INFECTION AND IMMUNITY, Sept. 1996, p. 3937–3941 Vol. 64, No. 9
0019-9567/96/$04.00+0

Apoptosis of Lymphocytes in Mice Induced by Infection with *Rickettsia tsutsugamushi*

SHIRO KASUYA,[1]* ISAO NAGANO,[1] TSUNEKO IKEDA,[2] CHITOSHI GOTO,[1] KUNIYASU SHIMOKAWA,[2] AND YUZO TAKAHASHI[1]

Department of Parasitology[1] and Department of Laboratory Medicine,[2] Gifu University School of Medicine, Tsukasa-machi 40, Gifu City 500, Japan

Received 8 April 1996/Returned for modification 23 May 1996/Accepted 28 June 1996

Histological examinations of mice infected with either a lethal (Karp) or a self-limitating (Gilliam) strain of *Rickettsia tsutsugamushi* were performed. Tingible body macrophages in the spleen and necrotizing lymphadenitis in regional lymph nodes were prominent only in the former. Apoptotic legions in the lymphocytes of these organs were clearly demonstrated by histochemical and electron microscopical examinations.

Scrub typhus, or tsutsugamushi disease, has become an important febrile illness in both Japan (15) and Korea (5) in recent years. Because of the vertical transmission of *Rickettsia tsutsugamushi* among specific species of trombiculid mites, it appears that rickettsial strains are reserved within a limited region (8). Although Karp, Kato, and Gilliam strains have been used as a set of standard strains for diagnosis and experimental studies, many isolated strains which are distinguishable from prototype strains both in antigenicity and in virulency have been identified in each region (24, 26–28). There have been several death cases reported each year in the northern part of Japan's main island of Honshu (1, 17); however, they have seldom been reported on the island of Kyushu (west of the main island), although this has been the most prevalent area for the disease in Japan (19). The latter is associated with infections by the Kawasaki or Kuroki strain (14, 26).

Disseminated intravascular coagulation (DIC) is a terminal stage of the clinical course of infections (1, 18). However, the mechanisms of developing DIC have been obscure. Immunosuppression at both humoral and cellular levels has been reported in experimental infections among animals (9, 10). In addition, histological changes such as increases in white pulp in the spleen, the prominence of Kupffer cells in the liver, and fibrous peritonitis have been reported (4). However, these findings do not appear to be significantly involved in shedding light on the differences between lethal infections and self-limiting ones; moreover, findings concerning DIC have not been detected in our experimental studies (unpublished data). In the present study, we further analyzed histological changes in BALB/c mice infected with the lethal Karp strain compared with the nonlethal Gilliam strain and clarified apoptotic death of lymphocytes in lethal infections of *R. tsutsugamushi.*

The Karp and Gilliam strains of *R. tsutsugamushi* supplied by the Toyama Prefectural Institute of Public Health were maintained by mouse passage as described previously (28). The 50% lethal dose for BALB/c mice infected intraperitoneally with the Karp strain was 0.25 ml of spleen homogenate diluted by SPG solution (3) to $1:10^{6.8}$ (wt/vol) calculated by the method described by Reed and Muench (16). Experimental mice (five in each group) were inoculated intraperitoneally with more than 100 50% lethal doses, i.e., 0.25 ml of $1:10^4$ (wt/vol) spleen homogenate from previously infected mice 10 to 12 days after infection. The Gilliam strain is nonlethal to BALB/c mice; therefore, experimental mice were infected with 0.25 ml of 1:10 (wt/vol) spleen homogenate from infected nude mice (ICI strain) also from 10 to 12 days after infection. Control mice were injected with 0.25 ml of normal spleen homogenate (1:10). Histological changes were examined at days 7, 14, and 21, respectively. The examinations were performed only during the survival period of the lethal infections.

