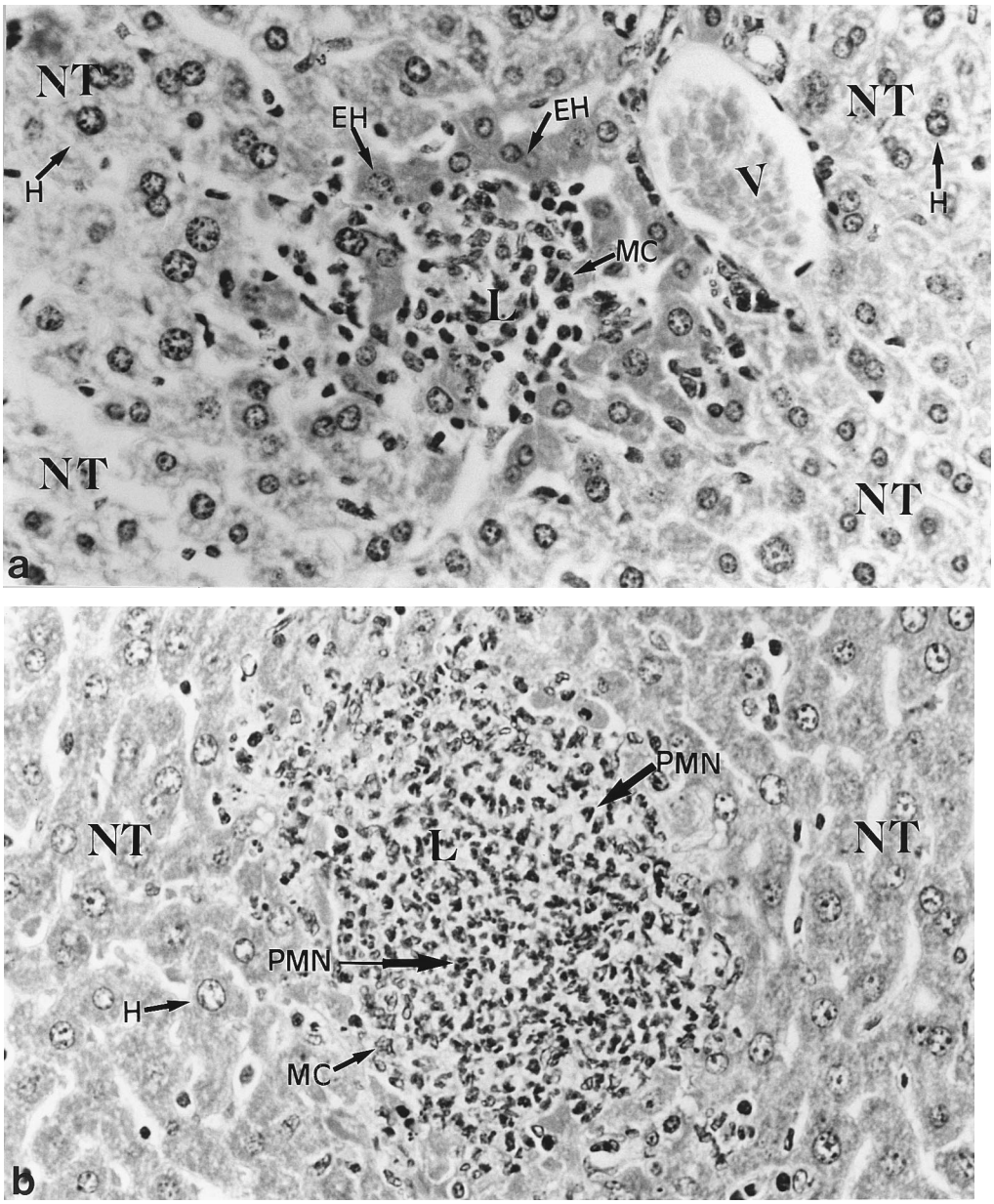


FIG. 2. Effect of anti-TNF-α antibodies on the histopathology of the livers of A/J mice during a primary sublethal infection with *S. typhimurium* C5. Similar results were obtained with BALB/c mice infected with *S. typhimurium* M525. (a) Day 1 controls or anti-TNF-α-treated mice. Magnification, ×200. (b) Day 4 controls or anti-TNF-α-treated mice. Magnification, ×200. (c) Day 7 controls. Magnification, ×400. (d) Day 7 anti-TNF-α-treated mice. Magnification, ×400. Abbreviations: NT, necrotic tissue; EH, eosinophilic hepatocytes; MC, mononuclear cells; L, lesion; V, venule; H, hepatocyte.

with the virulent C5 strain (Fig. 1). The anti-TNF-α treatment gave the expected exacerbation of the infection, preventing the formation of granulomatous lesions in the organs. Again, significant differences in bacterial counts and in the histological picture between controls and anti-TNF-α-treated mice appeared from day 4 onwards.

