JOURNAL OF VIROLOGY, Feb. 1991, p. 952–956
0022-538X/91/020952-05$02.00/0

Vol. 65, No. 2

NOTES

Replication of Aleutian Mink Disease Parvovirus in Lymphoid Tissues of Adult Mink: Involvement of Follicular Dendritic Cells and Macrophages

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Received 19 July 1990/Accepted 15 October 1990

By using strand-specific in situ hybridization and immunohistochemistry, evidence for replication of the Aleutian mink disease parvovirus was observed in cells resembling macrophages and cells resembling follicular dendritic cells at 10 days after infection but only in macrophages at 60 days. Sequestration of the Aleutian mink disease parvovirus in larger numbers of macrophages and follicular dendritic cells was noted at both 10 and 60 days.

Aleutian mink disease parvovirus (ADV) causes a persistent infection characterized by chronic viremia, extremely high levels of antiviral antibodies, polyclonal hypergammaglobulinemia, and plasmacytosis (2, 5, 6, 14). It has been proposed that the glomerulonephritis and polyarteritis observed in Aleutian disease (AD) are caused by deposition of immune complexes, and AD has been considered a typical immune complex disease (17–19). However, the relationship between the disturbances of the immune system and the persistent ADV infection has remained unclear.

One problem in the analysis of AD pathogenesis has been the difficulty in elucidating the cellular site for ADV replication in adult mink. In previous studies, we tried to identify the cells that support ADV replication in the mink by using strand-specific in situ hybridization (3–5) for specific intermediates of ADV replication.

ADV replicative-form (RF) DNA and mRNA were observed in association with germinal centers at 10 days after infection of adult mink coincident with the peak of viral replication, but the signal intensity was extremely low, and consequently, the cell type could not be convincingly identified (3). In the same specimens, ADV virion DNA was readily observed in locations surrounding germinal centers of the lymph node at 10 days after infection but was below the level of detection at later stages of infection (3). Therefore, although ADV replication was localized to lymphoid tissues, the specific cell supporting ADV replication and perhaps mediating the immune disturbances remain poorly characterized.

In order to better characterize these cells, we have employed in situ hybridization (13) and immunohistochemistry (13) to demonstrate specific viral nucleic acids and antigens. By using these techniques, ADV replication in lymphoid tissues of adult mink was localized to cells of dendritic morphology and macrophages.

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Initially, we performed immunohistochemical staining for ADV structural antigens or mink immunoglobulin (Ig) on 4-μm frozen sections of mesenteric lymph node using rabbit antisera prepared against purified ADV virions and mink Ig (4, 7). Sections were stained by the avidin-biotin-peroxidase complex immunoperoxidase technique (10, 13).

In brief, slides were blocked with normal goat serum, incubated with specific or control primary antisera, and incubated with a biotin-conjugated goat anti-rabbit IgG (heavy and light chain) antibodies (Vector Laboratories, Burlingame, Calif.) diluted with phosphate-buffered saline containing normal mink serum. Localization of the antigen was visualized by using the avidin-biotin-peroxidase complex (ABC kit PK-4000; Vector Laboratories).

The samples were taken from five normal sapphire mink (Aleutian genotype), seven mink 10 days after inoculation with 10^7 50% lethal doses of a standard ADV-Utah 1 inoculum (3), and seven mink 60 days after inoculation.

Lymph node sections prepared from the mink infected with ADV for 10 days showed the presence of ADV structural proteins (VP) in some individual cells with anti-VP antiserum (Fig. 1C and D) but showed no significant staining when normal rabbit serum was used as a negative control for the primary antibody (Fig. 1A).

Cells staining for VP antigens at 10 days after infection exhibited both nuclear and cytoplasmic staining and could be classified as one of two major morphological types. One type possessed long processes and little cytoplasm and exhibited a dendritic shape (Fig. 1C and D, arrows). These dendrite-shaped cells were found in the centers of the primary lymph follicles, the peripheries of the germinal centers, or both the peripheries and the centers of germinal centers (Fig. 1C). A population of dendrite-shaped cells with exactly the same distribution was also strongly positive for mink Ig (Fig. 1B, arrow). The location and morphology of these cells were consistent with their being follicular dendritic cells (FDC) (9, 22, 23, 25).