We could not find any specific changes in the preparation of lung, heart, kidney, adrenal capsula, cervical lymph node, thymus, bone marrow, and brain cells fixed with neutral 10% Formalin and stained with hematoxylin and eosin in any stage of each infection group, compared with control mice. Enlargement of white pulp and infiltrations of neutrophils and plasma cells into the spleen in each infection group on day 7 were observed. In the Karp infection group, tingible body macrophages, which are large cells with abundant cytoplasm containing phagocytized debris as described by Valk and Meijer (25), were prominent in the spleen from the 2nd week, while they were seldom observed in the group infected with the Gilliam strain and in the controls. Mononuclear cell infiltrations were prominent near vessels in the livers of mice infected with either the Gilliam or the Karp strain from the 1st week of the infection. Only the more virulent strain caused necrotizing lymphadenitis in mesenteric lymph nodes in the 2nd week; i.e., small round particles heavily stained with hematoxylin were scattered throughout (Fig. 1). No mice infected with the Karp strain survived until the 3rd week, while no changes in the organs of Gilliam-infected mice could be detected in the 3rd week.

Because tingible body macrophages and necrotizing lymphadenitis are thought to be evidence of apoptotic lesions (22), we used a method to detect fragmented nuclei histochemically using ApopTag (S7100-KIT; Oncor, Inc.) in the 2nd-week specimens. Briefly, 4-μm-thick sections were deparaffinized and treated with terminal deoxynucleotidyltransferase to add digoxigenin nucleotides to the 3′ ends of DNA. Then, the digoxigenin was immunostained with anti-digoxigenin antibody and peroxidase-labelled secondary antibody. Spleen and mesenteric lymph node cells from Karp-infected mice were strongly stained compared with controls (only a stained preparation of mesenteric lymph node is shown in Fig. 2), while these changes were not detected in the Gilliam-infected mice.

Details of affected cells in the spleens and mesenteric lymph nodes were further examined by transmission electron microscopy at the 5th and 10th days of infection. Changes in specimens from the 5th day of infection were few, while cells main-

* Corresponding author.

