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Lymphocyte Apoptosis During Early Phase of *Listeria* Infection in Mice

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During the acute phase of growth of Listeria monocytogenes in spleen and lymph nodes, the infective foci consist of macrophages and neutrophils accompanied by extensive death of lymphocytes. Many of the lymphocytes die by apoptosis. The lesions are found by 48 hours after infection and can regress with time. Depending on the dose, the infected foci can be restricted to the thymus-dependent areas or can occupy the entire lymphoid tissue. The *Listeria* in the lesions are primarily found inside macrophages, but a few are extracellular amid cellular debris. Lymphocyte death appears to be an obligatory step in primary *Listeria* infection, the extent of which is controlled by the early restriction of *Listeria* growth by the innate cellular system. *(Am J Pathol 1997, 151:785–792)*

Listeria monocytogenes is a facultative intracellular bacterium that induces strong T-cell activation. The infection is characterized by the early involvement of neutrophils and macrophages with the participation of a number of early cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), IL-12, and interferon-γ (IFN-γ) (reviewed in Refs.1 and 2). Indeed, non-T-cell-dependent mechanisms contribute to restrict the early growth of *Listeria*. However, T-cell involvement is required to bring about the complete elimination of *Listeria*. Thus, we were surprised to note, like others before us,[3–5] a marked deletion of lymphocytes primarily from the T-cell-dependent areas of lymphoid tissues during the early stages of the infection. In the present histopathological study, we have evaluated this early lesion for the purpose of characterizing it and establishing its pathogenetic significance. We found that infection of lymphoid organs is invariably accompanied by loss of lymphocytes and massive death by apoptosis. These lesions took place within the first 48 hours and then regressed with time.

Materials and Methods

Mice

BALB/c and C57BL/6 mice of either sex were obtained from The Jackson Laboratories (Bar Harbor, ME) and were used at 8 to 16 weeks of age. A variety of gene null mice were obtained from Dr. Robert Schreiber and Kathleen Sheehan at our institution. (Their origin is cited in Table 1.) All mice were clean of pathogens. CB.17 mice were bred at our facilities.

Basic Experimental Protocol

Mice were infected with *Listeria monocytogenes*, strain EGD, at various doses and killed at periodic intervals. Estimates of *Listeria* growth were made in tissue homogenates following conventional procedures. Histology was done on spleen and lymph nodes at periodic times after infection. In some experiments we tested a strain of *Listeria* (DP L2161) defective in the secretion of listeriolysin O. This strain was obtained from Dr. Daniel Portnoy (University of Pennsylvania School of Medicine).[6] Tissue slices were fixed in 10% formalin for 24 hours and then taken to phosphate-buffered saline solution and processed for routine histology. Sections were stained by hematoxylin and eosin. Sections were also stained for apoptotic nuclei using the terminal deoxynucleotide transferase-mediated dUTP-biotin dependent nick-end labeling (TUNEL) assay. Details on the procedure were given in our previous publication.[7] To obtain an estimate of the extent of the lesions, we report here on the number of spleen white pulp profiles that exhibited the lesion per tissue section (sections were approximately 1 cm in length). + are lesions in 10 to 25% of white pulp profiles; ++ are lesions in 26 to 50% of white pulp profiles; +++ are lesions in 51 to 75% of white pulp profiles; and ++++ are lesions in over 75% of white pulp profiles.

Localization of *Listeria* in tissues was done as before[7] using the Difco Laboratory rabbit anti-*Listeria* serum and

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Table 1. Experimental Manipulations that Evaluated the Development of Lymphocyte Apoptosis at 48 hours

No effect of the experimental manipulations: lesions present	Effect of the experimental manipulations: lesions not present
1. Depletion of neutrophils Use of RB6. 8C5 monoclonal antibody[11]	1. *Listeria*-immune mice Previously immune mice given a secondary challenge
2. Depletion of NK cells Use of anti NK 1. 1 antibody[13]	2. Use of listeriolysin O-negative *Listeria*
3. Neutralization of IL-1 Use of neutralizing antibody to IL-1R (JAMA-147)[10]	3. Use of heat-killed *Listeria*
4. Neutralization of IL-12 Use of neutralizing antibodies Tosh/Red T[12]	
5. Neutralization of TNF-α Use of neutralizing antibody to TNF-α (TN3-19.12)[9]	
6. Neutralization of IFN-γ Use of H-22 neutralizing antibody[8]	
7. Use of IFN-γ-α-receptor null mice[19]	
8. Use of Fas-negative mice Examination of BL/6 lpr mice	
9. Use of IL-6 null mice[22]	
10. Use of TNF receptor p55, p75 and p55, and p75 null mice[20,21]	
11. Use of T cells from transgenic mice for hen-egg white lysozyme (HEL) 3A9 T-cell receptor transgenic[24]	
12. Neutralization of NO$^{\cdot}$ production by the use of aminoguanidine[14]	