Thus, the anti-TNF-α treatment does not affect either spleen and liver CFU or the histological picture in the early phase of the infection regulated by the *Ity* gene (phase 2, days 1 to 4), but it prevents the establishment of the plateau and granuloma formation later in the infection (days 4 to 7).

Late administration of anti-TNF-α antibodies induced the regression of spleen and liver granulomas in primary *Salmonella* infections. A/J mice were infected as described above. Viable counts performed on days 5 and 7 revealed the expected suppression of bacterial growth in the RES and the formation of hepatic and splenic granulomas (Fig. 1).

One group of infected mice was injected with anti-TNF-α globulins on day 7 (after the suppression of bacterial growth and the formation of granulomas). Viable counts on day 10 confirmed the expected relapse of the infection (19) (Fig. 1). At this time, histology revealed the persistence of well-defined granulomatous lesions in the organs of control mice; the lesions observed on day 10 contained more lymphocytes than did lesions observed on day 7 (data not shown). At the same time (day 10), the histological picture in mice receiving anti-TNF-α globulins on day 7 showed widespread necrosis, mononuclear depletion, and thrombosis in the spleen and few necrotic le-

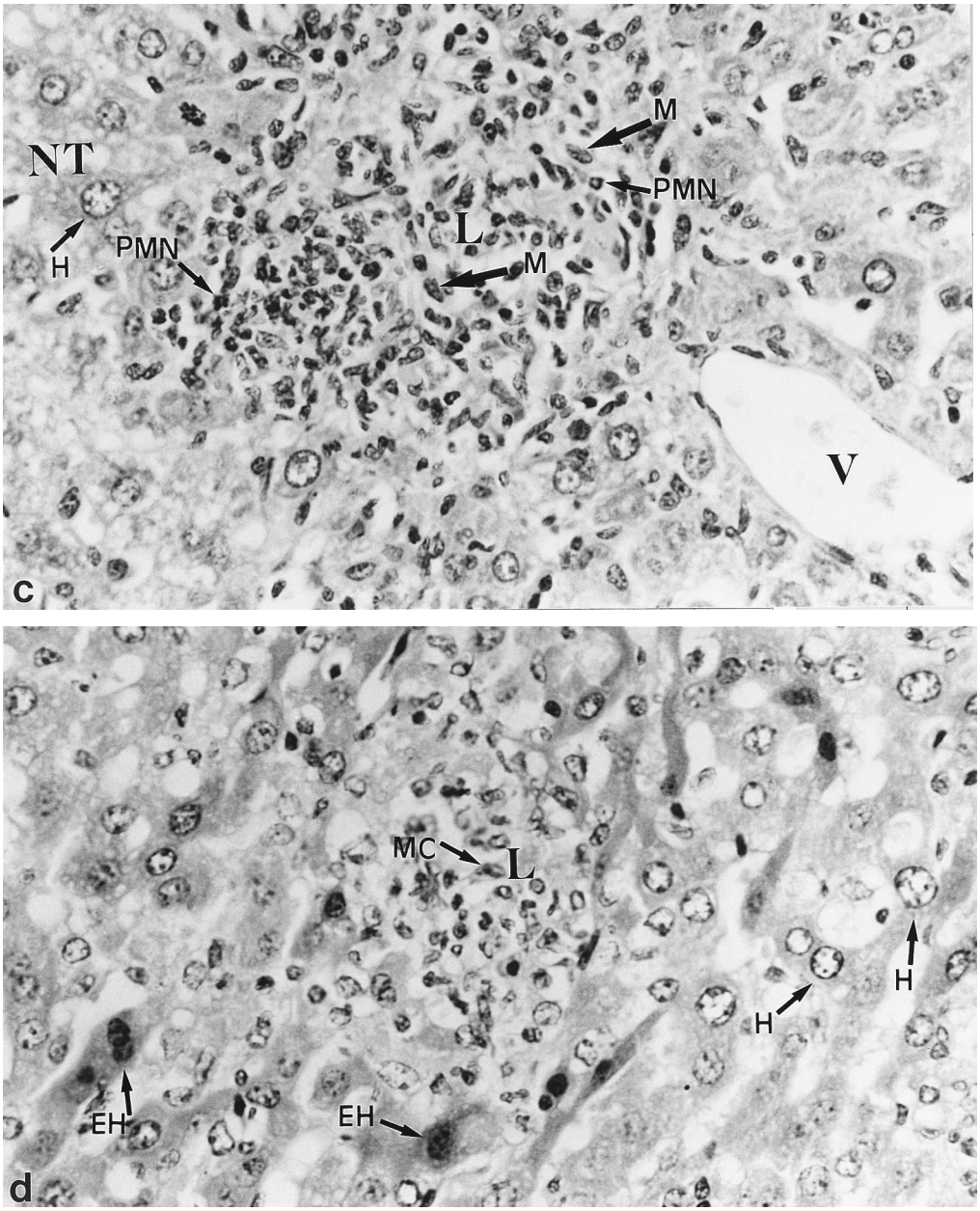


FIG. 2—*Continued.*

sions in the liver; the overall picture was reminiscent of the one observed after early administration of the anti-TNF-α globulins (Fig. 2d and Fig. 3c).

The histological picture observed in the organs of anti-TNF-α-treated A/J and BALB/c mice was, at any time of the infection, reminiscent of the one observed during the course of lethal infections (started with initial bacterial doses above the 50% lethal dose [LD_{50}]) performed with *S. typhimurium* C5 and *S. typhimurium* M525 in A/J and BALB/c mice, respectively.

Thus, late administration of anti-TNF-α antibodies (after the suppression of bacterial growth) induced a marked regression of already established granulomatous lesions and a relapse of the infection.

Presence of bacteria in the organs of anti-TNF-α-treated mice on day 7 of a sublethal primary infection. Individual bacteria were not visible in 5-μm-thick sections. However, they were visible in 1-μm-thick sections (data not shown) and in the 50-nm sections examined by transmission electron microscopy (Fig. 4).

A/J (*Ity*^{r}) mice were infected intravenously with a sublethal dose (10^3 CFU) of the virulent *S. typhimurium* C5. A group of six mice received 2×10^4 neutralizing units of anti-TNF-α antibodies intravenously 12 h prior to challenge and on day 3 of the infection. Controls received a similar amount (2 mg) of normal rabbit globulins. On day 7, one of the anti-TNF-α-treated mice was dead, one appeared very sick, and the remaining four appeared only moderately sick. Two of the latter

