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Defense Mechanisms in Peyer's Patches and Mesenteric Lymph Nodes against *Yersinia enterocolitica* Involve Integrins and Cytokines

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Adhesion molecules and cytokines are involved in regulation of cellular host responses in infection processes. In this study the roles of the integrins Mac-1 and VLA-4, as well as those of the cytokines tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), in defense mechanisms against *Yersinia enterocolitica* in Peyer's patches (PP) and mesenteric lymph nodes (MLN) were investigated by blocking these molecules with antibodies in vivo prior to orogastric *Yersinia* infection. Intestinal *Yersinia* infection caused abscesses composed of polymorphonuclear (Mac-1$^+$ VLA-4$^-$ Pgp-1$^+$ ICAM-1$^-$) and mononuclear (Mac-1$^+$ VLA-4$^+$ Pgp-1$^+$ ICAM-1$^+$) phagocytes and formation of MAdCAM-1$^+$ venules in PP and MLN. Blocking of Mac-1 or VLA-4 (i) inhibited phagocytosis of yersiniae by macrophages, (ii) reduced *Yersinia*-specific proliferation and IFN-γ production of T cells from PP and MLN, and (iii) caused increased bacterial growth in PP and MLN followed by profound tissue destruction. Neutralization of TNF-α or IFN-γ had comparable effects, suggesting that cell-mediated host responses including activated macrophages are required for control of yersiniae in intestinal tissues. The number of Mac-1$^+$ cells in PP and MLN increased after yersinia infection, and recruitment of these cells was not blocked by administration of anticytokine or anti-integrin antibodies. While anti-VLA-4, -TNF-α, or -IFN-γ antibody treatment caused an increased dissemination of yersiniae from PP to the spleen, systemic dissemination was reduced by anti-Mac-1 antibodies. The results of this study suggest that the cytokines IFN-γ and TNF-α as well as the integrins Mac-1 and VLA-4 are involved in protective cellular host defense mechanisms in PP and MLN against *Y. enterocolitica*, the latter probably being involved in both cell-cell and cell-pathogen interactions.

Yersinia enterocolitica is enteropathogenic for humans and rodents (22, 27, 29). Infection with this pathogen causes a wide range of clinical manifestations including enteritis, enterocolitis, and mesenteric lymphadenitis (18, 27). Furthermore, systemic infection with abscesses in spleen and liver as well as immunopathological sequelae such as reactive arthritis can occur (2, 3, 18, 19). A number of virulence factors of *Y. enterocolitica* which are encoded by a virulence plasmid (e.g., YadA and Yops) and by the chromosome (e.g., Ail and Yersiniabactin) mediate functions such as resistance against phagocytosis, complement lysis, and iron uptake and thus promote extracellular survival of *Y. enterocolitica* in infected host tissue (27, 28, 58, 79).

In the experimental mouse infection model it was shown that *Y. enterocolitica* binds to and invades the follicle-associated epithelium (FAE) of the Peyer's patches (PP), which are a part of the gut-associated lymphoid tissue (GALT) (6, 41, 44). Subsequently, this process leads to pyogenic lesions and production of interleukin-1 in PP (6, 13). Previous studies focusing on defense mechanisms against parenteral *Yersinia* infection have implicated both T cells and macrophages, including the cytokines tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin-12, as essential components in protective host responses against this pathogen (5, 8, 10, 17). In contrast, there are practically no data available about the mechanisms of local host defense in PP upon intestinal *Yersinia* infection.

Integrins are heterodimeric adhesion molecules which are involved in (i) binding of cells to extracellular matrix proteins, (ii) cell-cell adhesion processes, including binding of leukocytes to inflamed endothelial cells, and (iii) cell migration processes (76, 77). In addition, integrins are involved in interactions of host cells with microorganisms including, e.g., *Y. enterocolitica*, *Bordetella* spp., *Escherichia coli*, and *Borrelia burgdorferi*, although it is not yet clear for most of them whether this interaction is of significance during the infection process (53).

Yersiniae can bind to β1 integrins and thereby invade host cells (16, 50, 51). The Inv (invasin) protein of *Y. enterocolitica* accounts for this interaction and binds to the same integrin site as do host-synthesized integrin-ligands (34, 51). Moreover, Inv provides costimulatory activity for T cells in vitro by binding to α4β1 (VLA-4) integrin (21, 34), which is involved in cell trafficking to sites of inflammation by interaction with VCAM-1 on endothelial cells (42, 47, 60, 76). However, whether the interaction between Inv and VLA-4 is of significance during the infection process in vivo remains unclear.

The αMβ2 integrin Mac-1 (complement receptor 3) is expressed on phagocytes and plays an important role in binding to and phagocytosis of as well as killing of bacteria such as streptococci, listeriae, mycobacteria, and salmonellae (4, 32, 33, 45, 52, 60, 67, 72). Further, Mac-1 binds to ICAM-1 and mediates adhesion of monocytes to endothelial cells, which is required for the recruitment of phagocytes into inflamed tissues (60, 76, 77). Interestingly, the expression of Mac-1 and VLA-4 integrins as well as their ligands ICAM-1 and VCAM-1, respectively, is inducible by inflammatory mediators, including TNF-α and IFN-γ (54, 59, 76).

Because phagocytes are apparently involved in the host response against *Y. enterocolitica* in PP and because many functions of phagocytes are mediated by cytokines and integrins, the latter of which may even directly interact with yersiniae, we

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TABLE 1. Monoclonal antibodies used in this study

Target	Cell type	Hybridoma	Isotype	Reference(s) or source
IFN-γ		R4-6A2	Rat IgG1	75
		AN18.17.24		73
T cells	CD3-ε	145-2C11	Hamster IgG	56
Helper T cells	CD4	GK1.5	Rat IgG2b	82
Cytotoxic T cells	CD8a	53-6.72	Rat IgG2a	55
Mac-1 antigen on monocytes, macrophages, granulocytes, NK cells	CD11b/CD18	5C6	Rat IgG2b	69
		M1/70.15.11	Rat IgG2b	48
Pgp-1 on prothymocytes, memory T cells	CD44	IM7.8.1	Rat IgG2b	57
VLA-4 on T cells, thymocytes, monocytes, B cells	CD49d/CD29	PS2.3	Rat IgG2b	43, 49
ICAM-1 on monocytes, lymphocytes	CD54	YN1/1.7.4	Rat IgG2b	80
LFA-1 on myeloid and lymphoid cells	CD11a/CD18	FD441.8	Rat IgG2b	31
MAdCAM-1 on venules of mucosal tissues		MECA 367		63
Endothelial cells, smooth muscle cells		9F1		A. Hamann

wanted to investigate the roles of the integrins Mac-1 and VLA-4 as well as the cytokines TNF-α and IFN-γ in the protective host response in PP and mesenteric lymph nodes (MLN). For this purpose anti-integrin or anticytokine antibodies were injected into mice prior to orogastric *Y. enterocolitica* infection in order to block the functions of these molecules in vivo. Our results suggest that both TNF-α and IFN-γ are essential mediators of the protective host response in the GALT against yersiniae and that Mac-1 and VLA-4 are involved in cell-cell and cell-pathogen interactions rather than in migration of phagocytes into infected intestinal tissue.

MATERIALS AND METHODS

Mice. Six- to eight-week-old C57BL/6 mice were purchased from Charles River Wiga (Sulzfeld, Germany) and kept under specific-pathogen-free conditions in positive-pressure cabinets. Mice were provided with sterile food and water ad libitum.

Bacteria and infection of animals. Plasmid-harboring *Y. enterocolitica* WA-314 of serotype O8 (46) was passaged in mice and grown as recently described (5). Mice were orogastrically infected by injection, through a gastric tube, of 200 μl of a suspension containing 1×10^8 to 5×10^8 yersiniae. Mice were starved for 16 h prior to and for 4 h after the infection. The 50% lethal dose of the orogastric infection was 2×10^8 *Y. enterocolitica* bacteria. The actual number of administered bacteria was controlled for each experiment by plating 200 μl of serial dilutions of the suspension on Mueller-Hinton agar and counting the CFU after an incubation at 28°C for 36 h. The mice were weighed prior to and every day after the infection. At 5 days after infection, mice were killed and the spleen and the MLN of each mouse were aseptically removed. The entire small intestine was removed and extensively washed with cold phosphate-buffered saline (PBS), pH 7.4, containing 100 μg of gentamicin per ml and thereafter was washed with PBS to remove bacteria associated with the mucosal surface of the PP. Penetration of gentamicin into PP tissue was excluded by using a bioassay with *Bacillus subtilis* (data not shown). Then, the PP for each mouse were carefully excised and pooled. The number of bacteria present in infected PP, MLN, and spleens was determined by homogenization of these organs in PBS containing 0.1% Tergitol TMN 10 (Fluka, Buchs, Switzerland) and 0.1% bovine serum albumin (Merck, Darmstadt, Germany) and plating serial dilutions of the homogenates on cefsulodine-Irgasan-novobiocin agar as described above. The limit of detection was 25 CFU. All experiments were performed at least three times and revealed comparable results.