The sources of the cytokine gene null mice are given in the references. The monoclonal antibodies were developed in our laboratories from hybridomas initially produced by the laboratories indicated in the references. Antibodies were used usually at doses of 300 to 500 μg per mouse intravenously 1 day prior to *Listeria* infection. The lesions were examined at various times, but primarily at 48 hours.

fluorescein conjugated goat anti rabbit IgG. Ultrastructural analysis was performed by standard methods. In short, samples were fixed in 3% cacodylate-buffered glutaraldehyde, postfixed in OSO_4, stained *en bloc* with uranyl acetate, and embedded with a methacrylate resin. Thin sections were stained with lead citrate and examined in a Phillips CM10 electron microscope.

All the monoclonal antibodies used for neutralization of cytokines or cells were the same ones tested in previous papers. These include hamster anti-IFN-γ (H22),[8] hamster anti-TNF-α (TN3–19.12),[9] hamster anti-IL-1 receptor (JAMA-147),[10] rat anti-mouse neutrophil (RB6–8C5),[11] hamster anti-IL-12,[12] and mouse anti-NK1.1.[13] Neutralization of NO$^{\cdot}$ was done as before[14] by giving 6 mg of aminoguanidine intraperitoneally every 12 hours.

Results

The experiments describe the early pathology found in the lymphoid organs of *Listeria*-infected mice. The pathology consisted of massive depletion with death of lymphocytes. A number of experimental manipulations were done in order to study and analyze the pathogenesis of the lesions. The experimental manipulations, such as the use of cytokine-deficient mice or of mice depleted of cytokines or cells by using antibodies, have been studied before. However, these studies were never placed in the framework of this early pathology, that is to say whether a given cell or cytokine contributed either to the development or to the amelioration of the lesion. Thus, all the experimental manipulations reported here were centered exclusively on the examination of the lymphocyte deletion taking place at 48 hours. Clearly, cytokines and cells participate in various stages of *Listeria* infection, and it was not the purpose here to examine their participation beyond the context of the lymphocyte deletion at 48 hours.

Description of the Lesion

Mice were infected with *Listeria* at various doses and killed at different times. Spleen and lymph nodes were examined histologically. In the spleen the lesion was observed primarily in the white pulp and characterized by four features: 1) infiltration by mononuclear cells; 2) the presence of neutrophils, usually distributed in small clusters; 3) a massive depletion of lymphocytes (Figure 1); and 4) a large number of dead cells (Figure 1C), many of which were TUNEL-positive (Figure 2). The lesions were found in the three strains of mice C57BL/6, CB-17, and BALB/c. When *Listeria* was injected in the footpad of the mice, the lesions were found primarily in the deep cortex of the popliteal lymph nodes (Figure 1D).

The extent and localization of the lesions depended on the dose of *Listeria*. At doses below the LD_{50} (10^4 to 5×10^3, depending on the strain of mice), one or two profiles of red pulp were found per tissue section amid regular appearing areas. The general features of the lesion were the same when higher numbers of organisms were injected. The differences in the lesions with higher doses of *Listeria* were with regard to the degree of depletion of lymphoid areas; the number and the degree of macrophage and neutrophil infiltrates and lymphocyte depletion was more pronounced at doses over the LD_{50}. At the lower infective doses the lesions were usually confined to the periarteriolar lymphoid sheath of the spleen, sparing the B cell areas. Injection of larger doses of *Listeria* at the