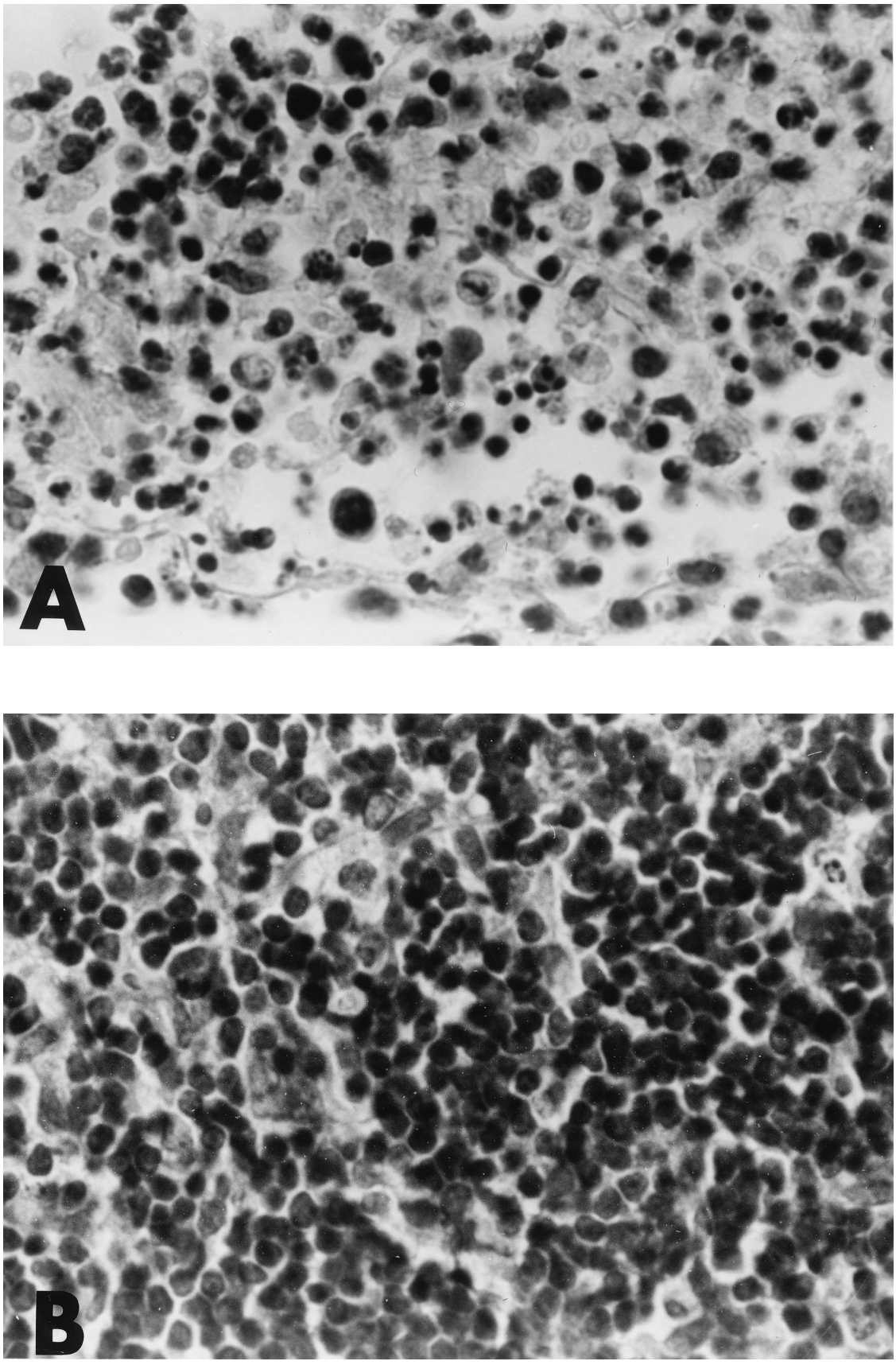

FIG. 1. (A) Mesenteric lymph node 14 days after Karp infection. Scattered particles, stained strongly with hematoxylin and suggested to be condensed nuclei, are prominent. (B) Control mesenteric lymph node. Original magnification of hematoxylin and eosin staining, ×400.