Antibodies. The nomenclature and recognized target structures of the antibodies used in this study are listed in Table 1. Antibodies used included the following: anti-IFN-γ (R4-6A2 and AN18.17.24); anti-CD3 (145-2C11); anti-CD4 (GK1.5; Becton Dickinson); anti-CD8 (53-6.72; Becton Dickinson); anti-CD11b/CD18 (Mac-1 or complement receptor 3; M1/70.15.11 and 5C6 [5C6 hybridoma was kindly provided by H. Rosen, MSD Research Laboratories]); anti-CD44 (Pgp-1; clone IM7.8.1; hybridoma cells were kindly provided by T. Hünig, Würzburg, Germany); anti-CD49d/CD29 (VLA-4; clone PS2.3; hybridoma cells were kindly provided by A. Hamann, Hamburg, Germany); anti-CD54 (ICAM-1; YN1/1.7.4); anti-CD11a/CD18 (LFA-1; FD441.8); anti-MAdCAM-1 (MECA 367; kindly provided by R. Hallmann, Erlangen, Germany), which is selectively expressed on venules of mucosal tissues (mucosal addressin); and culture supernatant of 9F1 antibody recognizing endothelial cells and smooth muscle cells (unpublished data), kindly provided by A. Hamann. Polyclonal anti-murine immunoglobulin G (IgG) (Dianova, Hamburg, Germany), rabbit anti-TNF-α (Genzyme Diagnostics, Cambridge, Mass.), and sheep anti-IFN-γ antibodies as well as polyclonal rabbit anti-*Y. enterocolitica* YadA antibodies have been recently described (7, 8, 81). Unless otherwise stated, antibodies were purified from hybridoma supernatants by using protein G-Sepharose 4 Fast Flow and fast-performance liquid chromatography (Pharmacia LKB). A portion of purified antibody was coupled to fluorescein isothiocyanate or biotin according to standard methods (40). Irrelevant monoclonal IgG2a (Dianova) and IgG2b antibody (EE5; kindly provided by W. Bohne, Würzburg, Germany), rat immunoglobulin (Dianova), and rabbit and sheep sera were used as control antibodies.

To block integrin molecules during infection, mice were injected intraperitoneally (i.p.) with 0.2 ml of PBS containing 0.3 mg of rat IgG or EE5 antibody (control group), anti-Mac-1 antibody (5C6), or anti-VLA-4 antibody (PS2.3) 4 h prior to and 2 days after infection. Alternatively, the cytokines TNF-α and IFN-γ were neutralized by administration of 0.5 mg of polyclonal rabbit anti-TNF-α or polyclonal sheep anti-IFN-γ antibodies prior to and after the infection as described previously (5, 8).

The polyclonal and monoclonal antibodies used in vivo were not cytotoxic for cells. Therefore, in uninfected control mice injection of the above-mentioned antibodies did not cause any tissue changes or alterations in cell populations in the spleen, MLN, and PP as determined by fluorescence-activated cell sorter (FACS) analysis and immunohistology (data not shown). However, administration of an isotype-identical antibody which is known to be cytotoxic for CD4 cells depleted $CD4^+$ cells from PP and MLN (not shown), suggesting that the antibodies injected reached intestinal tissues.

Immunohistology. For histological examinations, PP and MLN were excised, fixed in 4% buffered formalin, embedded in paraffin, cut, and stained as recently described (7). For immunohistological analysis the tissues were embedded in Tissue-Tek OCT compound (Nunc, Roskilde, Denmark), snap-frozen in liquid nitrogen, and stored at −80°C. Frozen sections were prepared and stained by an indirect three-stage immunoperoxidase method (peroxidase–anti-peroxidase [PAP]) including 3,3-diaminobenzidine-tetrahydrochloride acid (Sigma, Deisenhofen, Germany) as indicator as previously described (7). Nonspecific binding sites were blocked by incubation of the sections with PBS containing 25% sheep serum. Anti-Mac-1 (M1/70), anti-VLA-4, and anti-ICAM-1 antibodies diluted 1:10 were used as hybridoma cell culture supernatants. The secondary antibody was peroxidase-conjugated mouse $F(ab')_2$ fragment anti-rat IgG (Dianova; diluted 1:100), and the tertiary antibody was rat peroxidase-antiperoxidase complex (Dianova; diluted 1:100). The IgG fraction of rabbit anti-*Yersinia* YadA of serotype O8 serum (P1-8) (7, 81) was diluted 1:100, and this was followed by the addition of peroxidase-conjugated goat anti-rabbit antibodies (Dianova). Isotype-matched irrelevant antibodies were used in controls and revealed no staining signal. The sections were counterstained with Mayer's hemalaun, mounted, and assessed microscopically by two independent investigators.

Flow cytometry. Single-cell suspensions of PP, MLN, and spleens were prepared as recently described (5). A total of 2×10^6 cells per organ from each mouse of each group were pooled and stained with fluorescein isothiocyanate-conjugated monoclonal antibodies. Labeling procedures were carried out at 4°C in PBS containing 2% fetal calf serum for 20 min. Fluorescein isothiocyanate-conjugated irrelevant antibodies were included as controls. From each sample, 10,000 cells were analyzed by flow cytometry with a FACScan (Becton Dickinson) and FACScan research software. Dead cells were excluded from analysis by gating out propidium iodide (Sigma)-positive cells.

Phagocytosis assay. Mice were injected i.p. with 1 ml of 10% Proteose Peptone (Sigma) solution to elicit peritoneal exudate cells (PEC). Three days later, 0.5 mg of anti-Mac-1, anti-VLA-4, or control antibodies was injected i.p. After 30 min, 2×10^7 bacteria were injected i.p. After 45 min, mice were killed and a peritoneal lavage with 5 ml of ice-cold Hank's balanced salt solution (pH 7.4; Biochrom, Berlin, Germany) was performed. The harvested PEC were immediately centrifuged at $1,200 \times g$ for 10 min on glass slides by using a Cytocentrifuge

(Shandon, Pittsburgh, Pa.). Slides were air dried and stained with Giemsa stain (Merck) according to standard methods (35). Parallel samples were stained with double immunofluorescence to discriminate between intra- and extracellularly located bacteria as previously described (35). Briefly, extracellular bacteria were first stained with polyclonal rabbit anti-*Yersinia* antibody (anti-WA-v [81]). After methanol fixation, a Texas red-conjugated goat anti-rabbit antibody was used. Thereafter, intracellular bacteria were stained with a rabbit anti-*Yersinia* antibody followed by fluorescein-conjugated goat anti-rabbit antibody. At least 150 infected cells were examined by two independent investigators to calculate the average number of ingested bacteria. Further, the percentage of infected cells that ingested one or more bacteria was determined. The phagocytic index (PI) was calculated as follows: PI = mean number of ingested bacteria × percentage of infected cells. Blocking of Mac-1 and VLA-4 on the surface of PEC by the above-described procedure was controlled in parallel by staining the cells with fluorescein isothiocyanate-labeled anti-Mac-1, anti-VLA-4, or anti-rat IgG antibodies and performing flow cytometry analysis as described. In vitro phagocytosis assays including the cell lines $P388D_1$ and J774A.1 (kindly provided by H. Moll and J. Hacker, Würzburg, Germany) revealed comparable results (not shown).

Cell culture and proliferation assay. Cells were cultured in Click-RPMI 1640 medium (Biochrom) supplemented with 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5×10^{-5} M 2-mercaptoethanol, 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 10% heat-inactivated fetal calf serum. T cells were isolated from single-cell suspensions of PP, MLN, and spleen, and *Yersinia*-specific T-cell lines were prepared and established as described recently (9). Cells were stimulated either with concanavalin A (Pharmacia LKB; 3 μg/ml), anti-CD3 monoclonal antibody (2 μg per ml) or heat-killed yersiniae (10 μg/ml) in the presence of irradiated splenic antigen-presenting cells. After 2 or 3 days, cultures were pulsed with 1 μCi of [^{3}H]thymidine (Dupont, NEN Research Products, Homburg, Germany) per well for 8 h. Samples were collected with a cell harvester, and [^{3}H]thymidine uptake was measured with a liquid scintillation counter (Betaplate; Pharmacia LKB).

IFN-γ capture ELISA. Single-cell suspensions of PP, MLN, and spleens from mice 5 days after orogastric *Y. enterocolitica* infection were prepared, cultured, and stimulated with heat-killed yersiniae or concanavalin A as described above. The supernatants of these cultures were collected after 24 h and assayed for the presence of IFN-γ by a capture enzyme-linked immunosorbent assay (ELISA) as described recently (5, 9). Briefly, microtiter plates (Greiner, Frickenhausen, Germany) were coated with rat anti-mouse IFN-γ antibody (AN18.17.24). After blocking of nonspecific binding sites supernatants were added to the wells in duplicate. Then, a biotin-conjugated rat anti-mouse IFN-γ antibody (R4-6A2) was added. After wash steps, an avidin-alkaline phosphatase complex (Vectastain ABC-AP kit; Camon, Wiesbaden, Germany) was added and the signal was developed with *p*-nitrophenyl phosphate disodium (Sigma) in diethanolamine buffer. Optical density was measured with an ELISA reader (Dynatech, Stuttgart, Germany). Values were calculated from the straight-line portion of the standard curve by using recombinant murine IFN-γ (kindly provided by G. Adolf, Bender, Vienna, Austria).

Statistical analysis. Differences between mean values were analyzed by Student's *t* test. A P value of <0.05 was considered statistically significant.