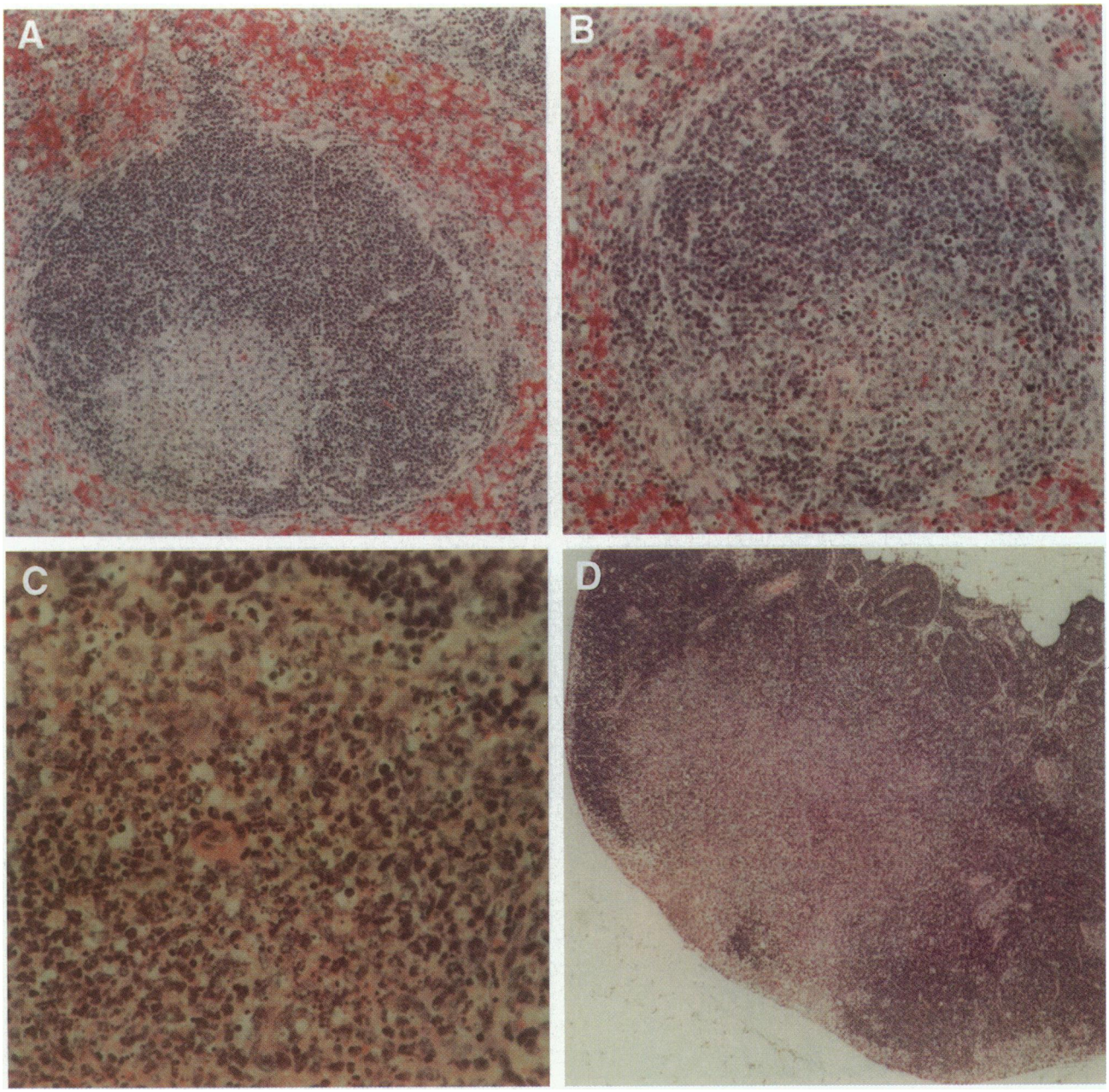

Figure 1. Early phase of *Listeria* infection results in lymphocyte depletion (**A** to **C**). Sections of spleen at 48 hours from *Listeria*-infected mice were stained with hematoxylin and eosin. **A:** Spleen section from a BALB/c mouse infected intravenously with 10^4 *Listeria* shows depletion of lymphocytes in the periarteriolar lymphoid sheaths. **B:** Section of the spleen from a BALB/c mouse infected intravenously with 5×10^4 *Listeria* shows a neutrophic infiltrate with more extensive depletion. **C:** Another area, but shown at a higher magnification. It shows marked infiltrate with neutrophils and macrophages, cellular debris, and apoptotic nuclei. **D:** Section of popliteal lymph node at 48 hours, from BALB/c mice injected with 10^4 *Listeria* into the footpad, shows extensive lymphocytic depletion confined to the deep cortical region (original magnifications of **A**, **B**, and **D**, ×100; **C**, ×400.)

LD_{50} or higher resulted in progressively extensive lesions that extended also to the entire white pulp.

The peak intensity of the lesions was usually at 48 hours after infection (Figure 1A). By 24 hours the changes were minor with a small increase in cellularity but without much infiltration by neutrophils and macrophages. For example, in one experiment CB.17 mice were given a 10× LD_{50} dose (10^5) and tissues were obtained at 12, 24, and 48 hours. The number of *Listeria* recovered in spleen in each of two mice were 3.1×10^4 and 1.4×10^4 at 12 hours, 2.6×10^5 and 5.4×10^5 at 24 hours, and 1×10^7 and 4×10^6 at 48 hours. At 12 hours there was minor depletion of T cells in about 40% of histological profiles, and no $TUNEL^+$ nuclei. By 24 hours the lesions had progressed only slightly with a few more $TUNEL^+$ nuclei. At 48 hours approximately 60% of white pulp profiles were involved with the changes noted above including abundant $TUNEL^+$ nuclei.