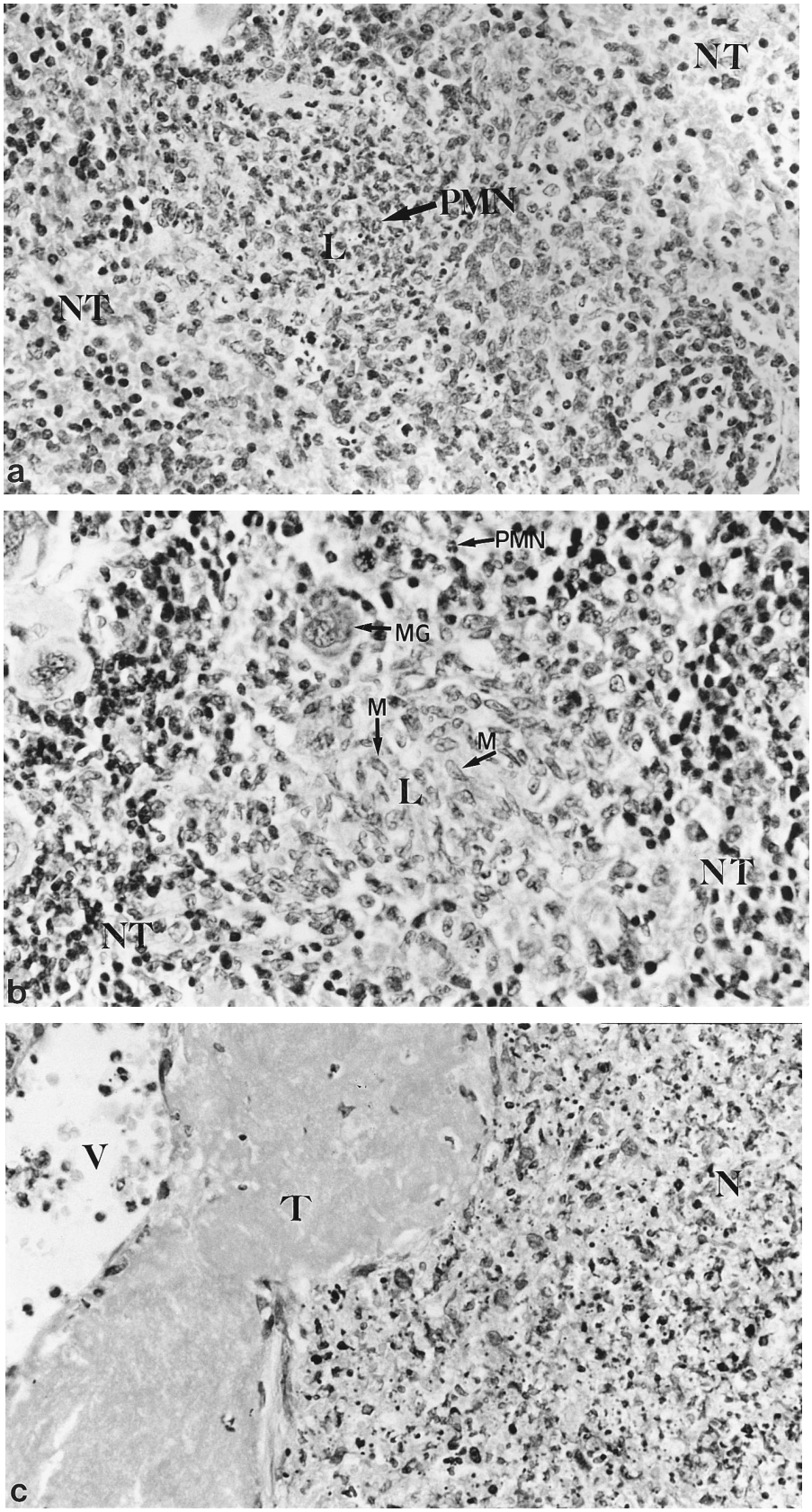


FIG. 3. Effect of anti-TNF-α antibodies on the histopathology of the spleens of A/J mice during a primary sublethal infection with *S. typhimurium* C5. Similar results were obtained with BALB/c mice infected with *S. typhimurium* M525. (a) Day 4 controls or anti-TNF-α-treated mice. Magnification, ×200. (b) Day 7 controls. Magnification, ×400. (c) Day 7 anti-TNF-α-treated mice. Magnification, ×200. Abbreviations: NT, necrotic tissue; L, lesion.

were killed, and their organs were processed for morphological studies. Spleen and liver counts were performed on the two remaining mice (which were moderately sick), and bacterial numbers of ca. 10^7 CFU per organ were detected. Preliminary studies with 1-μm-thick sections showed that most of the salmonellae in the liver of anti-TNF-α-treated mice were to be found within the resident Kupffer cells, PMNs, and hepatocytes (results not shown). Electron microscopy confirmed that in the livers of the anti-TNF-α-treated mice, most of the salmonellae were seen in Kupffer cells (Fig. 4a), PMNs (Fig. 4b and d), and degenerating hepatocytes located in close proximity to the sinusoids (Fig. 4c and d). The intracellular bacteria were seen rarely as single organisms and most commonly in small clusters. The majority of the organisms appeared to be intact when observed within hepatocytes, while bacteria often appeared in the process of degenerating when observed within PMNs or macrophages. The highest degree of degeneration was seen in bacteria within macrophages. The organisms were observed occasionally inside the few mononuclear cells present in the lesions described in the legend to Fig. 3d as well as extracellularly within these lesions (Fig. 4e).