RESULTS

Immunohistological and cytofluorometric characterization of PP cell populations. The cell populations of PP from normal uninfected C57BL/6 mice were analyzed by flow cytometry and revealed the following pattern: 11.7% $CD4^+$ and 7.9% $CD8^+$ T cells, 71.4% IgG^+ B cells, and 3.1% Mac-1^+, 88.1% ICAM-1^+, 86.6% VLA-4^+, 96.8% Pgp-1^+, and 98.0% LFA-1^+ cells (data not shown). These data were confirmed by immunohistology (data not shown) and were in keeping with those previously reported by others (20, 38, 71, 74). Only a few Mac-1^+ phagocytes could be observed parafollicularly and in the dome area of uninfected PP. Within the FAE of the PP, single VLA-4^+ and Mac-1^+ cells were found. Vessels with endothelial cells positively stained with anti-MAdCAM-1 and -9F1 antibodies were found parafollicularly (not shown).

***Yersinia*-induced tissue lesions in PP.** Orogastric administration of 5×10^8 *Y. enterocolitica* bacteria of serotype O8 in C57BL/6 mice caused an infection, in at least 50% of the PP (data not shown; see also reference 6), which disseminated to the MLN, spleen, liver, and lungs within 3 to 5 days (data not shown). *Yersinia* infection caused a considerable recruitment of phagocytes into the PP and, subsequently, the formation of abscesses composed of polymorphonuclear cells (ICAM-1^- Mac-1^+ VLA-4^- Pgp-1^+) in the center and a distinct cellular border made up of predominantly mononuclear cells (ICAM-1^+ Mac-1^+ VLA-4^+ Pgp-1^+) (Fig. 1). Moreover, at the edges of the abscesses, formations of small vessels (MAdCAM-1^+ 9F1^+) could be observed (Fig. 1).

Modulation of intestinal *Yersinia* infection by anti-Mac-1 and anti-VLA-4 antibodies. To investigate the role of the integrins Mac-1 and VLA-4 in intestinal *Yersinia* infection, anti-Mac-1 or anti-VLA-4 antibodies were injected i.p. into C57BL/6 mice prior to and after orogastric infection with 5×10^8 *Y. enterocolitica* bacteria. After administration of this inoculum, only a few clinical signs of yersiniosis, such as diarrhea and weight loss, were found for control mice, whereas anti-Mac-1- and anti-VLA-4-treated mice became compromised and showed significant weight loss (Fig. 2a). Bacterial numbers in PP (~10-fold) and in MLN (~100- to ~1,000-fold) of anti-Mac-1 or anti-VLA-4 antibody-treated mice were increased (Fig. 2b). However, only anti-VLA-4-treated animals exhibited increased bacterial numbers in the spleen (~1,000-fold) whereas reduced splenic infection was found in anti-Mac-1-treated mice (Fig. 2b). In keeping with these results, masses of bacteria and a complete destruction of the cytoarchitecture were observed in the PP of anti-Mac-1- or anti-VLA-4-treated mice (Fig. 3). *Yersinia*-specific immunostaining revealed bacteria scattered within the PP of anti-VLA-4-treated mice, while for anti-Mac-1-treated mice, the bacteria were located in microcolonies instead (Fig. 3). These results suggest that Mac-1 and VLA-4 are involved in protective cell-mediated host responses in PP and MLN. In an attempt to explain this observation, we investigated three events in which the integrins might be involved: (i) recruitment of effector cells, (ii) phagocytosis, and (iii) T-cell activation.

Recruitment of phagocytes into infected PP. To assess whether anti-VLA-4 or anti-Mac-1 antibodies had affected the recruitment of phagocytes into infected PP, MLN, and spleens, flow cytometric analyses were performed. The results show that the number of Mac-1^+ phagocytes increased from 3% in noninfected to 10% in *Yersinia*-infected PP whereas the number of T cells and IgG^+ cells decreased (from 20 to 14% and from 71.4 to 67%, respectively) after the infection (Fig. 4). In mice treated with anti-Mac-1 or anti-VLA-4 antibodies, even greater numbers of Mac-1^+ cells (24.7 and 32.7%, respectively) were found. These observations were confirmed by immunohistology (Fig. 3). Hence, some of the lymphoid follicles of PP were almost completely replaced by Mac-1^+ cells. Comparable results were found for the cell populations of MLN and spleens (data not shown). From these data we can conclude that administration of anti-Mac-1 or anti-VLA-4 antibodies did not inhibit the recruitment of phagocytes into *Yersinia*-infected tissues.

Role of Mac-1 and VLA-4 in phagocytosis of *Y. enterocolitica*. To determine whether Mac-1 or VLA-4 integrins are involved in ingestion of *Y. enterocolitica* by phagocytes, an in vivo phagocytosis assay was performed. Flow cytometry analysis revealed that Mac-1 and VLA-4 integrins on phagocytes were blocked by or internalized after the antibody pretreatment, although this effect was less pronounced after anti-VLA-4 treatment (Fig. 5; Table 2). At 45 min after infection, PEC were harvested and stained with double immunofluorescence or Giemsa stain and ingested bacteria were counted. The data (Table 3) indicate that both anti-Mac-1 and anti-VLA-4 treatment reduced the number of infected macrophages (17 and 13%, respectively) as well as the number of bacteria per infected cell (57 and 26%, respectively). Thus, there was a 65% reduction of the phagocytic index by anti-Mac-1 antibodies and a 35% reduction by anti-VLA-4 antibodies. The combination of both anti-Mac-1 and anti-VLA-4 antibodies further decreased the phagocytic index by 72%, suggesting that both integrins may independently be involved in phagocytosis of yersiniae (Table

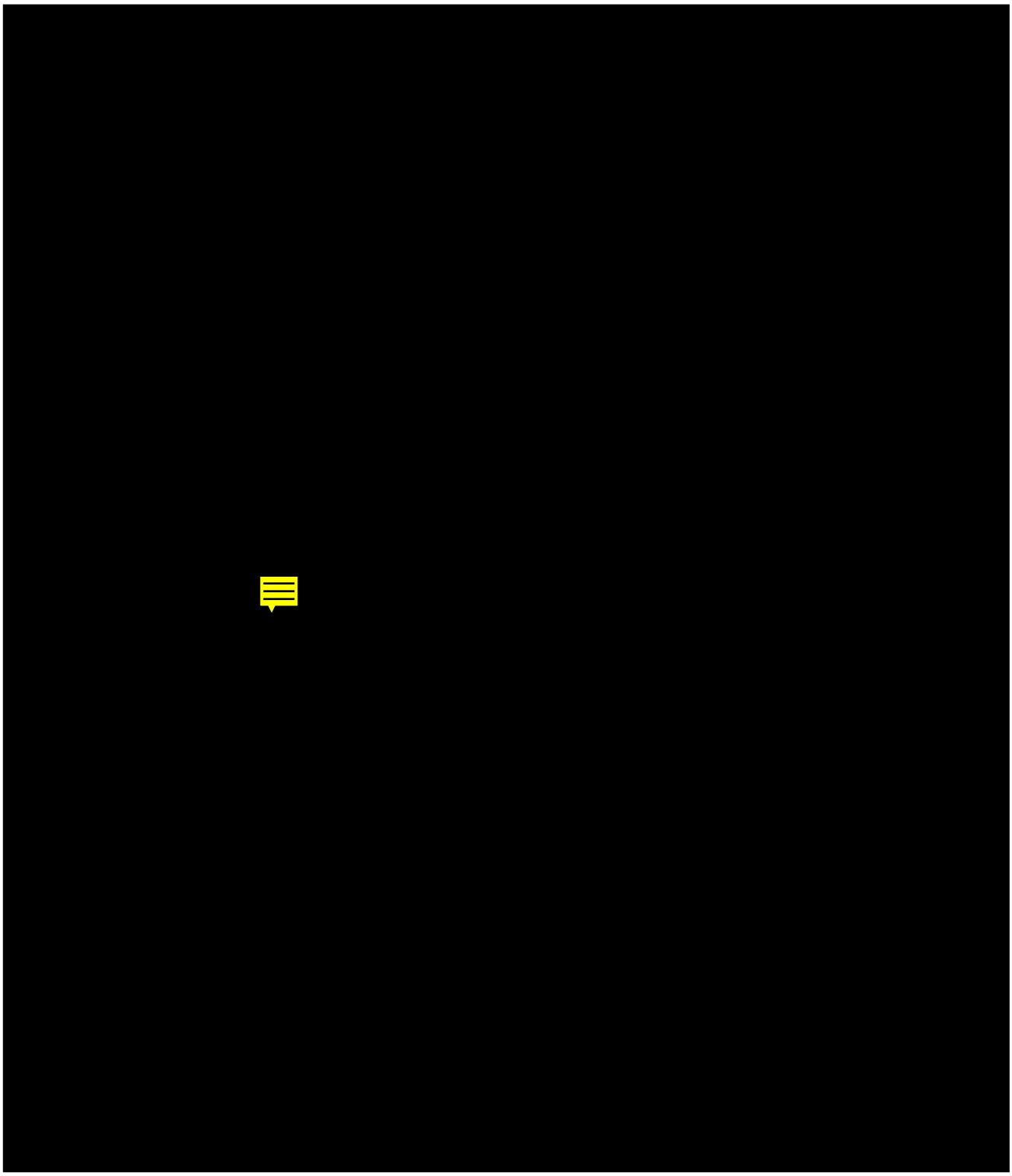

FIG. 1. PP of C57BL/6 mice 7 days after infection with 5×10^8 *Y. enterocolitica* bacteria. An abscess in the dome area with erosion of the FAE is shown. Lymphoid follicles (triangles), cellular abscess border (bold arrows), and centers (a's) of the abscesses are indicated. (a) Hematoxylin and eosin staining. Immunohistology with anti-Mac-1 (b), anti-VLA-4 (c), anti-ICAM-1 (d), anti-MAdCAM-1 (e), and 9F1-antibodies (f) is shown. Frozen sections prepared by the PAP method (brown signal) and counterstained with Mayer's hemalaun are shown. Magnification, ×147.