After their development at 48 hours, lesions receded by 96 hours (Figure 3) with the disappearance of neutrophils, marked reduction in lymphocyte apoptosis, and

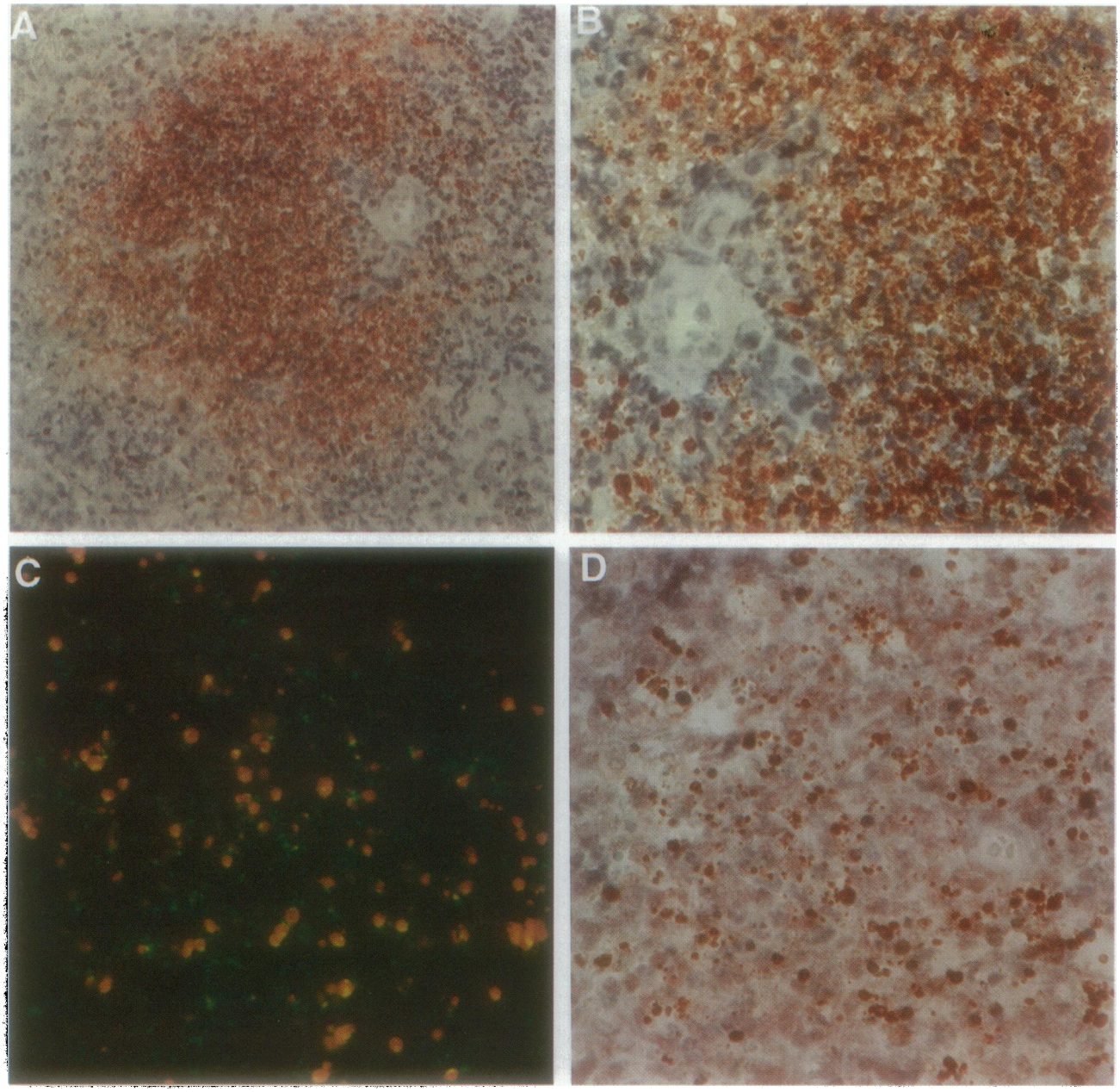

Figure 2. Analysis of the splenic lesions shows numerous apoptotic cells in the infected areas. In **A** to **C** BALB/c mice were infected with 10^4 *Listeria* and in **D**, C57BL/6 mice were infected with 5×10^4 organisms. Shown are sections from spleen examined at 48 hours. In **A**, **B**, and **D** slides were incubated for TUNEL. **C** shows localization of *Listeria* using fluorescein isothiocyanate-conjugated rabbit antibodies to *Listeria* (**green**) and apoptotic nuclei detected by TUNEL using fluorolink Cy3-UTP reagents (**red**). The *Listeria* growth colocalized with apoptotic cells in the depleted areas.

progressive reappearance of lymphocytes. However, macrophages persisted for at least 5 to 8 days.