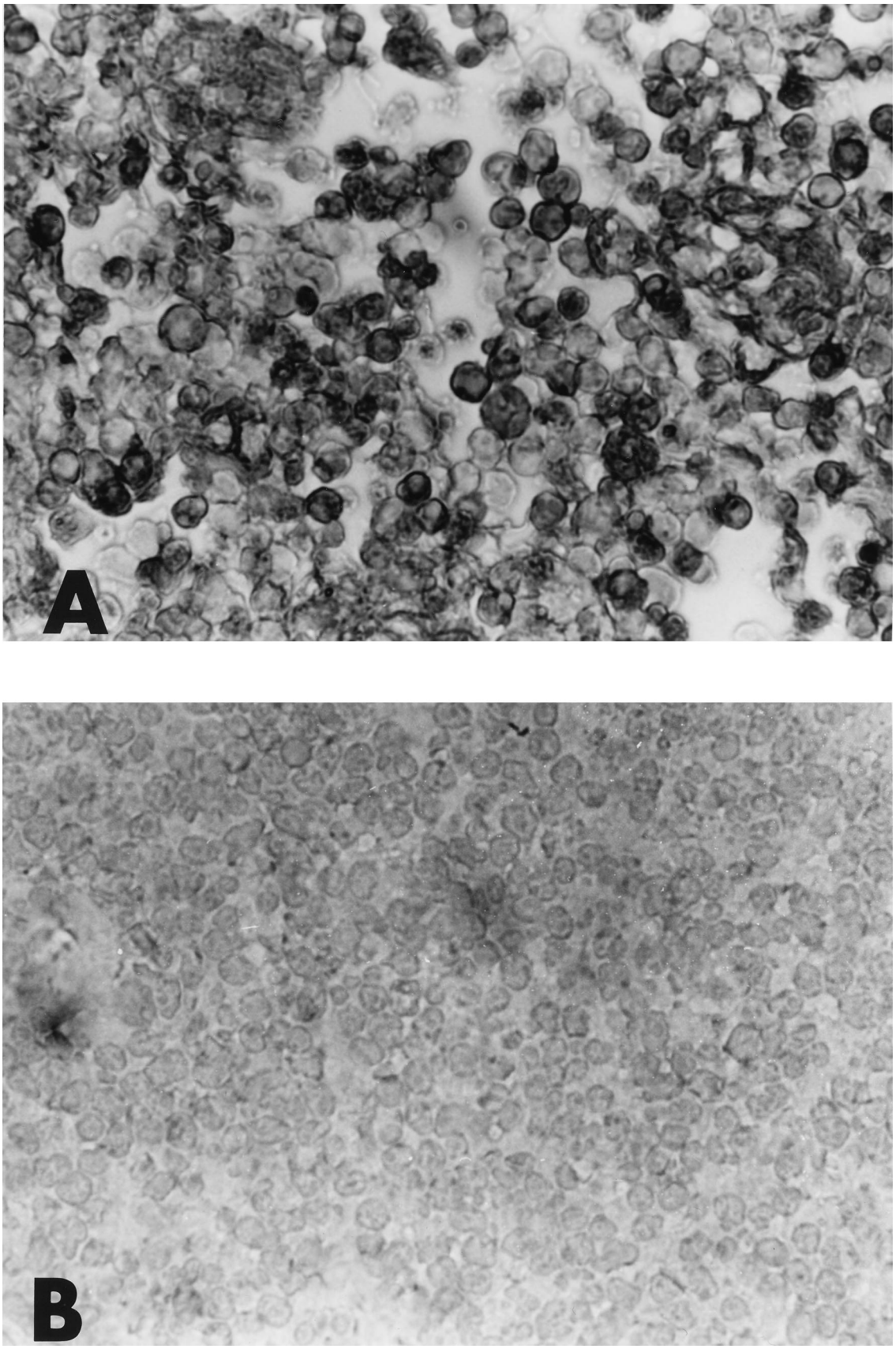

FIG. 2. (A) Mesenteric lymph node 14 days after infection. Staining for apoptosis is remarkable in lymphoid cells. (B) Control mesenteric lymph node. Original magnification of ApopTag staining, ×400.

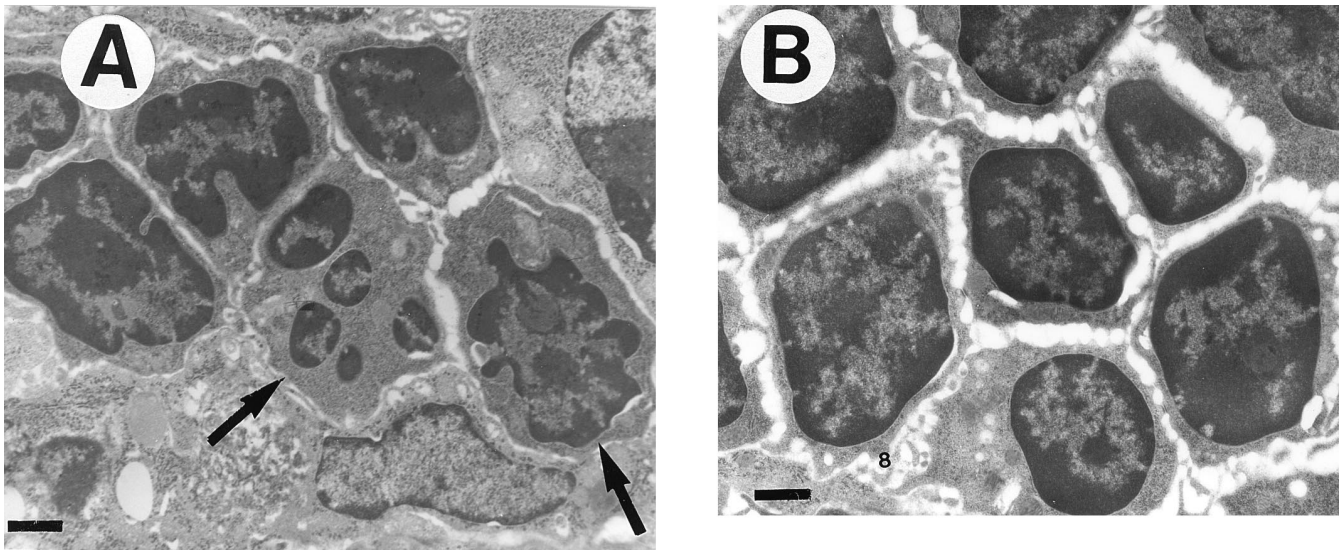

FIG. 3. Electron micrograph of spleen. (A) Affected lymphocytes on 10th day of Karp infection (arrows). Segmentation of nuclei is observed, but the cytoplasm maintains its integrity. Bar, 1 μm. Magnification, ×7,000. (B) Control. Bar, 1 μm. Magnification, ×8,000.