The other type of cells staining for VP at 10 days had irregular shapes and abundant cytoplasm but lacked the prominent long processes. These cells were most often

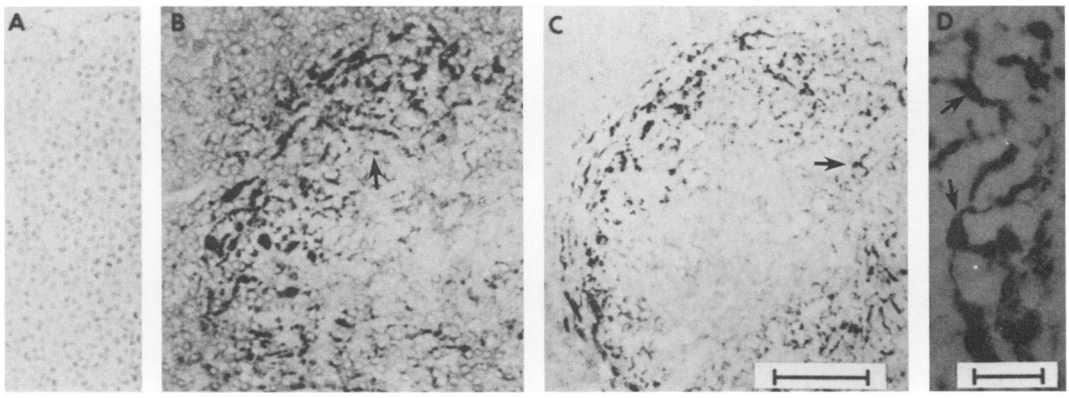

FIG. 1. Immunohistochemical staining on parallel sections of a lymph follicle in the lymph node obtained from a mink 10 days after ADV infection. Staining was done with normal rabbit serum (negative control) (A), anti-mink Igs (B), and anti-VP antiserum (C). Bar = 85 μm. (D) Higher-magnification micrograph taken from the periphery of a germinal center stained for VP. Bar = 25 μm. The sections were counterstained with methyl green. Sections prepared from the control mink consistently gave no significant staining with anti-VP antiserum or with normal serum. Arrows indicate dendrite-shaped cells.

observed in the medullary, intermediate, and marginal sinuses and in the germinal centers. They showed weaker VP staining than the dendrite-shaped cells, and mink Ig staining was not convincingly elevated above the background level. On the basis of morphology and distribution, these cells were likely to be macrophages (23, 25).

In contrast, staining for VP in lymph node sections from ADV-infected mink at 60 days postinoculation was either weak or undetectable. Furthermore, although the number of positive cells was fewer at 60 days than at 10 days, the distribution and morphology of such weakly VP-positive cells were the same.

We next analyzed parallel sections from the same mink by in situ hybridization using the strand-specific in situ hybridization protocol previously reported. ^{35}S-labeled strand-specific RNA probes were prepared as described previously (4). Sections were probed either with the plus-sense probe to detect virion DNA or with the minus-sense probe to detect RF DNA and viral mRNA (3, 8). The in situ hybridization protocol was optimized for higher sensitivity with these probes (13). In brief, sections were refixed in 5% paraformaldehyde for 20 min at room temperature, acetylated twice, denatured by heating slides at 65°C in 95% formamide in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min, and prehybridized at 45°C for 4 h. Hybridization proceeded under sealed coverslips with 6×10^4 cpm of ^{35}S-labeled RNA probe per μl at 45°C for 15 h in the dark. Slides were then washed, dehydrated, and autoradiographed. Some sections were treated with RNase for 2 h prior to hybridization to verify that signals were deriving from ADV mRNA and not from the low level of complementary virion DNA strands (3).

Sections prepared from the lymph node and spleen of the control mink consistently showed no significant grain production with either the plus- or the minus-sense probe (Fig. 2A, B, E, and F).

The lymph node sections prepared from the ADV-infected mink at 10 days showed significant grain production with both probes.