3). However, a significant number of bacteria were still internalized under these conditions, suggesting that in addition to VLA-4 and Mac-1, alternative receptors play a role in phagocytosis of yersiniae. Nevertheless, these results suggest that both integrins may be involved in interaction of phagocytes with *Y. enterocolitica.*

Modulation of T-cell responses by anti-Mac-1 and anti-VLA-4 antibodies. Since T cells play an important role in over-

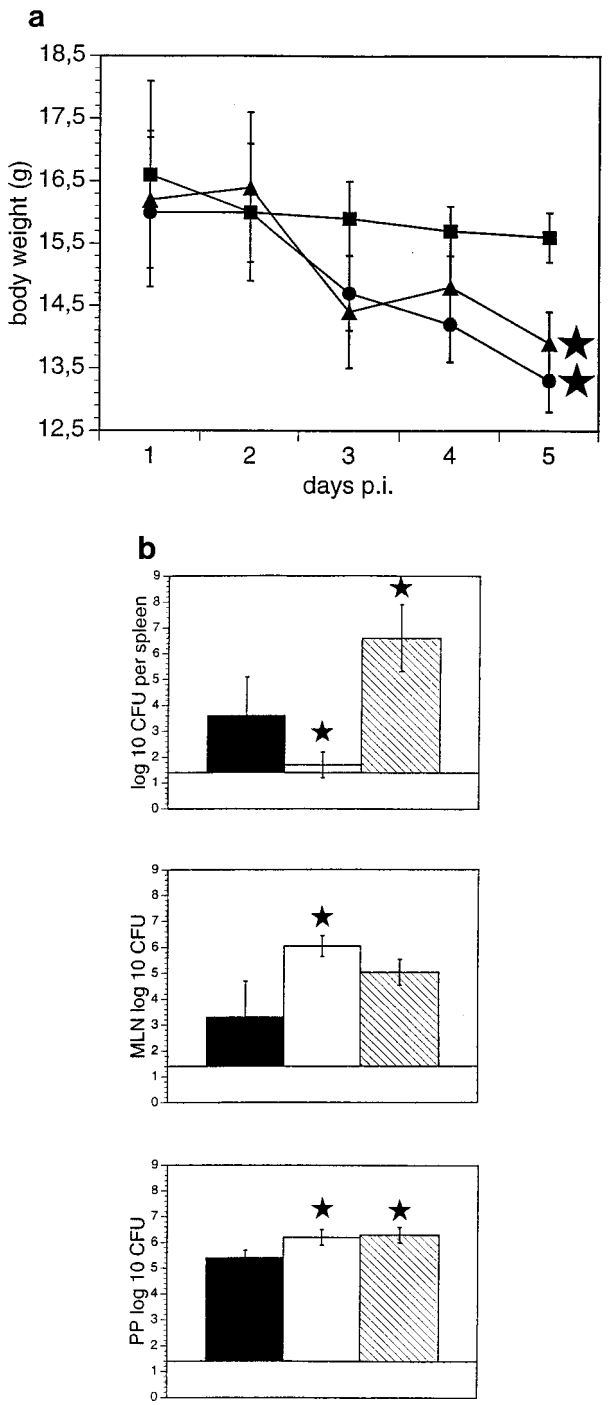

FIG. 2. Body weight (a) and number (b) of *Y. enterocolitica* bacteria in PP, MLN, and spleens of C57BL/6 mice 5 days after infection with 5×10^8 *Y. enterocolitica* bacteria. One day prior to the infection, mice were i.p. injected with 0.5 mg of irrelevant antibodies (control) (solid bar), anti-Mac-1 antibodies (open bar), or anti-VLA-4 antibodies (hatched bar). The values are the means for 10 mice per group. Error bars show standard deviations. Stars indicate values that differ from those of the control group at a P of <0.05.

coming systemic *Y. enterocolitica* infection, the effect of anti-VLA-4 and anti-Mac-1 antibodies on proliferative responses of splenic T cells and *Yersinia*-specific PP T-cell lines were investigated. As is evident from Table 4, neither anti-Mac-1 nor anti-VLA-4 antibodies inhibited CD3-triggered splenic T-cell proliferation in vitro. In contrast, anti-LFA-1 antibodies reduced T cell proliferation in a concentration-dependent manner as previously shown (30). In parallel experiments a *Yersinia*-specific T-cell line (GLP92.2) derived from *Yersinia*-infected PP was used. This cell line produced IFN-γ and was $CD3^+$ $CD4^+$ $CD8^-$ ICAM-1^+ VLA-4^+ Mac-1^- (data not shown). The results in Table 4 indicate only slight inhibition of the proliferative response of this T-cell line by anti-Mac-1 or anti-VLA-4 antibodies in vitro, suggesting that anti-VLA-4 or anti-Mac-1 antibodies do not significantly modulate *Yersinia*-specific proliferative T-cell responses in vitro.

To investigate whether anti-Mac-1 or anti-VLA-4 antibody treatment modulates T-cell functions in vivo, mice were orogastrically infected with *Y. enterocolitica* and treated with control, anti-Mac-1, or anti-VLA-4 antibodies. Five days later, T cells were isolated from PP, MLN, and spleens and were stimulated with heat-killed yersiniae. Then, proliferative responses and IFN-γ production were determined. The results (Fig. 6) indicate that administration of anti-Mac-1 or anti-VLA-4 antibodies in vivo reduced *Yersinia*-triggered IFN-γ production in the PP (30 and 50% inhibition, respectively), MLN (90 and 50%, respectively), and spleen (75 and 10%, respectively) ex vivo. In terms of *Yersinia*-specific T-cell proliferation, comparable effects were found, which suggests that Mac-1 as well as VLA-4 integrins are involved in *Yersinia*-specific T-cell responses in vivo.

Modulation of intestinal *Y. enterocolitica* infection by anti-TNF-α and anti-IFN-γ antibodies. The observations described above suggest that cell-mediated host responses including macrophages are required to control yersinia infection in PP and MLN. As the cytokines TNF-α and IFN-γ may be involved in such processes, mice were treated with neutralizing anti-TNF-α or anti-IFN-γ antibodies prior to and after orogastric *Y. enterocolitica* infection to investigate whether these cytokines are involved in protective host responses against this pathogen in PP and MLN. As is evident from Fig. 7, neutralization of TNF-α or IFN-γ caused a dramatic increase of bacterial numbers in PP, MLN, and spleens. Immunohistology revealed that the PP were completely destroyed by the fulminant infection and that the lymphoid follicles were often replaced by masses of bacteria and Mac-1^+ cells (Fig. 8; control results are shown in Fig. 3). The number of phagocytes in PP increased from 3 to 18% after anti-IFN-γ treatment and up to 33% after anti-TNF-α treatment, as revealed by flow cytometry analysis (data not shown). Comparable results were found for MLN and spleens (not shown). Thus, administration of neutralizing anti-TNF-α or anti-IFN-γ antibodies did not affect the recruitment of Mac-1^+ phagocytes into infected PP and MLN but abolished local host defense against *Y. enterocolitica* in intestinal tissues.

DISCUSSION

In our approach, mice were injected with antibodies against integrins or cytokines prior to orogastric *Yersinia* infection in order to investigate and dissect the roles of these components in defense mechanisms in PP and MLN against yersiniae. The data presented herein suggest that cellular inflammatory host responses including phagocytes are involved in local intestinal defense mechanisms against *Y. enterocolitica*. The multifunctional integrins VLA-4 and Mac-1 play an essential role during this process, as blocking of these molecules inhibited phagocytosis of yersiniae by macrophages and reduced *Yersinia*-specific T-cell responses while recruitment of Mac-1^+ cells was not affected. The fact that neutralization of the cytokines TNF-α and IFN-γ caused comparable effects supports the hy-