In the spleens of the anti-TNF-α-treated mice, the bacteria were seen inside macrophages and PMNs in the red pulp and extracellularly in the areas where necrosis was more prominent. Bacteria were seen to grow in large colonies in the sinusoids associated with thrombi. No bacteria were present in intact white-pulp follicles. Again, extracellular bacteria were most often intact morphologically, while a large proportion of the intracellular organisms within macrophages and PMNs (often seen in clusters) appeared to be in the process of degenerating (data not shown).

Similar observations were made with A/J and BALB/c mice in the late stages of lethal infections performed with *S. typhimurium* C5 and *S. typhimurium* M525, respectively.

Anti-TNF antibodies do not affect spleen and liver histology in already-lethal infections. A/J and BALB/c mice were lethally infected with ca. 10^5 CFU of *S. typhimurium* C5 and *S. typhimurium* M525, respectively. In either case, one group of mice received two injections of anti-TNF globulins on days −1 and +3 of the infection. As expected (16), the anti-TNF treatment did not affect the course of the rapidly lethal infections, i.e., in mice that were sick on day 4 as a result of high bacterial loads in the liver and spleen (ca. 10^7 CFU per organ). No histological differences were observed between control and anti-TNF-treated mice. In both groups, histology revealed multiple necrotic foci with degenerating PMNs in the spleen and liver, with few mononuclear cells. The lesions were similar to the ones described in Fig. 2d and 3c, but necrosis was more evident.

DISCUSSION

The present results show that the exacerbation of a *Salmonella* infection observed in mice treated with anti-TNF-α antibodies is due to an increase in bacterial numbers accompanied by a lack of granuloma formation and extensive growth of bacteria predominantly within macrophages, PMNs, and hepatocytes. Bacteria were also seen growing extracellularly more often in the spleen and seldom in the liver. Salmonellae were often in clusters and appeared morphologically unaltered when in the extracellular space or within hepatocytes, whereas both healthy and degenerating bacteria (also in clusters) were seen within phagocytes.

With anti-TNF-α-treated mice, we observed a striking reduction in the presence of mononuclear cells in the liver and spleen and a severe impairment in granuloma formation. We believe that in the absence of biologically active TNF-α, the reduced recruitment of mononuclear cells to the RES is one of the causes of the unrestrained bacterial growth in the organs. Macrophages are in fact essential for the suppression of bacterial growth in mouse typhoid (plateau phase), which seems to be mediated by a localized response (probably granuloma formation) rather than a systemic response (15). The plateau phase coincides with an increase in spleen and liver weight, requires an influx of radiation-sensitive bone-marrow-derived cells, and can be transferred adoptively by macrophages but not by T cells (10, 12a, 15). TNF-α-mediated enhancement of the influx of monocytes in the organs due to increased expression of adhesion molecules on the vascular endothelium (6) and to a chemotactic effect (20) has been reported.

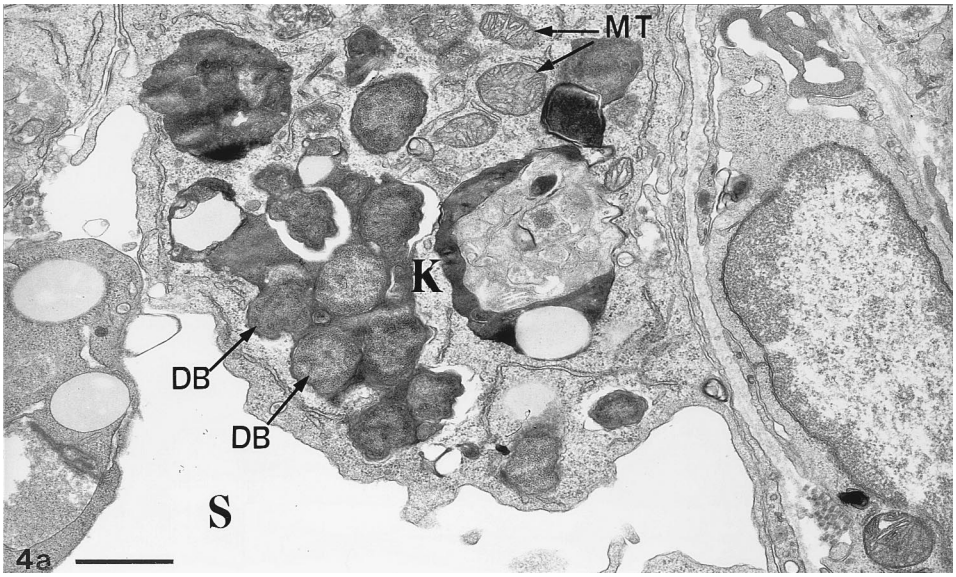
TNF-α neutralization in *Salmonella*-infected mice did not prevent the migration of PMNs in the organs in the early stages of infection. The role of TNF in PMN migration in the tissues is controversial, the cytokine having been implicated in the margination of PMNs but less clearly in migration and chemotaxis (6). In our model, it appears that TNF-α is not required for the migration of PMNs in the livers and spleens of infected mice.