Cells reactive with the minus-sense probe and thus containing ADV RF DNA-mRNA (3, 4) were readily identified in lymph nodes from mink at 10 days (Fig. 2G and H) in association with primary and secondary lymphoid follicles. The distribution of positive cells appeared to mirror that of the dendrite-shaped cells containing VP antigen; however, autoradiographic grain scatter made it impossible to identify these cells unequivocally even at low grain density. In addition, some cells located in the medullary cords and in the lymphoid sinus system were also positive with the minus-sense probe. Because RNase treatment of the samples prior to hybridization greatly reduced the amount of grain production (data not shown), this signal was due mostly to viral mRNA, and thus, ADV replication was occurring in these cells. The density of grain production with the minus-sense probe and hence the level of resolution were substantially higher than in former studies (3). The higher resolution resulted from superior retention of the ADV mRNA signal by the modified in situ hybridization procedure (13).

Cells reactive with the plus-sense probe (thus containing virion DNA [3, 4]) were also primarily found in association with primary and secondary lymph follicles, often ringing germinal centers (Fig. 2C). In some instances, the grain deposition appeared to be along processes of dendrite-shaped cells. Other positive cells were located along lymph node sinuses and occasionally in the medullary cords (Fig. 2D). The distribution of these cells exactly mirrored that of cells positive for the VP antigen. RNase treatment prior to hybridization did not affect grain development by the plus-sense probe (data not shown).

The distribution of grains with the plus-sense probe at 60 days was essentially identical to that seen at 10 days with the same probe (Fig. 3A and B), although the level of grain production was substantially lower at 60 days.

The number of cells positive with the minus-sense probe was much lower at 60 days than at 10 days. Furthermore, the level of grain production in individual cells was also much reduced, especially in the lymph follicles (Fig. 3C). Nevertheless, occasional cells exhibited strong signals with the minus-sense probe in the sinuses or medullary cords (Fig. 3D), suggesting that some cells continued to support replication of ADV.

Lymphoid organs contain, in addition to lymphocytes, a variety of other cells such as macrophages and FDC (9, 22, 23, 25). Although many of these cells remain poorly characterized, some are thought to be involved with antigen processing or presentation. FDC express Fc and C3 recep-