taining integrity in the cytoplasm with a condensed or fragmented nucleus were easily detected both in the spleens (Fig. 3) and in the mesenteric lymph nodes (Fig. 4) in only the Karp-infected mice from the 10th day of infection. These are ultrastructural changes characteristic of apoptosis (7), and the affected cells were lymphocytes both in the spleens and in the mesenteric lymph nodes by the ultrastructures of the cytoplasm. Rickettsial bodies could not be found in these affected cells by this point.

Kikuchi has reported histiocytic necrotizing lymphadenitis (HNL) observed mainly in cervical lymph nodes in humans (11). The characteristics of histological features reported by Kikuchi are quite similar to those of our necrotizing lymphadenitis in the mesenteric lymph nodes from mice infected with highly virulent *R. tsutsugamushi*. The mesenteric lymph nodes are regional lymph nodes in our experimental system, i.e., intraperitoneal infections, while no change was observed in the cervical lymph nodes. Furthermore, Liu et al. clearly demonstrated that apoptotic processes were prominent in HNL (13). The cause of HNL is not confirmed yet; however, infections such as those caused by herpes virus (20) and Epstein-Barr virus (21) have been suspected. Also, evidence of infections by *Toxoplasma gondii* (12) or *Yersinia enterocolitica* (6) were demonstrated in HNL patients. Our report may offer a new view of the cause of HNL.

In addition, we demonstrate for the first time that prominent apoptotic changes occur in lymphocytes in the regional lymph nodes and spleens in rickettsial infections. Apoptotic death is believed to be an important process in human immunodeficiency virus infection or AIDS (7). It has become clear that expanded T lymphocytes induced by viral infection are susceptible to apoptotic cell death (2). Enlargement of the white pulp of the spleen or regional lymph nodes, which suggests proliferative processes, is observed equally for BALB/c mice infected with either the Karp or the Gilliam strain. However, prominent apoptosis begins only in Karp-infected mice. Strain-

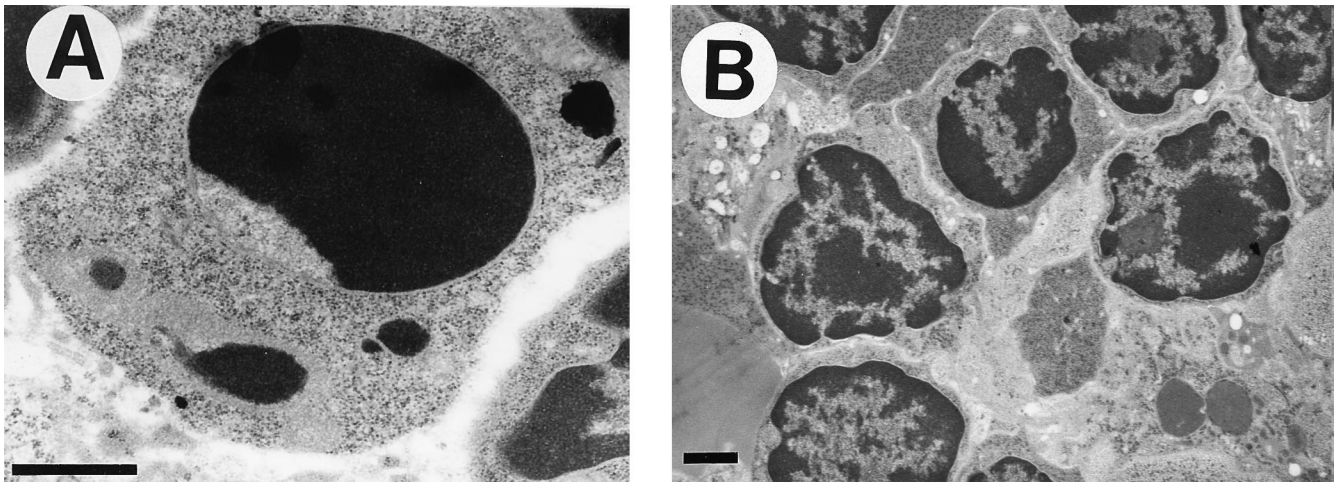

FIG. 4. Electron micrograph of mesenteric lymph node. (A) Affected lymphocyte on 10th day of Karp infection. Condensation of nucleus is prominent. Bar, 1 μm. Magnification, ×17,000. (B) Control. Bar, 1 μm. Magnification, ×6,000.