With anti-TNF-α-treated mice and lethally infected mice, we observed clusters of intracellular bacteria in macrophages, PMNs, and hepatocytes. Direct evidence for the presence of bacteria in all of the cell types mentioned above has been provided, but the ability of *Salmonella* spp. to grow within phagocytes has been questioned (11, 12). It is now clear that salmonellae can grow inside hepatocytes (12). Our observation of clusters of intracellular bacteria in macrophages and PMNs indicates that intracellular bacterial growth occurs not only in parenchymal cells but also in phagocytes. In fact, the presence of heavily infected phagocytes near uninfected phagocytes is most likely to be the result of intracellular growth rather than uptake of high numbers of organisms by selected phagocytes within the organs.

Whether the presence of salmonellae in parenchymal cells is due to the high bacterial numbers reached in the organs during a lethal infection or whether salmonellae would normally grow in hepatocytes during the course of a sublethal infection with low bacterial numbers remains to be established; unfortunately, bacteria are difficult to locate unless they are present in high numbers in the tissues. Noticeably, with our model (with a low initial inoculum and TNF-α neutralization or an initial bacterial challenge above the LD_{50}), we observed that intracellular growth of salmonellae was damaging to hepatocytes. Infected hepatocytes often appeared dramatically affected by the presence of intracellular organisms.

The mechanisms by which hepatocytes degenerate require further investigation. We often observed *Salmonella*-infected PMNs in close proximity to the degenerating hepatocytes, a picture observed also by other authors who envisaged the lysis of *Salmonella*-infected hepatocytes by phagocytes as a mechanism of early resistance (3). If this is the case, TNF-α does not seem to be involved in the latter phenomenon since they still occur in anti-TNF-α-treated mice. Heavily infected degenerating hepatocytes were seen in areas devoid of PMNs, indicating that the degeneration of liver parenchymal cells is not always strictly dependent on phagocytes but can also be due to the presence of high numbers of bacteria.