In three experiments we injected mice (two or three per experiment) with 10^8 dead *Listeria* in the footpads or 10^{10} dead *Listeria* intravenously. The lesions were not present. The lesions were not found in mice that were given up to 10^7 *Listeria* monocytogenes defective in listeriolysin O production. *Listeria* numbers in spleen were 10^3 or less.

The lymphocyte depletion was not observed in mice previously immunized with *Listeria* and then given a secondary challenge. In two experiments, CB-17 mice were primed with 10^4 *Listeria* and 2 weeks later challenged with 10^5 organisms. The *Listeria* counts at day 2 never exceeded 10^2 organisms. The spleen did not show depletion or lymphocyte apoptosis.

TUNEL$^+$ Cells

The lesions were characterized by abundance of TUNEL-positive cells (Figure 2). Many of the TUNEL$^+$ cells had the typical appearance of lymphocytes (Figure 2). At doses of *Listeria* at approximately the LD_{50}, when the lesions were prominent in the T-cell-dependent areas of

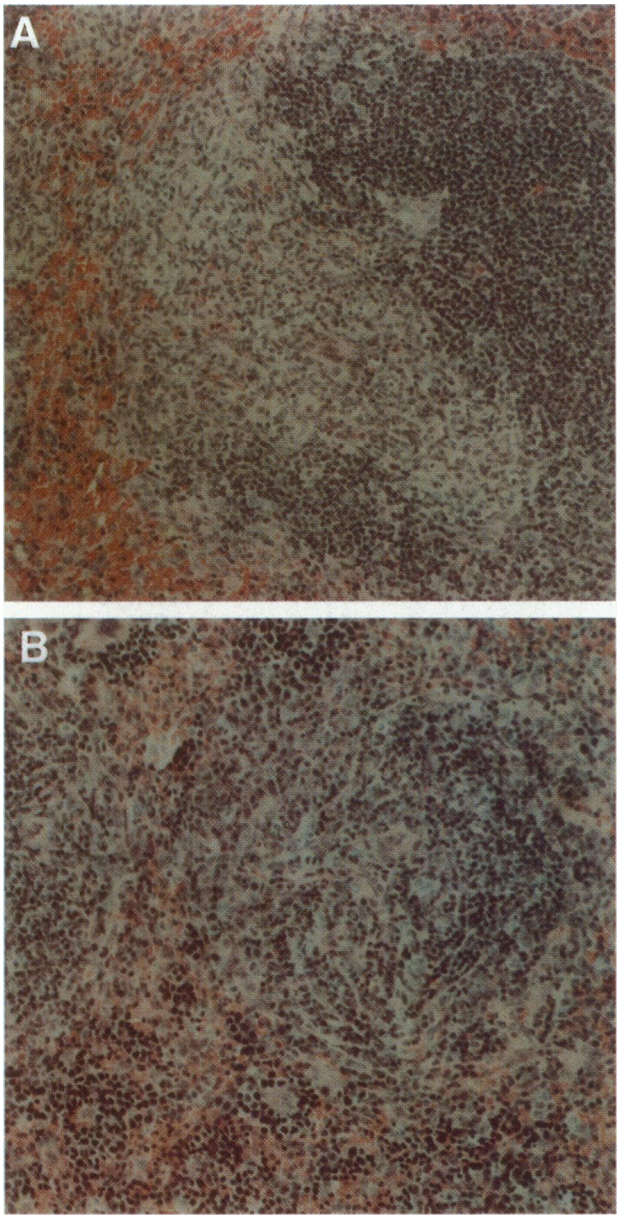

Figure 3. Recovery of the lesions. **A:** Section of spleen from BALB/c mouse 96 hours after infection intravenously with 10^4 *Listeria*. More macrophages and a few neutrophils are present in the depleted periarteriolar area, which now contain more lymphocytes. **B:** Spleen section at 144 hours after infection. It indicates that the repopulation of the periarteriolar lymphoid sheath is more marked.