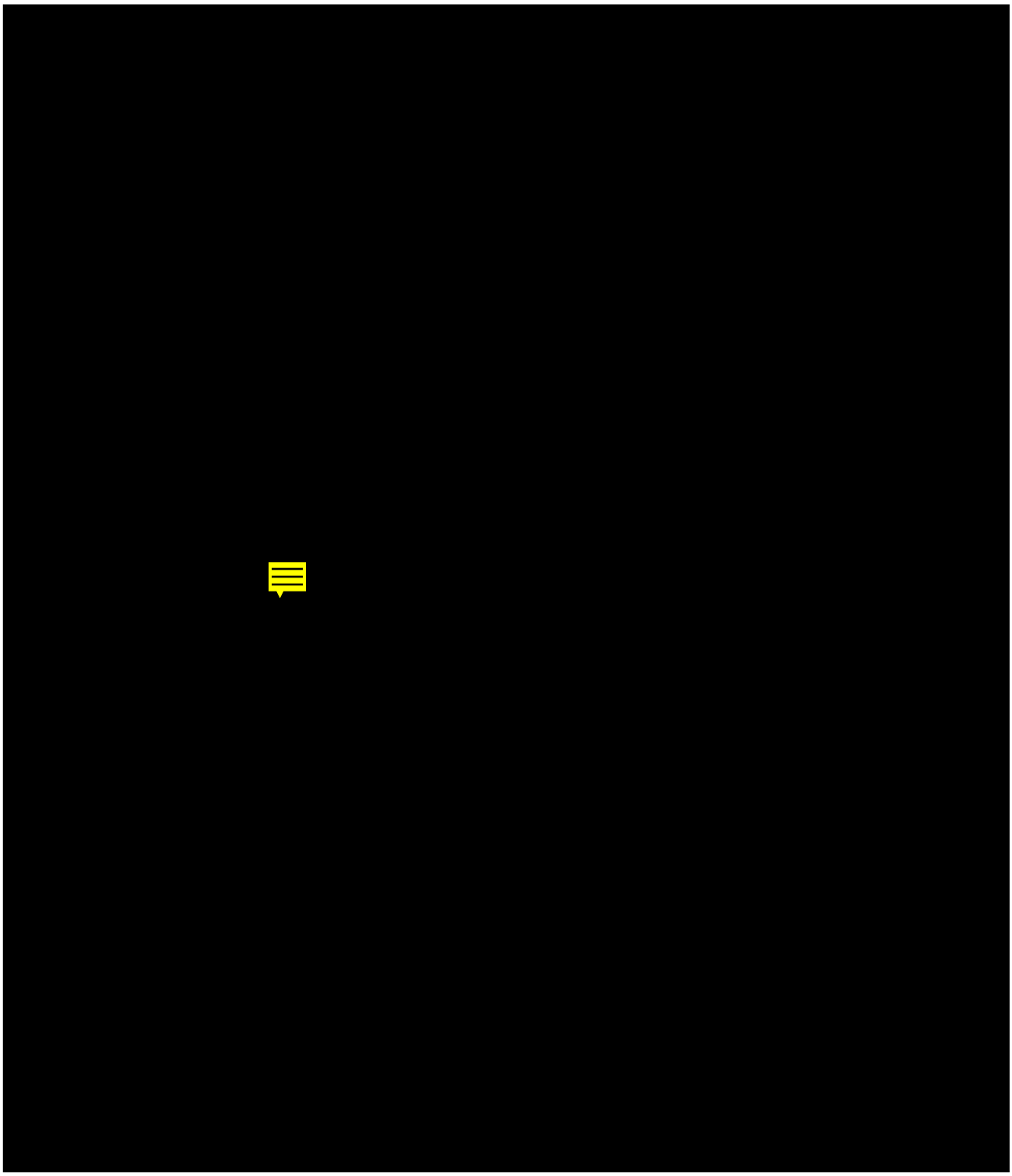

FIG. 3. Immunohistological staining of abscesses in PP from C57BL/6 mice after orogastric *Y. enterocolitica* infection and i.p. injection of control antibodies (a and b), anti-Mac-1 antibodies (c and d), or anti-VLA-4 antibodies (e and f). Immunostaining was performed with anti-Mac-1 (a, c, and e; magnification, ×92) or anti-*Yersinia* antibodies (b, d, and f; magnification, ×147). Frozen sections stained by the PAP method (brown signal) and counterstained with Mayer's hemalaun are shown. (f) Arrowheads indicate the distribution of bacteria in a sample from an anti-VLA-4 antibody-treated mouse. Asterisks indicate remaining intact lymphoid tissue.

pothesis that control of yersinia infection in PP and MLN is mediated by activated macrophages.

Y. enterocolitica infects the small intestine by penetration of M cells of the FAE of the PP (6, 41, 44). The Inv protein of *Yersinia* spp. plays an important role in the entry into PP (68). Inv binds to β1 integrins and thereby mediates invasion into eukaryotic cells in vitro (50, 61). On the other hand, Inv triggers T-cell activation by interaction with VLA-4 (α4β1) and

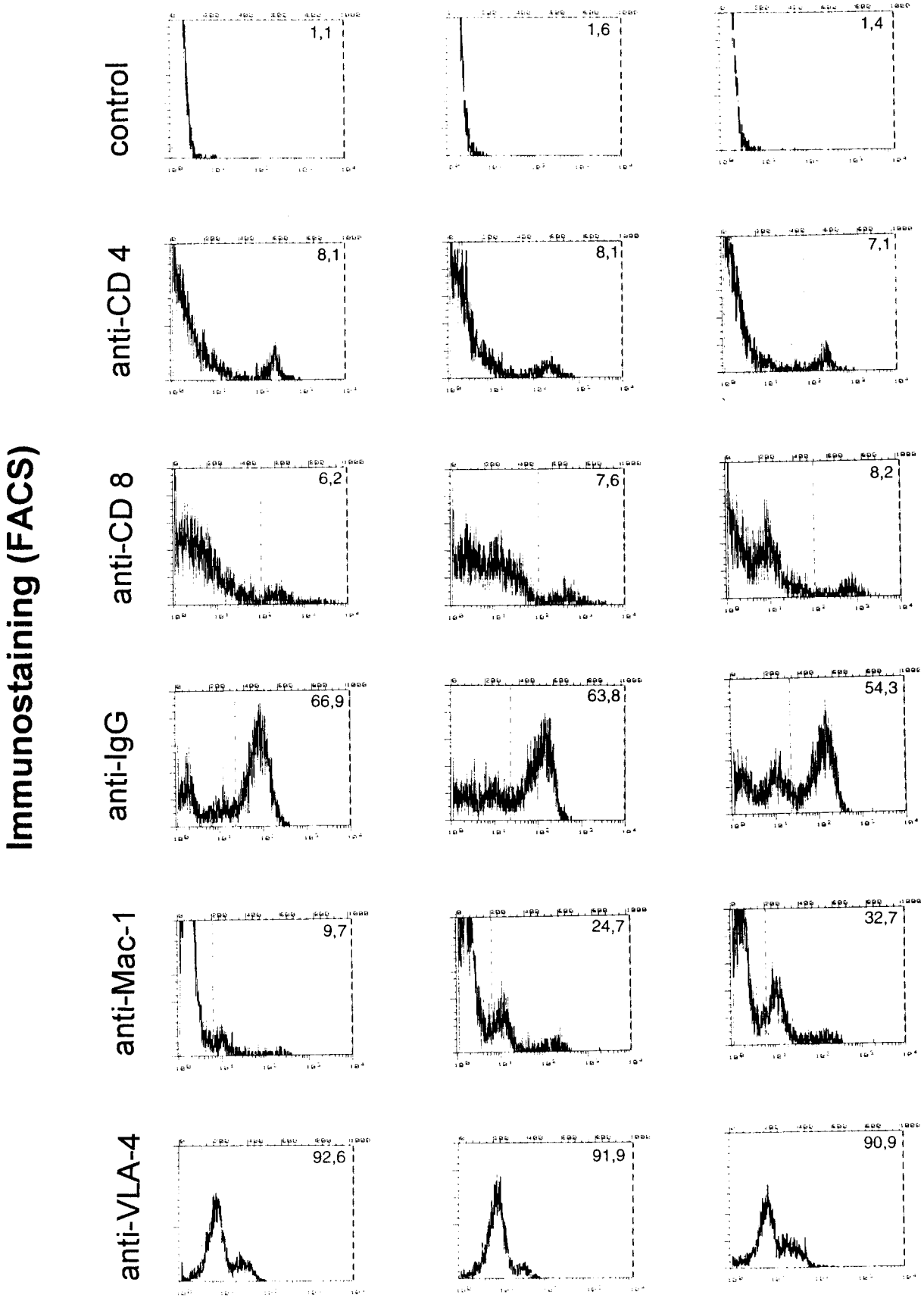

FIG. 4. Flow cytometric analysis of cell populations in PP from C57BL/6 mice 5 days after orogastric administration of 5×10^8 *Y. enterocolitica* bacteria and immunomodulation with anti-Mac-1 (middle column) or anti-VLA-4 (right-hand column) antibodies. The left-hand column shows results for cells from control mice. *y* axis, relative number of cells; *x* axis, intensity of staining signal. The values indicated in the corner of each graph are the numbers of positively stained cells.

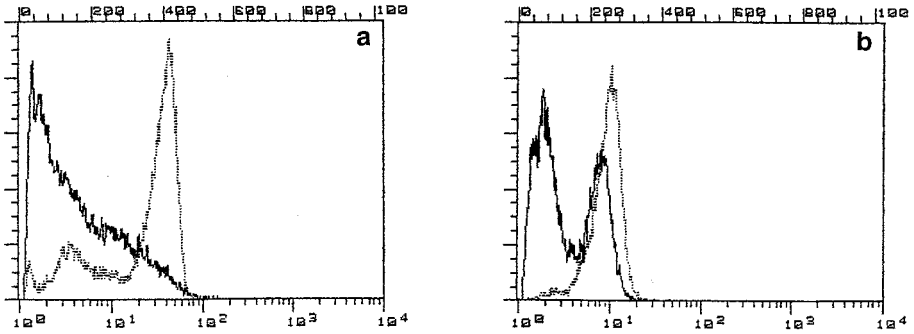

FIG. 5. Flow cytometry analysis of PEC prepared 45 min after i.p. injection of mice with control antibodies or anti-Mac-1 or anti-VLA-4 antibodies. (a) Anti-Mac-1 immunostaining of PEC from a control mouse (thin line) and an anti-Mac-1 antibody-treated mouse (bold line). (b) Anti-VLA-4 immunostaining of PEC from a control mouse (thin line) and an anti-VLA-4 antibody-treated mouse (bold line). *y* axis, relative number of cells; *x* axis, intensity of staining signal.

thus might interfere with cellular immune responses (21, 34, 60). We found that VLA-4 is expressed on cells within the epithelium of the FAE. Therefore, it will be interesting to characterize the VLA-4$^+$ cells within the FAE and to elucidate whether these cells are involved in the invasion process of yersiniae. Whether M cells express integrins on their apical membranes remains to be established.