The intracellular bacteria within hepatocytes were intact morphologically, while both healthy and degenerating bacteria were seen within PMNs and macrophages. Under our experimental conditions, despite the fact that the bactericidal activity of (resident) phagocytes is not abrogated (degenerating bac-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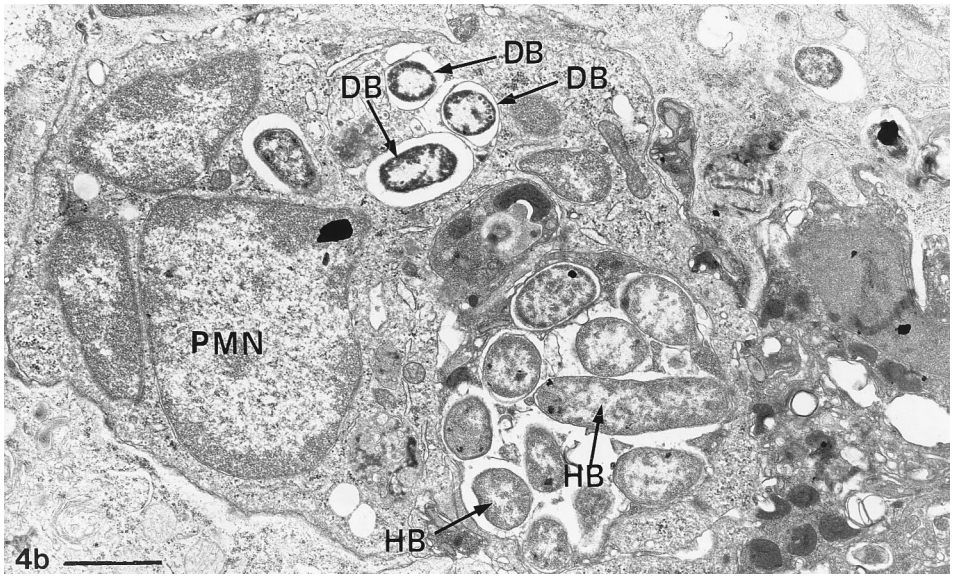
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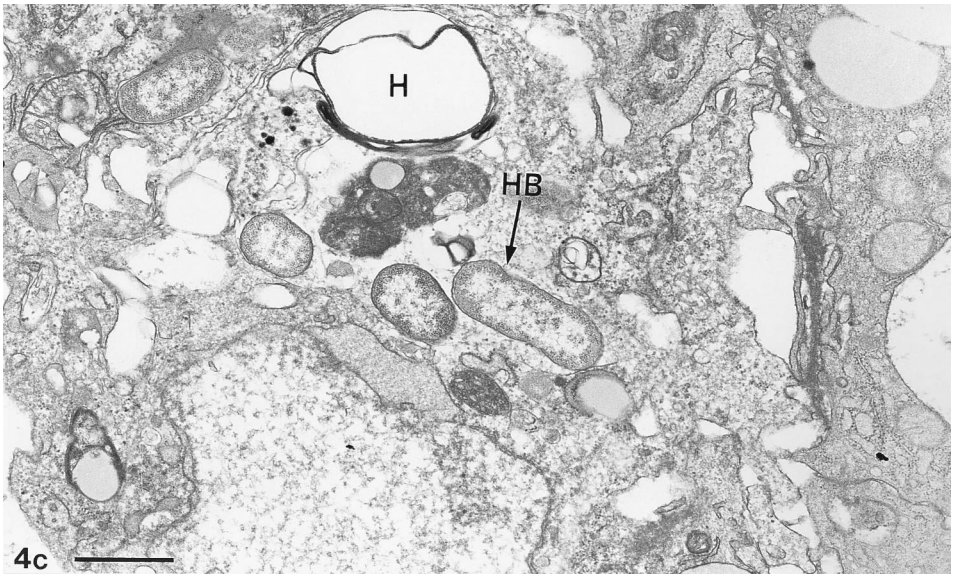
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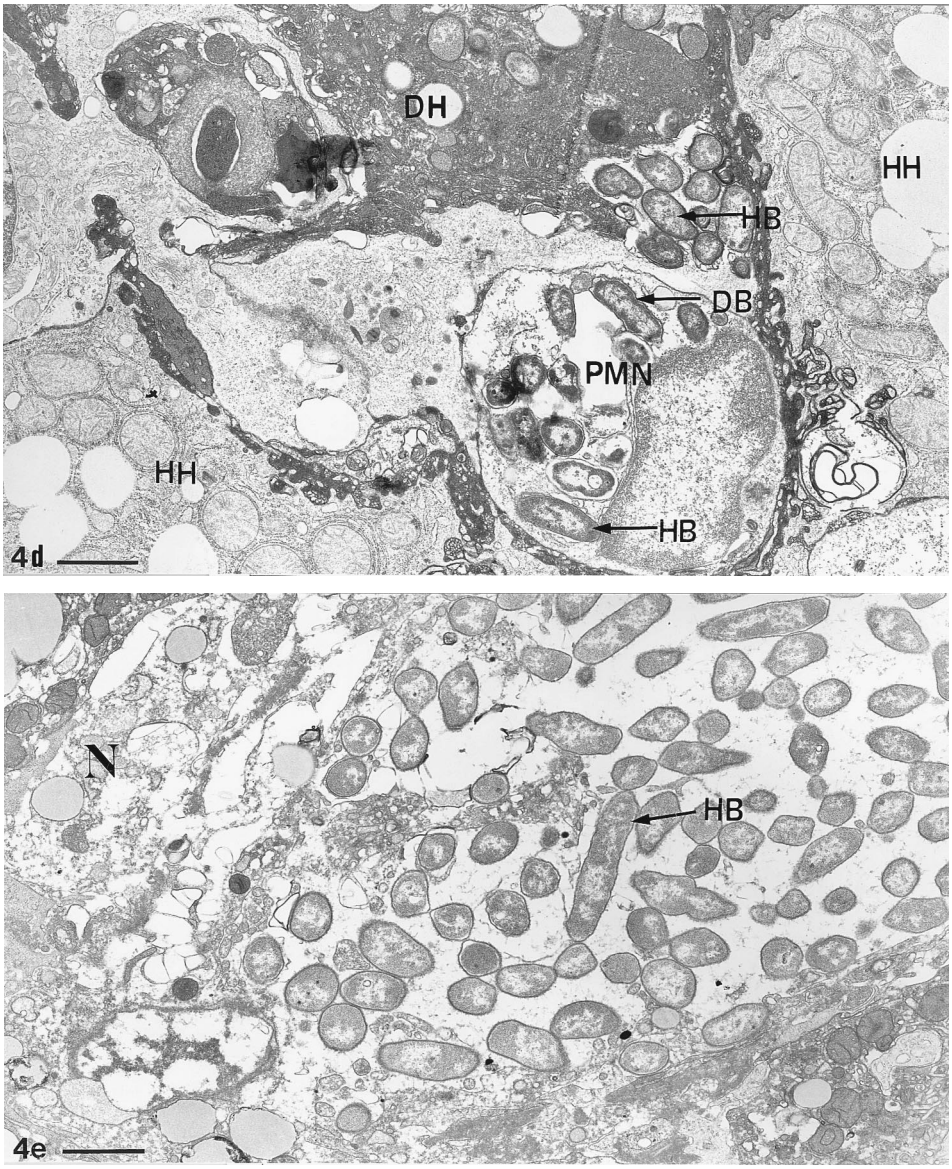