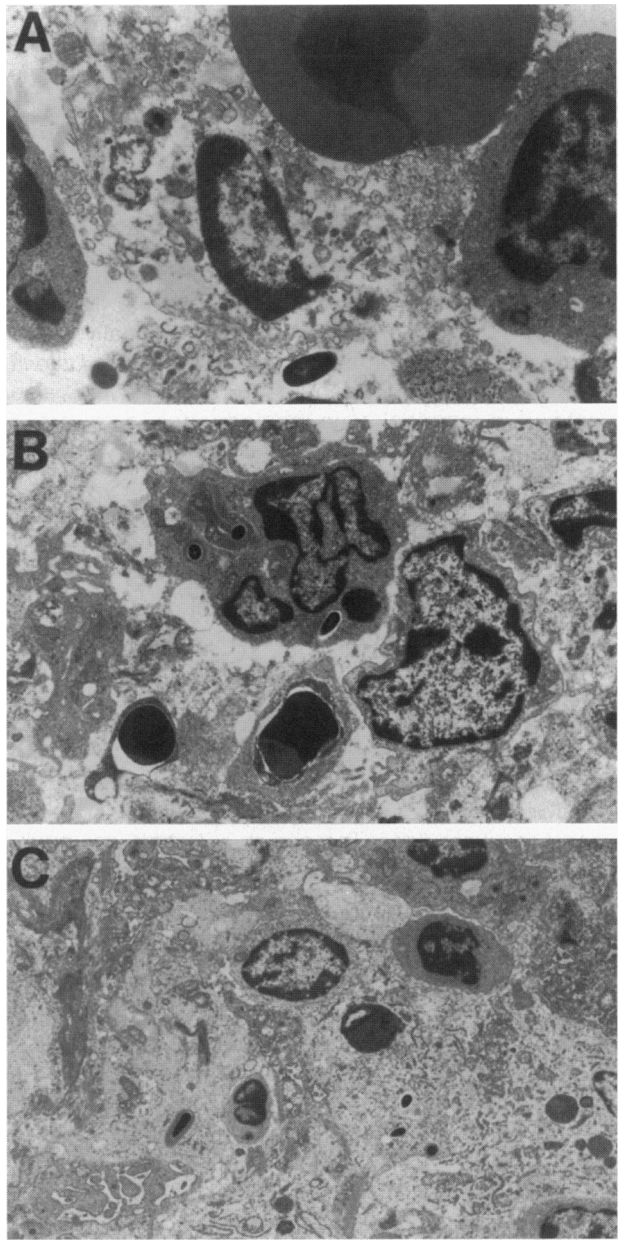

Figure 4. Localization of *Listeria* by electron microscopy. **A** shows an area containing cellular debris and an extracellular *Listeria* (original magnification ×5160). **B** shows apoptosis of lymphocytes in close proximity to *Listeria*-laden macrophages. The lymphocytes do not carry the organism (×3900). **C** shows macrophages found in the periarteriolar lymphoid sheath containing *Listeria* organisms in membrane-bound vacuoles or in the cytoplasm. The latter are surrounded by actin filaments (×2200). All micrographs are from spleens obtained 48 hours after infection of CB.17 mice with 10^4 organisms.

the spleen, so were the profiles of TUNEL$^+$ cells (Figures 1 and 2). The B cell follicles were usually spared of dead cells. However, at a higher dose, TUNEL$^+$ cells were found throughout the entire white pulp. Thus, the involvement of one particular zone depended entirely on the dose of organisms. As mentioned above, few TUNEL$^+$ cells were found at 24 hours. They were shown most abundantly at the 48-hour time point decreasing rapidly after that. Thus, the lymphocyte apoptosis developed abruptly in the 24- to 48-hour period of time.

Localization of Listeria

By immunofluorescence the *Listeria* organisms were found only in the lesions and in close proximity to the TUNEL$^+$ cells (Figure 2C). Many appeared to be extracellular. The optimal time for detection was at 48 hours, after which there was a marked decline in visible organisms.

Ultrastructural studies were made of the lesions at 48 hours (Figure 4). The majority of intracellular *Listeria*

resided in phagocytic vacuoles or in the cytoplasm of macrophages (Figure 4, B and C). A minority of macrophages were necrotic. A few extracellular organisms were found in areas of cell deletion amid cellular debris (Figure 4A). The latter comprised approximately 5% of the *Listeria* found in the samples. An occasional neutrophil contained *Listeria* in vacuoles. Profiles of apoptotic lymphocytes were abundant, which confirmed that the majority of TUNEL$^+$ cells were lymphocytes dying by apoptosis (Figure 4B). However, *Listeria* was never localized in either viable or apoptotic lymphocytes. The apoptotic lymphocytes were usually found in close proximity to macrophages containing *Listeria* (Figure 4B). *Listeria*-laden macrophages were also identified in marginal zone macrophages, as in previous reports[4] (not shown).