specific proteins among *R. tsutsugamushi* strains were detected in the 54- to 56-kDa molecular mass range (23); therefore, these strain-specific regions of the rickettsial body may also be associated with apoptotic mechanisms. Furthermore, an apoptotic process in lymphocytes may explain immunosuppression in rickettsial infections; however, it is still unclear whether the process contributes to the production of DIC.

REFERENCES

1. **Akai, H., M. Ito, T. Shindo, H. Yamazaki, and Y. Ishizuka.** 1982. An autopsy case of tsutsugamushi disease complicated with disseminated intravascular coagulation syndrome. Jpn. J. Med. **71:**59–64. (In Japanese.)
2. **Akbar, A. N., N. Bothwick, M. Salmon, W. Gombert, M. Bofill, N. Shamsadeen, D. Pilling, J. E. S. Pett, and G. Janossy.** 1993. The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infection: the role of apoptosis in T cell memory. J. Exp. Med. **178:**427–438.
3. **Bovarnick, M. R., J. C. Miller, and J. C. Snyder.** 1950. The influence of certain salts, amino acids, sugars, and proteins upon the stability of rickettsiae. J. Bacteriol. **59:**509–522.
4. **Catanzaro, P. J., A. Shirai, P. K. Hilderbrandt, and J. V. Osterman.** 1976. Host defenses in experimental scrub typhus: histopathological correlates. Infect. Immun. **13:**861–875.
5. **Chang, W.-H., J.-S. Kang, W.-K. Lee, M.-S. Choi, and J.-H. Lee.** 1990. Serological classification by monoclonal antibodies of *Rickettsia tsutsugamushi* isolated in Korea. J. Clin. Microbiol. **28:**685–688.
6. **Feller, A. C., K. Lennert, H. Stein, H. D. Bruhn, and H. H. Wuthe.** 1983. Immunohistology and aetiology of histiocytic necrotizing lymphadenitis. Report of three instructive cases. Histopathology **7:**825–839.
7. **Gougeon, M. L., and L. Montagnier.** 1992. New concepts in the mechanisms of $CD4^+$ lymphocyte depletion in AIDS, and the influence of opportunistic infections. Res. Microbiol. **143:**362–368.
8. **Iwasa, M., S. Kasuya, N. Noda, A. Hioki, A. Ito, and H. Ohtomo.** 1990. Trombiculid mites (Acari:Trombiculidae) and *Rickettsia tsutsugamushi* isolated from wild rodents in a new endemic area of Japan. J. Med. Entomol. **27:**501–508.
9. **Jerrells, T. R.** 1985. Immunosuppression associated with the development of chronic infections with *Rickettsia tsutsugamushi*: adherent suppressor cell activity and macrophage activation. Infect. Immun. **50:**175–182.
10. **Jerrells, T. R., and J. V. Osterman.** 1983. Development of specific and cross-reactive lymphocyte proliferative responses during chronic immunizing infections with *Rickettsia tsutsugamushi*. Infect. Immun. **40:**147–156.
11. **Kikuchi, M.** 1972. Lymphadenitis showing focal reticulum cell hyperplasia with nuclear debris and phagocytes: a clinicopathological study. Acta Haematol. Jpn. **35:**379–380. (In Japanese.)
12. **Kikuchi, M., T. Yoshizumi, and H. Nakamura.** 1977. Necrotizing lymphadenitis: possible acute toxoplasmic infection. Virchows Archiv A Pathol. Anat. Histopathol. **376:**247–253.
13. **Liu, Q., K. Ohshima, T. Shinohara, and M. Kikuchi.** 1995. Apoptosis in histiocytic necrotizing lymphadenitis. Pathol. Int. **45:**729–734.