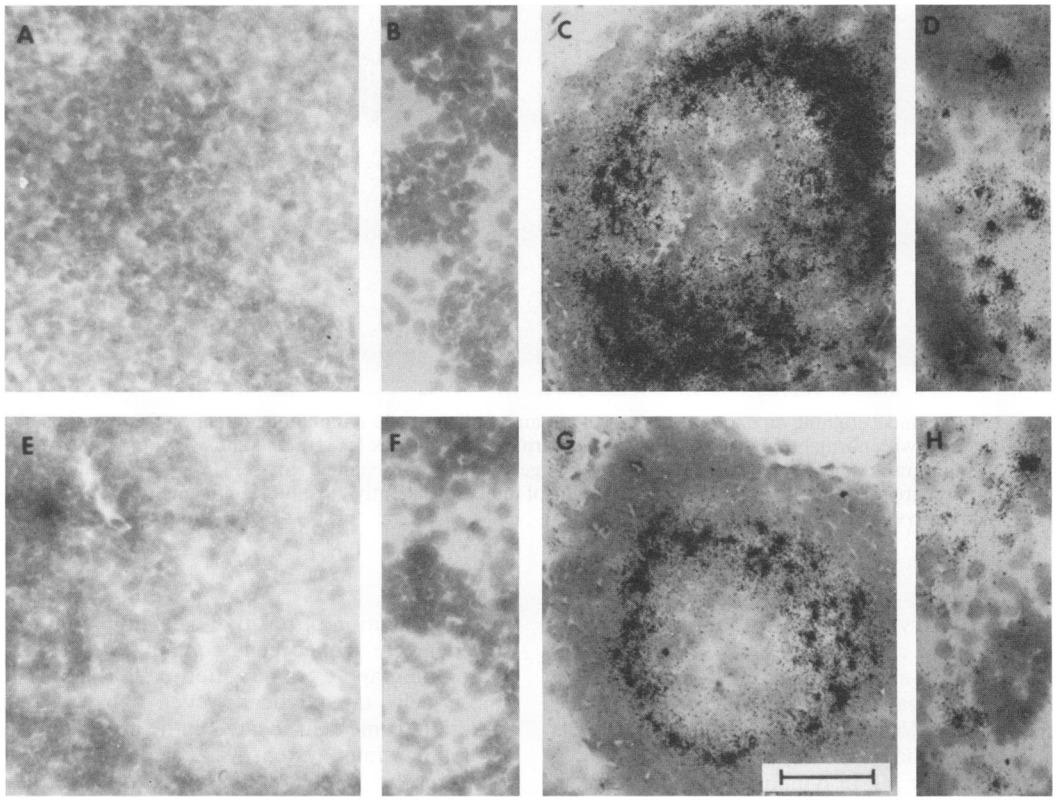

FIG. 2. In situ hybridization on parallel sections from mesenteric lymph node. (A, B, E, and F) Lymph follicle (A and E) and medullary area (B and F) of lymph node from an uninfected mink. Probes were plus sense to detect virion DNA (A and B) and minus sense to detect RF DNA-viral mRNA (E and F). (C, D, G, and H) Lymph follicles (C and G) and the medullary area (D and H) of the lymph node from a mink 10 days after ADV infection. Probes were plus sense (C and D) and minus sense (G and H). The sections were counterstained with hematoxylin. Bar = 85 μm. RNase treatment prior to hybridization did not affect grain development by the plus-sense probe but greatly reduced the amount of grain production by the minus-sense probe.

tors and can retain immune complexes on their surfaces for extended periods (21–23, 25). It has been speculated that the complexes are slowly released from the FDC and thus provide a constant source of antigenic stimulation. Furthermore, FDC have been shown to present antigens and stimulate the proliferation of B lymphocytes (24). The work presented in this note has suggested that FDC in mink lymphoid organs may be targets for ADV replication.

When sections of infected lymph node taken at the peak of ADV replication (10 days postinoculation) were studied by immunohistochemistry and in situ hybridization, ADV components were found in two types of cells.

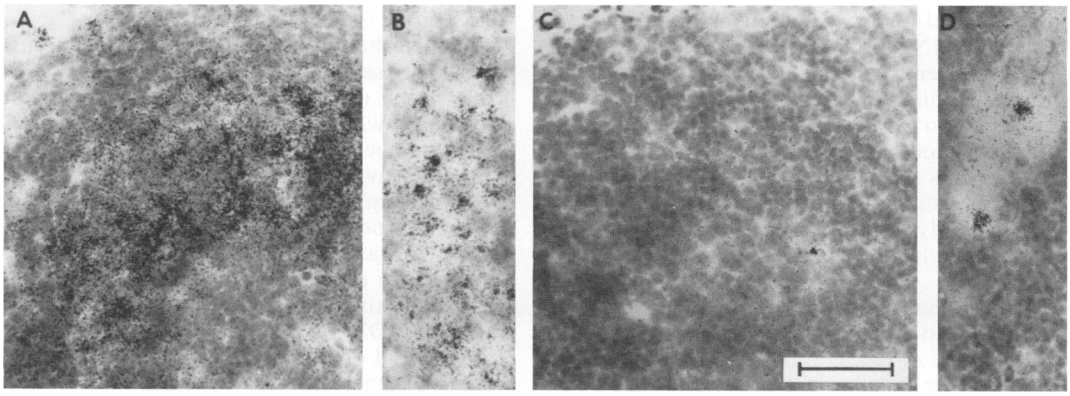

FIG. 3. In situ hybridization on parallel sections from a mink 60 days after ADV infection. (A and B) Lymph follicle and medullary area, respectively, with the plus-sense probe. (C and D) Lymph follicle and medullary area, respectively, with the minus-sense probe. The sections were counterstained with hematoxylin. Bar = 85 μm.

One cell type positive for ADV was primarily observed in the follicles and had morphology and distribution characteristic of FDC (Fig. 1C and D, arrows) (9, 23, 25), i.e., dendritic morphology with scant cytoplasm and long processes. ADV virion antigen was present in the nucleus and cytoplasm. As would be expected for FDC sequestering immune complexes, mink Ig was also readily found on these cells (Fig. 1B). In earlier studies (14, 17, 20), staining for ADV antigen in a crescentic cap or ring around germinal centers of the spleen and lymph nodes obtained from ADV-infected Aleutian mink were noted. The reported distribution of the viral antigen in those studies is consistent with our observation of VP-positive FDC.