Administration of anti-Mac-1 antibodies prior to *Yersinia* infection caused increased bacterial numbers in the PP and MLN but decreased bacterial numbers in the spleen. Hence, dissemination of yersiniae to the spleen (via the bloodstream) but not to MLN (via the lymphatics) was reduced by anti-Mac-1 antibodies. This observation may give a clue to understanding the unknown dissemination mechanisms and suggests that dissemination of yersiniae from the PP to the spleen depends on Mac-1 interactions while dissemination to the MLN is rather Mac-1 independent. Although this assumption is quite speculative, it may well be that direct interaction of *Yersinia* adhesin YadA with its putative ligand Mac-1 (23) may account for this dissemination mechanism. This might also explain the organotropism of yersiniae, which tend to infect exclusively organs of the reticuloendothelial system (spleen, liver, lymph nodes) (7, 36). Hence, Mac-1 might function as a homing receptor for yersiniae. On the other hand, we cannot exclude the possibility that yersiniae may disseminate in a cell-associated fashion or (transiently) intracellularly, which may require pathogen-host cell interactions during which Mac-1 may be engaged.

Infection of PP with yersiniae was followed by a marked recruitment of Mac-1$^+$ phagocytes into PP. Human monocytes have been demonstrated to use either Mac-1 or VLA-4 integrins to cross endothelium in vitro (60). The recruitment of phagocytes into liver lesions induced by *Listeria monocytogenes* was reported to be inhibited by administration of anti-Mac-1 (clone 5C6) antibodies (25, 69, 70). In contrast, administration of this antibody had no effect on recruitment of phagocytes into *Yersinia*-infected PP and MLN tissue. Likewise, administration of anti-VLA-4 antibodies did not decrease recruitment of phagocytes into PP and MLN. Thus, alternative mechanisms, similar to that described recently (26), are likely to be involved in recruitment of these cells into infected intestinal tissues. On the other hand, we cannot exclude the possibility that the recruitment of a particular subpopulation of Mac-1$^+$ phagocytes was inhibited in our experiments.

Administration of either anti-Mac-1 or anti-VLA-4 antibodies decreased *Yersinia*-triggered T-cell proliferation and IFN-γ production. This observation is consistent with the observation that both Mac-1 and VLA-4 are involved in the recruitment or expansion of antigen-specific T cells into inflamed tissues (1, 11, 12, 24, 37, 53, 66, 83). After intravenous *Yersinia* infection, IFN-γ is mainly produced by both NK cells and CD4$^+$ T cells (17). At present, however, it is not clear which cell types contribute to *Yersinia*-induced IFN-γ production in PP and MLN.

The number of cells expressing the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) increased in PP after *Yersinia* infection because of the formation of vessels which surrounded the abscesses. MAdCAM-1 is selectively expressed on GALT and is an endothelial receptor for the lymphocyte homing receptor α4β7 (76). It will be interesting to investigate which factor(s) mediates the new formation of vessels or up-

TABLE 2. Modulation of Mac-1 and VLA-4 expression on PEC by treatment with antibodies

Pretreatment[a]	FACS staining result[b] with:					
	Anti-Mac-1		Anti-VLA-4		Anti-rat IgG	
	%	Median	%	Median	%	Median
Control	70.2	404	94.2	253	14.4	398
Anti-Mac-1	18.9	345	92.6	233	82.4	443
Anti-VLA-4	63.0	522	41.4	228	83.1	251

[a] Three days after stimulation of PEC by Proteose Peptone, mice were injected with PBS containing 0.5 mg of control antibodies or anti-Mac-1 or anti-VLA-4 antibodies. After 45 min, PEC were harvested, stained with anti-Mac-1, anti-VLA-4, or anti-rat IgG antibodies, and analyzed with a FACScan.

[b] Results are expressed as relative percentages and median fluorescence intensities of positively stained cells.

TABLE 3. Phagocytosis of *Y. enterocolitica* by PEC[a]

Pretreatment	No. of bacteria/ macrophage[b]	% Infected macrophages[c]	Phagocytic index[d] ± SD
Control	14.74	30	4.42 ± 0.40
Anti-Mac-1	6.6	25	1.57 ± 0.29[e]
Anti-VLA-4	10.97	26	2.85 ± 0.49[e]
Anti-Mac-1 plus anti-VLA-4	5.89	21	1.24 ± 0.36[e]

[a] Mice were injected i.p. with Proteose Peptone and antibodies prior to i.p. infection with *Y. enterocolitica*. PEC were harvested after 45 min, centrifuged onto slides, and stained with Giemsa stain or by double immunofluorescence (see Materials and Methods).

[b] Mean number of intracellular bacteria per 150 counted macrophages.

[c] Percentage of macrophages infected with one or more yersiniae.

[d] Phagocytic index = mean number of intracellular bacteria per macrophage × number of infected macrophages/100.

[e] Differs from the control value at a P of <0.05.

TABLE 4. Modulation of T-cell proliferation by anti-integrin antibodies

T-cell origin[a]	Amt of antibody added (mg/well)	[^{3}H]thymidine uptake (cpm)[b]			
		Control	Anti-Mac-1	Anti-VLA-4	Anti-LFA-1
Spleen					
	0	76.816			
	0.1	75.089	78.507	74.699	57.703
	1	77.256	71.718	74.737	15.649
	10	79.656	79.263	78.248	15.129
Yersinia-specific PP					
	0	48.365			
	0.1	46.429	49.399	47.322	30.609
	1	46.919	47.702	44.564	25.059
	10	49.565	41.931	41.079	26.556

[a] Splenic T cells or *Yersinia*-specific T cells derived from PP (T-cell line GLP92.2) (2×10^5 per well) were stimulated with 2 μg of anti-CD3 antibodies in the presence of various concentrations (0.1, 1, or 10 μg) of control (irrelevant antibody), anti-Mac-1, anti-VLA-4, or anti-LFA-1 antibodies.

[b] Proliferative T-cell responses were determined by [^{3}H]thymidine uptake and expressed as counts per minute (see Materials and Methods).

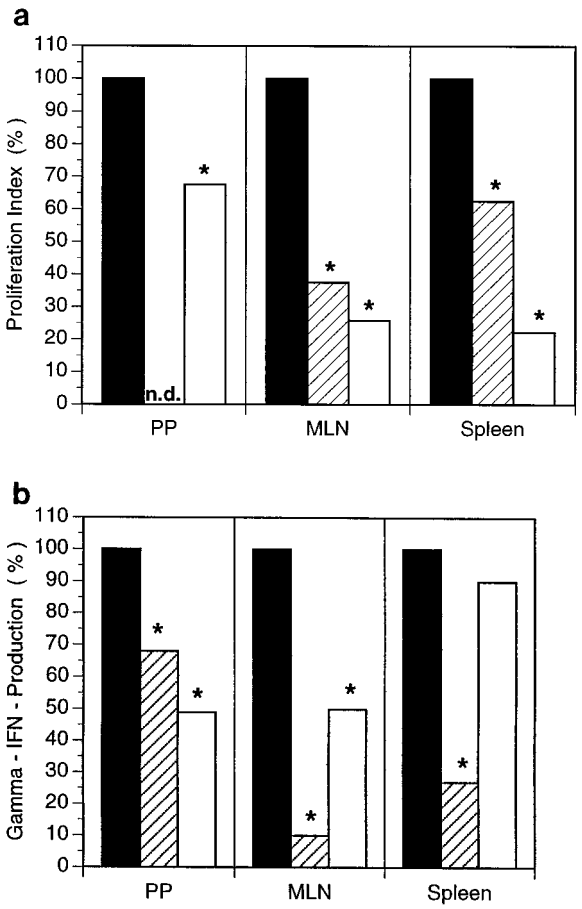

FIG. 6. Proliferative response (a) and IFN-γ production (b) of PP, MLN, and spleen T cells isolated from C57BL/6 mice 5 days after treatment with control antibodies (solid bar), anti-Mac-1 antibodies (hatched bar), or anti-VLA-4 antibodies (open bar) prior to and after orogastric infection with 5×10^8 *Y. enterocolitica* bacteria. Cells were stimulated with 10 μg of heat-killed yersiniae per ml. IFN-γ production was determined in the culture supernatants by ELISA. The values of the control group were taken as 100% and represent the means from 5 to 10 animals. Asterisks indicate values that differ from those of the control group at a P of <0.05. n.d., not determined.

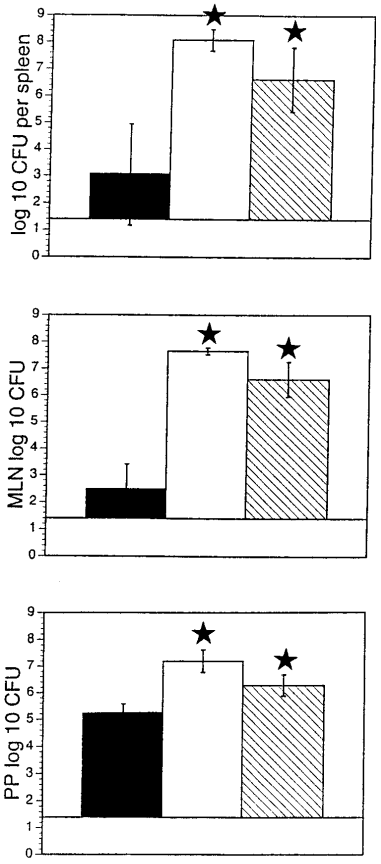

FIG. 7. Number of bacteria in PP, MLN, and spleens of C57BL/6 mice after orogastric infection with 5×10^8 *Y. enterocolitica* bacteria. One day prior to infection, mice were treated with 0.5 mg of control antibodies (solid bar), anti-TNF-α antibodies (open bar), or anti-IFN-γ antibodies (hatched bar). Values are the means for 10 animals per group. Error bars show standard deviations. Stars indicate values that differ from those of the control group at a P of <0.05.

regulation of MAdCAM-1 and whether this process is a prerequisite for the recruitment of appropriate quantities of *Yersinia*-specific IFN-γ-producing T cells.