14. **Ohashi, N., A. Tamura, H. Sakurai, and S. Yamamoto.** 1990. Characterization of a new antigenic type, Kuroki, of *Rickettsia tsutsugamushi* isolated from a patient in Japan. J. Clin. Microbiol. **28:**2111–2113.
15. **Rapmund, G.** 1984. Rickettsial diseases of the Far East: new perspectives. J. Infect. Dis. **149:**330–338.
16. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
17. **Sakai, H., K. Honda, A. Oowada, and N. Shimada.** 1987. A fatal case of probable tsutsugamushi disease in Saitama Prefecture. With particular reference to 8 lethal cases occurring in Japan from 1962 to 1985. Jpn. J. Med. **76:**736–739. (In Japanese.)
18. **Sassa, H., T. Shibata, M. Oba, M. Okubo, T. Niwa, and E. Matsui.** 1983. An autopsy case of the first occurrence of tsutsugamushi disease in Gifu Prefecture. Jpn. J. Med. **73:**401–407. (In Japanese.)
19. **Shishime, E., N. Tachibana, A. Okayama, J. Ishizaki, T. Yokota, and K. Tsuda.** 1987. Clinical study on tsutsugamushi disease in Miyazaki District. J. Jpn. Assoc. Infect. Dis. **61:**297–302. (In Japanese.)
20. **Sumiyoshi, Y., M. Kikuchi, K. Ohshima, S. Yoneda, S. Kobari, M. Takeshita, Y. Eizuru, and Y. Minamishima.** 1993. Human herpesvirus-6 genomes in histiocytic necrotizing lymphadenitis (Kikuchi's disease) and other forms of lymphadenitis. Am. J. Clin. Pathol. **99:**609–614.
21. **Takada, K., M. Iwanaga, T. Osato, C. Mikuni, I. Maekawa, T. Kawamura, and T. Miyake.** 1980. Elevated antibody titers to Epstein-Barr virus in subacute necrotizing lymphadenitis. Igaku No Ayumi. **112:**194–196. (In Japanese.)
22. **Takano, Y., M. Saegusa, and M. Okudaira.** 1993. Pathologic analysis of non-overt necrotizing type Kikuchi and Fujimoto's disease. Acta Pathol. Jpn. **43:**635–649.
23. **Tamura, A., N. Ohashi, H. Urakami, K. Takahashi, and M. Oyanagi.** 1985. Analysis of polypeptide composition and antigenic components of *Rickettsia tsutsugamushi* by polyacrylamide gel electrophoresis and immunoblotting. Infect. Immun. **48:**671–675.
24. **Tange, T., N. Kanemitsu, and Y. Kobayashi.** 1991. Analysis of immunological characteristics of newly isolated strains of *Rickettsia tsutsugamushi* using monoclonal antibodies. Am. J. Trop. Med. Hyg. **44:**371–381.
25. **Valk, P. V. D., and C. J. L. M. Meijer.** 1992. Reactive lymph nodes, p. 233–251. *In* S. S. Sternberg (ed.), Histology for pathologists. Raven Press, Ltd., New York.
26. **Yamamoto, S., N. Kawamura, A. Tamura, H. Urakami, N. Ohhashi, M. Murata, Y. Yoshida, and A. Kawamura, Jr.** 1986. Immunological properties of *Rickettsia tsutsugamushi*, Kawasaki strain, isolated from a patient in Kyushu. Microbiol. Immunol. **30:**611–620.
27. **Yamashita, T., S. Kasuya, S. Noda, I. Nagano, and J.-S. Kang.** 1994. Transmission of *Rickettsia tsutsugamushi* strains among humans, wild rodents, and trombiculid mites in an area of Japan in which tsutsugamushi disease is newly endemic. J. Clin. Microbiol. **32:**2780–2785.
28. **Yamashita, T., S. Kasuya, S. Noda, I. Nagano, S. Ohtsuka, and H. Ohtomo.** 1988. Newly isolated strains of *Rickettsia tsutsugamushi* in Japan identified by using monoclonal antibodies to Karp, Gilliam, and Kato strains. J. Clin. Microbiol. **26:**1859–1860.

Editor: A. O'Brien