A population of cells with an identical distribution was also positive for virion DNA, RF DNA, and mRNA by in situ hybridization. These results strongly suggested that a significant fraction of the presumed FDC were supporting viral replication at this time point. However, the number of cells positive for virion DNA was greater than the number positive for RF DNA-mRNA, implying that some of these cells were merely sequestering ADV or ADV-containing immune complexes as previously suggested (3, 5). In fact, by 60 days after infection, replication and VP staining were barely detectable in such FDC-like cells, although virion DNA was still evident. Thus, although our data implied that some FDC may be targets for replication early after infection, continued viral replication within these cells may not be obligatory. Alternatively, as we propose elsewhere (3), the infection may be so severely restricted that replication is undetectable.

The other population of ADV-positive cells was most likely composed of macrophages. Located in the sinuses and germinal centers, they had irregular shapes with abundant cytoplasm but lacked long processes (25). At 10 days, these macrophages also were supporting replication of ADV as evidenced by the presence of ADV RF DNA-mRNA. Furthermore, at 60 days, they were the only cells in the lymph node in which replication was demonstrable (Fig. 3C and D). Nevertheless, there was an excess of macrophages containing only virion DNA, implying that, as for the FDC, not every cell containing ADV DNA was engaged in active replication and that some cells merely sequestered ADV.

The source of the sequestered virions and the viral antigens in the immune complexes is unclear. One possibility is that virions produced in the FDC and macrophages in early stages of infection are retained as immune complexes on FDC and macrophages for a long time. It is well established that ADV remains infectious even in immune complexes (15), and virion DNA is detectable even in renal glomeruli (3). Another possibility is that although the production of ADV in FDC and most macrophages is highly restricted by 60 days, the small population of macrophages in which replication is ongoing continues to produce virus or viral degradation products (1). Finally, we have also observed that a few cells in other organs such as the spleen, liver, kidney, and bone marrow support low-level ADV replication throughout the course of infection (3, 12). Small amounts of virus and viral antigens may also be released from these cells. Virus or virus antigens from these sources might make up the sequestered viral components observed in the immune complexes (15) or in the FDC and macrophages.

Interestingly, Porter et al. (17) described that maximal viral titers were recovered from the spleen, lymph nodes, and liver of the mink 10 days after inoculation with ADV-Utah 1. The titers slowly decreased and plateaued by 60 days after infection (17). Thus, the initial burst of ADV replication may be in both FDC and macrophages but may then be restricted markedly so that only a small population of macrophages continues to produce the virus at later times.

Replication and sequestration of ADV in FDC may have biological significance when the pathogenesis of ADV infection is considered. The work reported here extended previous results (3) and strongly suggested that ADV replication and sequestration occur in cells that govern the differentiation of memory B lymphocytes into plasma cells (24). FDC present antigens to and stimulate the proliferation of B cells (11, 22, 23). The presence of ADV in the FDC may abrogate normal control mechanisms and at the same time provide a chronic ADV antigenic load. This might lead to an exaggerated stimulation of B-lymphocyte maturation and to the subsequent channeling of Ig production into a poorly controlled polyclonal response largely directed against ADV antigens (2, 6, 14). The hypergammaglobulinemia that is so characteristic of this persistent infection might then result. Studies are currently under way to characterize more fully the presumed FDC in vitro (21) and to assess their function in infected mink.

We acknowledge the assistance of Robert Evans and Gary Hettrick in preparation of the photomicrographs.