The Role of Neutrophils

We examined whether the lesions were associated with the presence of neutrophils. Deletion of neutrophils was accomplished as in our previous studies[7] by the injection of 300 μg of the monoclonal antibody RB6–8C5.[15–18] Two CB.17 mice that only received 10^4 organisms showed 5.5×10^6 and 4.4×10^7 at 48 hours with about one-third of white pulp profiles showing depletion in the periarteriolar lymphoid sheath. Similar results were obtained in a second experiment where time sequence evaluations were done. The growth of *Listeria* was markedly increased by neutrophil depletion (more than 10^9 organisms at 48 hours in each of the two mice). The time of development was similar to controls not injected with RB6–8C5 antibody, peaking at 48 hours. However, the lesions were more extensive including both of the entire white pulp areas containing apoptotic nuclei. We concluded that neutrophils were responsible for the control of *Listeria* growth and by doing this, established the degree of lymphocyte apoptosis.

The Role of Cytokines

We asked whether the extensive apoptosis was associated with the acute release of cytokines. For this, we used a panel of neutralizing anticytokine antibodies that we had experience with from our previous work. We also used mice lacking genes for a given cytokine. From Table 1 we can note that neutralizing IFN-γ, TNF, IL-1, or IL-12 had no effect on the development of the lesions. As we had shown before, the early 48-hour growth of *Listeria* after intravenous injection was not affected. The depletion of T-cell areas was still very evident.

The lesions were found in mice lacking IFN-γ receptor α-chain,[19] the TNF receptors,[20,21] or the IL-6 gene[22] (Tables 1 and 2). Mice lacking the p55 TNF receptor had severe infection by 48 hours (mean of 1.4×10^8 organisms in the spleen). The lymphocyte depletion was extensive, comprising all areas of the white pulp and also foci of infection in the red pulp. The same extensive lesions were found in mice lacking both the p55 and p75 receptor (mean of *Listeria* counts was 3.9×10^7). Normal

Table 2. Neutralization of Cytokines and NO· Does Not Affect the Development of Lymphocyte Apoptosis

Manipulation L2.309	*Listeria* counts	% lesions
Experiment 1		
Irrelevant antibody:	2.1×10^6	+
L2309 (300 μg)	0.5×10^6	+
	3.5×10^6	++
Anti-IFN-γ: H22 (300 μg)	2.6×10^6	++
	2.8×10^6	++
Anti-TNF-α: TN319.12 (300 μg)	9.5×10^6	+++
	8.3×10^6	++
Anti-IL-1R: JAMA 147 (300 μg)	1.5×10^6	++
	2.8×10^6	+
Anti-IL-12: Tosh/Red T	2.3×10^6	+
(250 μg of each)	4.8×10^6	+
NO· neutralization:	5.5×10^5	+
aminoguanidine	7.3×10^5	+
Experiment 2		
IFN-γ α-chain receptor	1.6×10^7	+++
null mice	8.8×10^6	+++
Normal C57BL/6 mice	3.9×10^5	++
	1.2×10^5	+
Experiment 3		
BL6/lpr	6.4×10^5	++
	6.0×10^5	++
	7.5×10^5	++
3A9 T-cell receptor transgenic	0.96×10^5	++
(B10.BR)	1.2×10^5	+
Normal mice	7.5×10^5	++
	2.7×10^5	++
	1.3×10^5	++
Experiment 4		
TNF p55 receptor null mice	2×10^8	++++
	2.7×10^7	++++
	2.1×10^8	++++
	1.3×10^8	++++
TNF p75 receptor null mice	3.5×10^6	++
	9.8×10^6	++
	1.0×10^6	++
	1.5×10^6	++
TNF p55 and p75 receptor	1.5×10^7	++++
null mice	2.3×10^7	++++
	8×10^7	++++
Normal mice	8.3×10^6	++
	0.6×10^6	++
	1.5×10^6	++

The table shows four representative experiments. Experiment 1 used CB.17 or BALB/c mice infected with 10^4 *Listeria* organisms intravenously. Experiments 2 to 4 used C57BL/6 mice given 5×10^4 *Listeria* intravenously. All *Listeria* numbers represent spleen determinations after 48 hours of infection. Each number represents results from a single mouse. +, 10 to 25%; ++, 26 to 50%; +++, 51 to 75%; ++++, over 75%.

mice and mice lacking the p75 receptor showed 3.9×10^6 and 9.6×10^6 *Listeria* counts and the lesions were more restricted to the periarteriolar lymphoid sheath of the white pulp. TUNEL$^+$ cells were found with all, but more extensively in the p55 null and p55 plus p75 null mice.

We had previously reported that NO$^\bullet$ was involved in *Listeria* resistance.[1,14] Our protocols consisted of injecting the drug aminoguanidine, an inhibitor with selectivity for the inducible nitric oxide synthase. In mice given aminoguanidine the lesions developed in the spleen. In other experiments to be reported elsewhere we found the development of the lesion in mice lacking the gp91^{phox} gene of the cytochrome *b* oxidase. Thus, neither reactive oxygen or nitrogen intermediates contributed to its development.