TNF-α and IFN-γ are involved in expression of adhesion molecules ICAM-1 and VCAM-1, which are the ligands for Mac-1 and VLA-4, respectively, on endothelial cells (54, 59, 60, 62, 76, 77). Moreover, both cytokines are produced upon *Yersinia* infection and mediate protective cellular host responses against yersiniae in the spleen and liver (5, 8). Therefore, analysis of the roles of TNF-α and IFN-γ during intestinal *Yersinia* infection was included in this study. Neutralization of TNF-α and IFN-γ in vivo, however, had no effect on the recruitment of inflammatory cells into PP and MLN, suggesting that other cytokines might have caused expression of the above-mentioned adhesion molecules. On the other hand, alternative mechanisms may be involved in transmigration of phagocytes into *Yersinia*-infected PP and MLN. Nevertheless, treatment with anti-IFN-γ, and particularly with anti-TNF-α antibodies, caused a dramatic increase of bacterial growth in infected organs followed by morphological tissue alterations, including necrosis of *Yersinia*-infected PP. These observations suggest that both cytokines play a central and essential role in local defense mechanisms in PP and MLN, possibly by activation of macrophages. However, a recent report provided evidence that TNF-α production in PP may be suppressed by virulence factors expressed by yersiniae, implicating TNF-α in

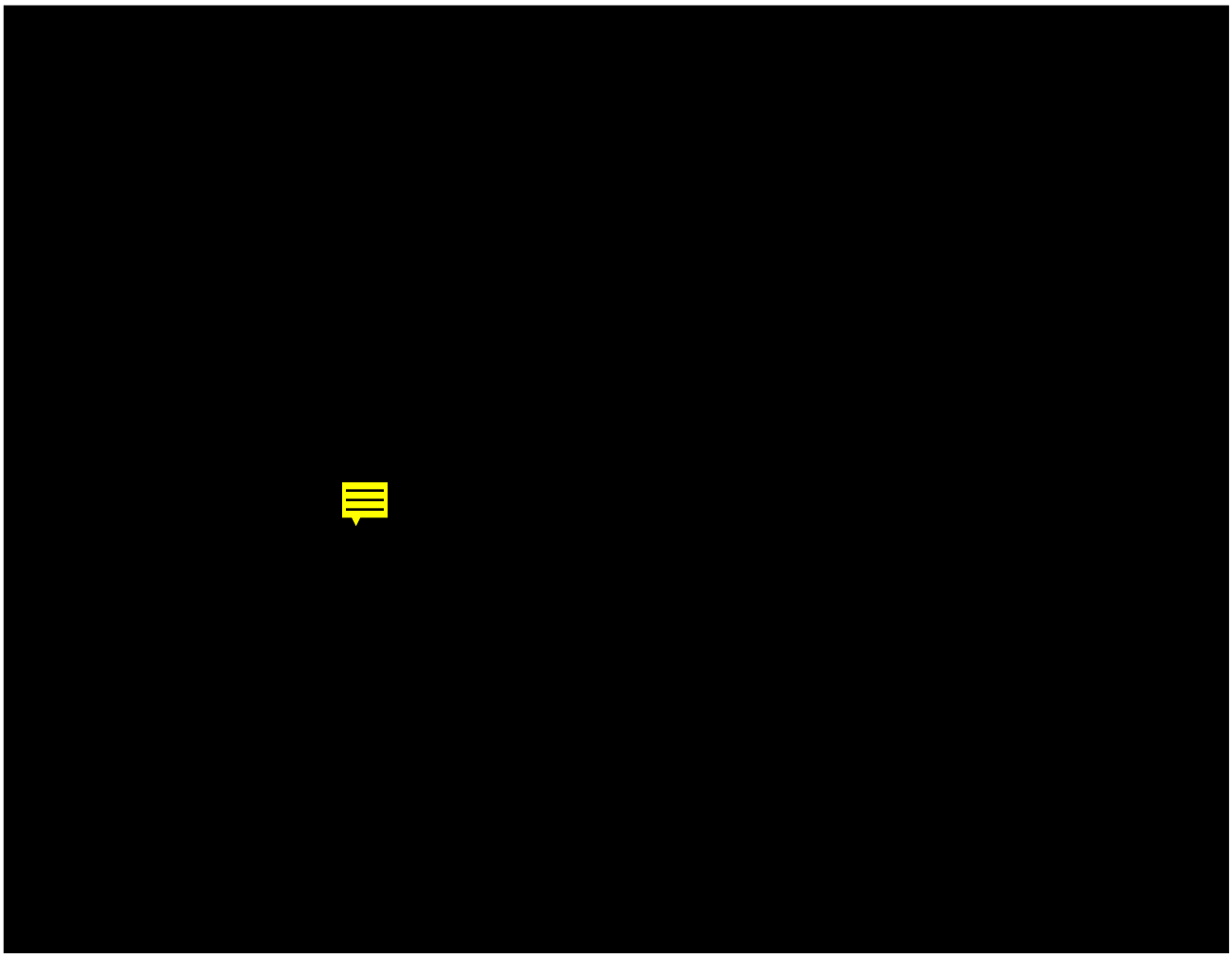

FIG. 8. Immunohistological staining of abscesses in PP from C57BL/6 mice after orogastric *Y. enterocolitica* infection and i.p. injection of anti-TNF-α (a and b) or anti-IFN-γ (c and d) antibodies (control results are shown in Fig. 3). Immunostaining with anti-Mac-1 (a and c; magnification, ×92) and anti-*Yersinia* YadA (b and d; magnification, ×147) antibodies is shown. Frozen sections stained by the PAP method (brown signal) and counterstained with Mayer's hemalaun are shown. Asterisks indicate remaining intact lymphoid tissue.

a minor role at the site of the infection (14). Likewise, interleukin-12, which in addition to TNF-α and IFN-γ is a protective key cytokine in yersiniosis in the spleen and liver, plays only a minor role in defense mechanisms against yersiniae in PP (17). Hence, the cellular source and the mode of action of IFN-γ and TNF-α in intestinal tissues remain to be established.

Currently, it is believed that the lymphoid tissue of the PP represents predominantly the afferent limb of the GALT (15, 39, 64, 65, 78). The results presented in this work, however, indicate that yersinia-induced inflammatory host reactions in PP resemble those observed in the spleen and liver (5, 7, 10). Hence, in addition to the afferent functions of the GALT, PP tissue may generate reactions that represent cellular effector functions.

Taken together, the results of this study suggest that phagocytes play a crucial role in the local host defense in PP against yersiniae and that this reaction is mediated by the cytokines TNF-α and IFN-γ. Moreover, the integrins Mac-1 and VLA-4 are involved in this process by a multifunctional role comprising both cell-cell and cell-pathogen interactions.