S. Mori and M. Miyazawa were participants in the NIH Visiting Program.

REFERENCES

1. **Aasted, B., R. E. Race, and M. E. Bloom.** 1984. Aleutian disease virus, a parvovirus, is proteolytically degraded during in vivo infection in mink. J. Virol. **51**:7–13.
2. **Aasted, B., G. S. Tierney, and M. E. Bloom.** 1984. Analysis of the quantity of antiviral antibodies from mink infected with different Aleutian disease virus strains. Scand. J. Immunol. **19**:395–402.
3. **Alexandersen, S., M. E. Bloom, and J. Wolfinbarger.** 1988. Evidence of restricted viral replication in adult mink infected with Aleutian disease of mink parvovirus. J. Virol. **62**:1495–1507.
4. **Alexandersen, S., M. E. Bloom, J. Wolfinbarger, and R. E. Race.** 1987. In situ molecular hybridization for detection of Aleutian mink disease parvovirus DNA by using strand-specific probes: identification of target cells for viral replication in cell cultures and in mink kits with virus-induced interstitial pneumonia. J. Virol. **61**:2407–2419.
5. **Bloom, M. E., S. Alexandersen, S. Mori, and J. B. Wolfinbarger.** 1989. Analysis of parvovirus infections using strand-specific hybridization probes. Virus Res. **14**:1–26.
6. **Bloom, M. E., R. E. Race, W. J. Hadlow, and B. Chesebro.** 1975. Aleutian disease of mink: the antibody response of sapphire and pastel mink to Aleutian disease virus. J. Immunol. **115**:1034–1037.
7. **Bloom, M. E., R. E. Race, and J. B. Wolfinbarger.** 1982. Identification of a nonvirion protein of Aleutian disease virus: mink with Aleutian disease have antibody to both virion and nonvirion proteins. J. Virol. **43**:608–616.
8. **Bloom, M. E., R. E. Race, and J. B. Wolfinbarger.** 1987. Analysis of Aleutian disease of mink parvovirus infection using strand specific hybridization probes. Intervirology **27**:102–111.
9. **Chen, L. L., J. C. Adams, and R. M. Steinman.** 1978. Anatomy of germinal centers in mouse spleen, with special reference to "follicular dendritic cells." J. Cell Biol. **77**:148–164.
10. **Hsu, S., L. Raine, and H. Fanger.** 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. **34**:39–43.
11. **Mandel, T. E., R. P. Phipps, A. Abbott, and G. Tew.** 1980. The follicular dendritic cell: long term antigen retention during immunity. Immunol. Rev. **53**:29–59.
12. **Mori, S., and M. Bloom.** Unpublished data.
13. **Mori, S., J. B. Wolfinbarger, N. Dowling, W. Wei, and M. E.**

Bloom. Microb. Pathog., in press.
14. **Porter, D. D.** 1986. Aleutian disease: a persistent parvovirus infection of mink with a maximal but ineffective host immune response. Prog. Med. Virol. **33**:42–60.
15. **Porter, D. D., F. J. Dixon, and A. E. Larsen.** 1965. Metabolism and function of gammaglobulin in Aleutian disease of mink. J. Exp. Med. **121**:889–900.
16. **Porter, D. D., and A. E. Larsen.** 1967. Aleutian disease of mink: infectious virus-antibody complexes in the serum. Proc. Soc. Exp. Biol. Med. **126**:680–682.
17. **Porter, D. D., A. E. Larsen, and H. G. Porter.** 1969. The pathogenesis of Aleutian disease of mink. I. In vivo viral replication and the host antibody response to viral antigen. J. Exp. Med. **130**:575–589.
18. **Porter, D. D., A. E. Larsen, and H. G. Porter.** 1973. The pathogenesis of Aleutian disease of mink. III. Immune complex arteritis. Am. J. Pathol. **71**:331–344.
19. **Portis, J. L., and J. E. Coe.** 1979. Deposition of IgA in renal glomeruli of mink affected with Aleutian disease. Am. J. Pathol. **96**:227–236.
20. **Race, R. E., B. Chesebro, M. E. Bloom, B. Aasted, and J. Wolfinbarger.** 1986. Monoclonal antibodies against Aleutian disease virus distinguish virus strains and differentiate sites of virus replication from sites of viral antigen sequestration. J. Virol. **57**:285–293.
21. **Schnizlein, C. T., M. H. Kosco, A. K. Szakal, and J. G. Tew.** 1985. Follicular dendritic cells in suspension: identification, enrichment, and initial characterization indicating immune complex trapping and lack of adherence and phagocytic activity. J. Immunol. **134**:1360–1368.
22. **Schnizlein, C. T., A. K. Szakal, and J. G. Tew.** 1984. Follicular dendritic cells in the regulation and maintenance of immune responses. Immunobiology **168**:391–402.
23. **Tew, J. G., R. P. Phipps, and T. E. Mandel.** 1980. The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. Immunol. Rev. **53**:175–201.
24. **van Roojien, N.** 1990. Direct intrafollicular differentiation of memory B cells into plasma cells. Immunol. Today **11**:154–157.
25. **Van Voorhis, W. C., M. D. Witmer, and R. M. Steinman.** 1983. The phenotype of dendritic cells and macrophages. Fed. Proc. **42**:3114–3118.