FIG. 4. Transmission electron micrographs demonstrating the location of salmonellae in the livers of anti-TNF-α-treated mice on day 7 postchallenge. (a) Degenerating bacteria within a Kupffer cell underlying a capillary sinusoid. Magnification, ×13,000; bar, 1 μm. (b) A small cluster of bacteria within a PMN. Magnification, ×13,000; bar, 1 μm. (c) Bacteria within the cytoplasm of a hepatocyte showing moderate signs of degeneration. Magnification, ×13,000; bar, 1 μm. (d) Bacteria visible within the cytoplasm of a PMN and also within the cytoplasm of an adjacent hepatocyte demonstrating the features of extreme degeneration. Magnification, ×11,000; bar, 1 μm. (e) Bacteria within the acellular core of a necrotic lesion. Magnification, ×11,000; bar, 1 μm. Abbreviations: K, Kupffer cell; DB, degenerating bacteria; S, sinusoid; H, hepatocyte; DH, degenerating hepatocyte; N, necrosis; MT, mitochondria; HB, healthy bacterium; HH, healthy hepatocyte.

teria were seen inside phagocytes), bacterial growth in the organs still proceeds in an unrestrained manner. This further suggests that, in the absence of TNF-α, it is the lack of recruitment of inflammatory macrophages rather than the impairment of bacterial killing by phagocytes which accounts for the exacerbation of the disease.

Salmonellae were seen to grow extracellularly more often in the spleen than in the liver. The presence of extracellular salmonellae in the splenic tissue is not surprising given the severe impairment of the cellularity of the organ, which leads to widespread necrosis. The extracellular bacteria were not a recurrent finding in the liver. It must be assumed that, since the bulk of salmonellae in the liver are seen inside the Kupffer cells and in the hepatocytes in close proximity to the sinusoids, the eventual lysis of the phagocytes would release the organisms directly into the bloodstream to be carried to distant sites, being captured eventually by other sinusoid-lining phagocytes. Noticeably, the extracellular bacteria in the liver were in the necrotic areas within the lesions, deep in the hepatic tissue.

TNF-α is known to be involved in the immunopathology of infection and inflammation (reviewed in reference 17) as well as in the physiopathology of acute, lethal, gram-negative infections. In our study, necrosis, edema, thrombosis, and PMN degeneration occurred in the livers and spleens of anti-TNF-α-treated mice. These preliminary observations suggest that the cytokine is not required for the development of the histological picture observed in mice that die of a rapidly evolving *Salmonella* infection.

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