In one experiment we infected C57BL/lpr mice.[23] The spleen lesions were evident, identically as in the normal C57BL/6 mice (ie, 20 and 40% of profiles of white pulp contained lesions). TUNEL$^+$ cells were evident (Table 2).

We concluded that although cytokines participate in the curbing of the infection, these molecules were not involved in lymphocyte depletion nor was lymphocyte apoptosis caused by a Fas/Fas ligand interaction.[23]

Role of NK Cells

The role of NK cells in the development of the lesion was examined in C57BL/6 mice treated with monoclonal antibody NK1.1. In the five mice treated with antibodies we did not find an effect in *Listeria* number at 48 hours compared with the control mice (9.8×10^6, 9.9×10^6, 2.9×10^6, 4.7×10^6, and 0.9×10^6 compared with 2.3×10^6 and 2.5×10^6 in the two control mice). The lesions were identical in the experimental histological sections. The injected mice showed 65, 42, 57, 72, and 71% with depleted profiles; controls showed 47 and 76%. TUNEL$^+$ cells were found in both. Of note is that by 7 days the control mice had cleared the infection, while five controls still maintained from 1.3×10^4 to 1.1×10^5 organisms.

Specificity of T Cells

Mice expressing the T-cell receptor transgene for HEL[24] and showing about 70% of T cells specific for HEL were injected with 5×10^3 *Listeria* and examined at 48 hours. The mice developed the spleen pathology described here (Table 2). Thus, there was no lymphocyte specificity in the development of this lesion.

Discussion

In summary, as part of the infectious process with *Listeria* there is an acute lesion in the lymphoid organs characterized by massive lymphocyte apoptosis at sites where macrophages and neutrophils infiltrate and where *Listeria* is localized. The focal depletion of lymphocytes that accompanies the macrophage and neutrophil infiltration is likely accounted for by *in situ* death of the cells. We cannot prove, however, that some lymphocytes may have left the site of infection. Peripheral blood counts did not give support to this and were within the normal range at 48 hours (8 to 11,000 per mm^3). Another point is that clearly there was no selectivity of the lesion for one or another lymphocyte subset. The lesion could extend to the entire white pulp affecting B and T cells or be circumscribed to the periarteriolar lymphoid sheath depending on *Listeria* dose. The lack of selectivity was confirmed by flow cytometry of CD4 or CD8 T cells, which showed no particular skewing in the infected mice (Ref. 3 and our own data).

We cannot at this time attribute the lesion to a response of the host. As noted, all attempts to attribute the development of the lesion to a given cytokine, to NO$^\bullet$, to reactive oxygen intermediates, or to the Fas/L-Fas interaction failed (Table 1). It is likely that the lesions are caused by a reaction of the lymphocyte with a soluble product of *Listeria*. Although listeriolysin O is a strong candidate, at this time we have no direct proof, but experiments are in progress. *Listeria* organisms not expressing listeriolysin O were not virulent, showed little growth in tissues, and were not accompanied by lymphocyte death.

Our interpretation is that the development of the lesions takes place at a time of rapid growth of *Listeria* before macrophages and neutrophils are able to fully restrict their growth. At such a time, some extracellular *Listeria* can be found amid cellular debris. Thus, during this period there could be contact with extracellular products of *Listeria*. The predominance of the lesion in the T-cell areas may be accounted for by the distribution of *Listeria* at those sites. Indeed, our ultrastructural analysis surprisingly disclosed organisms in the T-cell area. Our expectation was that much of the *Listeria* would have been taken by macrophages of the marginal zones (in the case of the spleen), as reported by Conlon.[5] Once *Listeria* is confined to the phagocytes, the extracellular release of this putative apoptotic molecule is most likely reduced, and then lymphocyte immunity can rapidly follow. Indeed, after the 48-hour period, it is not easy to detect *Listeria* organisms.

We believe that lymphocyte immunity cannot effectively develop during the early period and that resistance becomes entirely dependent on the macrophage and the neutrophil. Thus, neutrophils and macrophages must reduce the early growth of *Listeria* in order for lymphocyte immunity to take place. The exacerbation of the lesions in neutrophil-depleted mice gives credence to this interpretation. Finally, the participation of apoptosis in the early stages of listeriosis is important. We previously reported extensive apoptosis of *Listeria*-infected cells as a major component of the acute liver infection.[7] Although lymphocytic death was not the result of intracellular *Listeria*, the present findings of apoptosis indicate that a common organism-associated pathway of cell death may be at work.

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