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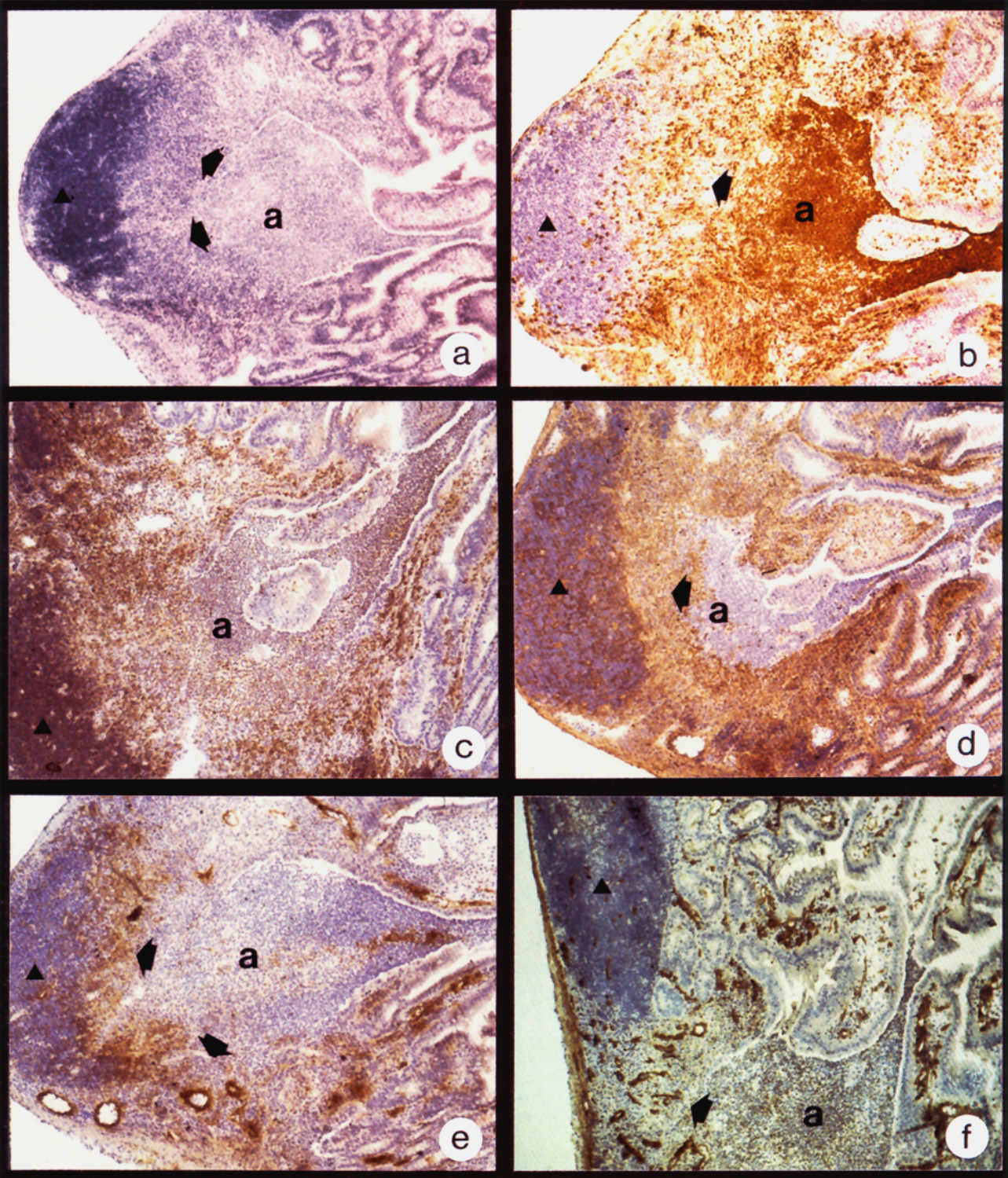
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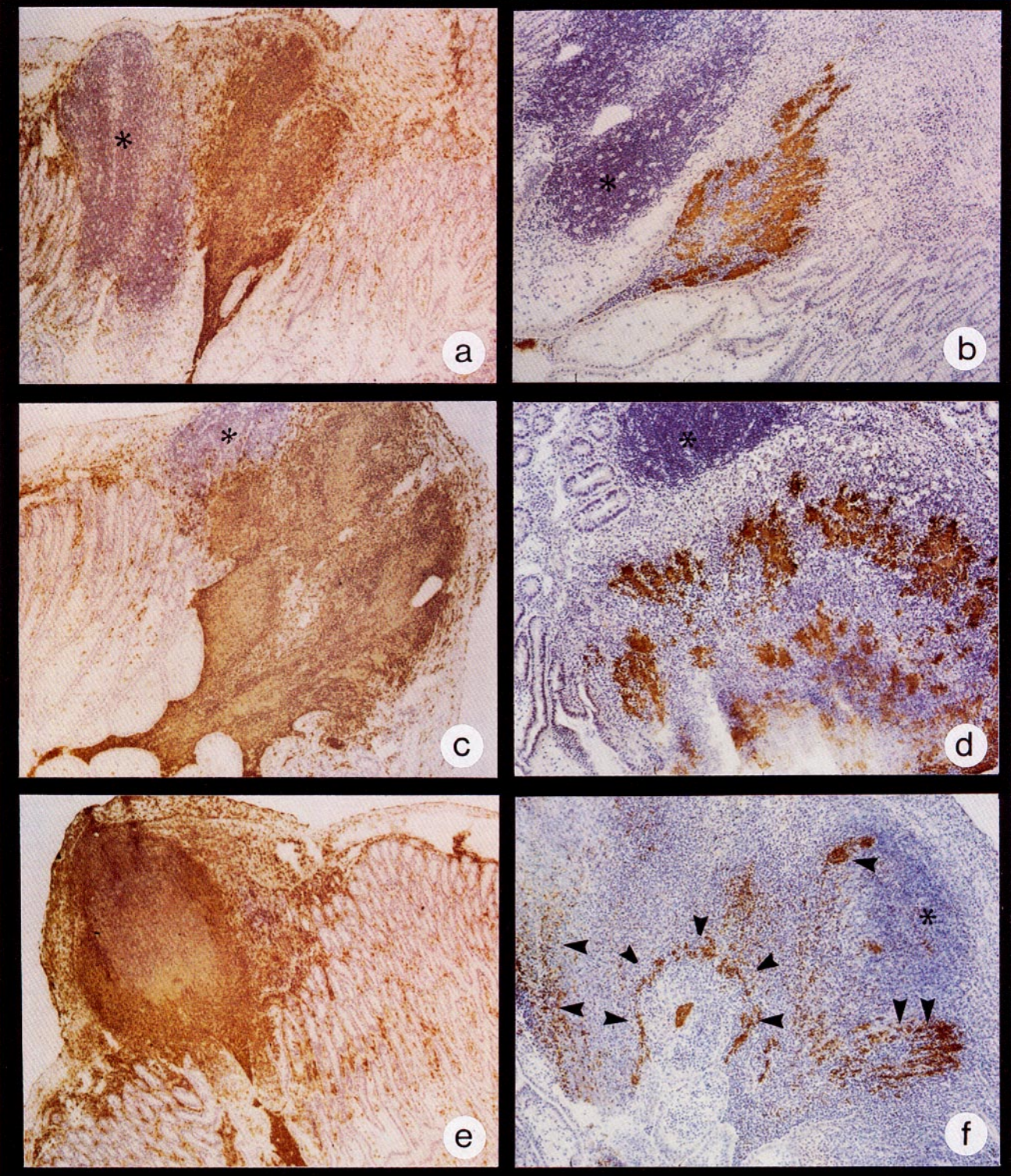
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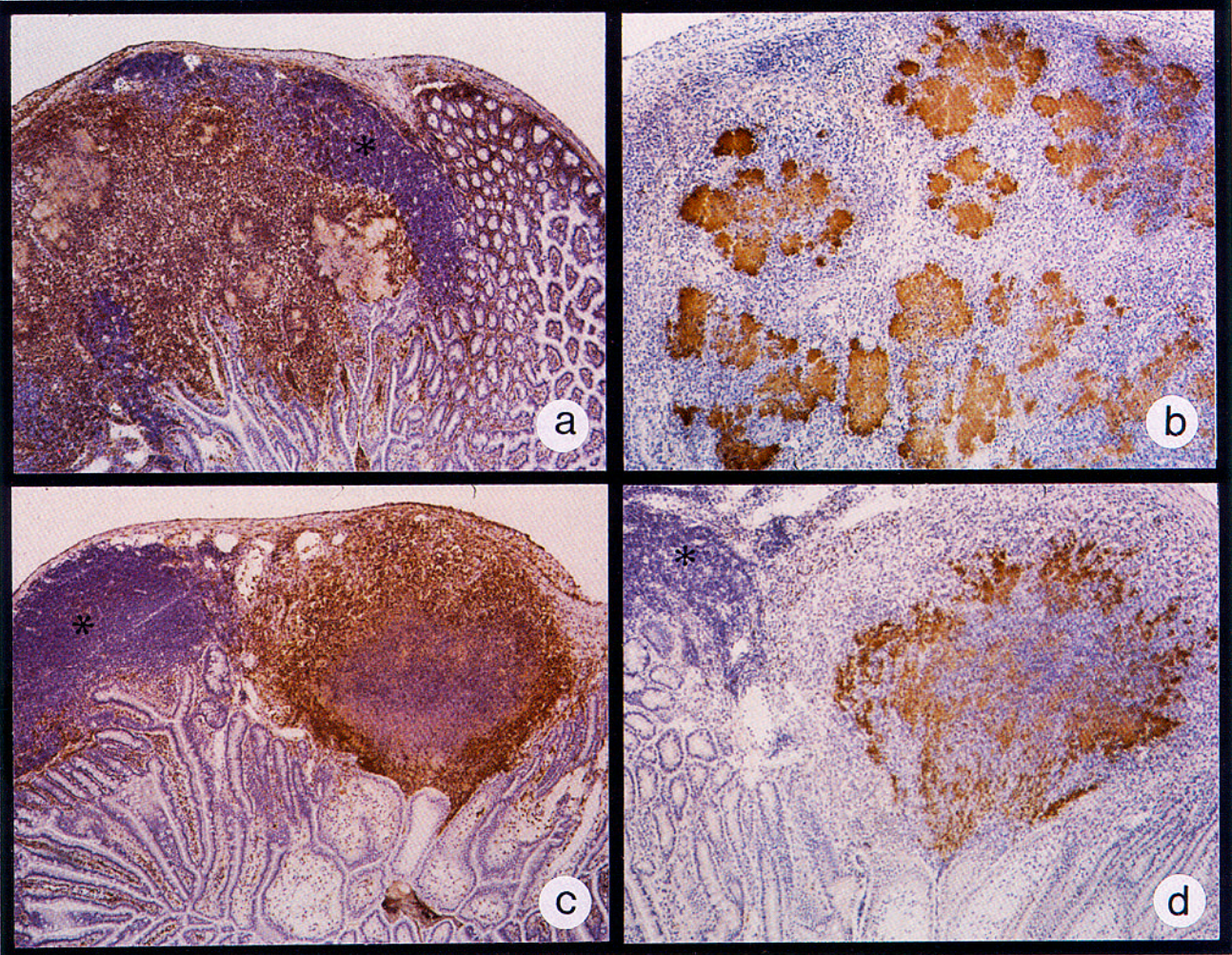
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