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Original Article

In Situ Observation of Germinal Center Cell Apoptosis During a Secondary Immune Response

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Germinal centers are highly organized anatomic structures essential for the clonal expansion of germinal center (GC) B-cells and associated somatic hypermutation, isotype switching, selection of the high-affinity B-cells (affinity maturation), and elimination of irrelevant or autoreactive clones. The identification of cellular interactions and regulatory mechanisms controlling apoptosis within GCs is essential for a complete understanding of the cellular and molecular dynamics of the GC reaction. We performed a kinetic analysis of the apoptotic activity occurring within GCs of draining lymph nodes of mice immunized with sheep red blood cells (SRBC) after secondary stimulation. The apoptotic activity of GC cells can be divided into three distinct phases : 1) initial phase (within the first days after immunization), 2) reactive phase (from the 5th day to 15th day after secondary immunization), and 3) late phase (after the 15th day). Apoptosis decreased shortly after secondary immunization followed by an increase to peak after an additional 10 days. Finally, apoptosis of GC cells decreased to basal levels. Administration of apoptosis inhibitors decreased the amount of apoptosis during the reactive phase. These results suggest that the reactive phase may be the critical period in which clonal selection and cellular differentiation to antibody forming cells take place. 〔*J Clin Exp Hematopathol 46(2) : 73-82, 2006*〕

Keywords: mouse lymph node, germinal center B-cell, secondary immune response, apoptosis, follicular dendritic cell

INTRODUCTION

Apoptosis, programmed cell death, is morphologically characterized by chromatin condensation, nuclear disintegration, cytoplasm shrinkage, and budding and separation of both the nucleus and cytoplasm into multiple, small, membrane-bounded apoptotic bodies[1-3]. Additionally, the cleavage of cellular DNA into oligonucleosomal fragments of multiples of 180 - 200 base pairs is a hallmark of apoptosis[4]. Apoptosis is an integral process involved in a wide variety of biological systems, including cellular self-renewal, embryonic development, metamorphosis, hormone-dependent atrophy, and immunopathologic reactions such as the cytotoxic T-cell response[5,6].

Germinal centers (GCs) are prominent anatomic sites in secondary lymphoid tissues primarily composed of proliferating B-cells, with a small number of T-cells, follicular dendritic cells (FDCs), and tingible body macrophages (TBMs)[7-9]. During the GC response a number of cellular and molecular events take place in this highly organized anatomic area including somatic hypermutation of IgV region, isotype switching, positive selection of the high-affinity B-cells and the generation of memory B-cells, positive selection of high affinity B-cell clones (affinity maturation), and negative selection of irrelevant or autoreactive clones of B-cells[7-11]. Indeed, large numbers of the GC B-cells undergo apoptosis and die rapidly to eliminate low-affinity or autoreactive B-cells generated by somatic mutation[12]. However, the kinetics, cellular interactions, molecular pathways, and regulatory mechanisms controlling cell death within the GC remain unclear.

In the present study, we histologically examined the draining lymph nodes of mice immunized with sheep red blood cells (SRBC) at various time points after primary and secondary stimulation and examined the extent of apoptosis kinetically.

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MATERIALS AND METHODS

Animals

BALB/ c female mice aged 8 weeks were purchased from Japan SLC (Shizuoka, Japan). The animals were kept in standard cages with filter tops and housed in a specific pathogen free environment, and mice were allowed sterilized food

and water *ad libitum*.

Antigen and immunization of animals

Active immunization : Animals were injected subcutaneously in the footpads and behind the nape of the neck with 1.0 × 10^8 SRBC (Toyo Bio, Tokyo, Japan). Five or 10 weeks later, mice were boosted with the same antigen by the same immunization route to elicit a secondary immune response. The interval was determined by preliminary observations concerning apoptotic activity in GC at 5, 8, and 10 weeks after primary immunization as indicated in Fig. 5. Mice without boosting were used as day 0 mice. The draining lymph nodes including both sides of popliteal lymph nodes (PLNs), inguinal lymph nodes (ILNs), axillary lymph nodes (ALNs), and brachial lymph nodes (BLNs) were excised on 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 days after secondary antigen injection and thus designated as day 1, day 3, day 5, day 7, day 9, day 11, day 13, day 15, day 17, day19, and day 21 lymph nodes, respectively. These lymph nodes were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and frozen without fixation at −80℃. At least three mice were examined at each time point.

Passive immunization : For passive immunization, mice were injected intraperitoneally with 1.0 ml SRBC-specific rabbit antisera (Inter-Cell Technologies, Hopewell, NJ) one day before antigenic challenge. The following day, the mice were subcutaneously injected with 1.0 × 10^8 SRBC in the footpads and behind the nape of the neck. The draining lymph nodes were excised at day 0 (non-antigenic challenge), day1, day 7, and day 15 after antigen administration identically to actively immunized mice. At least three mice were examined at each time point.

Immunoperoxidase labeling for detection of apoptotic cells in situ

Apoptotic cells were detected immunohistochemically *in situ* by indirect immunoperoxidase labeling using a rabbit polyclonal antibody against single-stranded DNA (ssDNA) as previously described[13,14]. Cryostat sections (3-μm thick) were prepared from the obtained lymph nodes. The sections were mounted on adhesive coated glass slides (Matsunami Glass, Osaka, Japan), air-dried, fixed using absolute acetone for 10 min at room temperature, and rinsed three times with phosphate-buffered saline (PBS, pH7.4). The sections were then incubated in 5 mM periodate solution (#266-05, Nacalai Tesque, Kyoto, Japan) for 20 min to block endogenous peroxidase activity, if it was needed. Samples were then rinsed with PBS three times and incubated in 5% bovine serum albumin (BSA) to inhibit non-specific antibody binding for at least one hour. Sections were then incubated overnight at 4℃ in rabbit polyclonal antibody against ssDNA (A4506, DAKO, Carpinteria, CA) according to the manufacturer's instructions. The sections were washed three times with PBS and incubated with goat anti-rabbit Ig conjugated to peroxidase labeled-dextran polymer (DAKO EnVision+™, peroxidase, rabbit, ready-to-use ; K4002, DAKO). The sections were then washed twice in PBS followed 0.05 M Tris-HCl buffer (pH7.6) and submerged into 0.03% 3,3'-diaminobenzidine tetrahydrochloride (DAB ; Dojin Chemical, Kumamoto, Japan) solution in 0.05 M Tris-HCl buffer with 0.01% H_2O_2 for development. Following counterstaining by 0.5% methyl green, the sections were dehydrated in a graded series of ethanol, cleared by xylene and mounted using Permount (Fisher Scientific, Fair Lawn, NJ) for observation by light microscopy.

As a specificity control, normal rabbit serum was used as the primary antibody. Additionally, sections were examined for reactivity with secondary antibodies and endogenous peroxidase activity.

Detection of antigen localization in the draining lymph nodes

Briefly, cryostat sections were mounted on adhesive coated glass slides, air-dried, fixed using absolute acetone for 10 min at room temperature, and rinsed three times with PBS. Samples were then incubated in 5% BSA to inhibit non-specific antibody binding for at least one hour and incubated with rabbit anti-SRBC specific antibody (Inter-Cell Technologies) for two hours. Sections were then washed three times using PBS and incubated with an affinity-purified, FITC-conjugated swine anti-rabbit Ig (F0205, DAKO) for one hour. Sections were then washed three times with PBS and mounted using buffered glycerol for observation by fluorescence microscopy (BX60 ; Olympus Optical, Tokyo, Japan).

As a specificity control, a non-specific rabbit antibody was used as the primary antibody. Additionally, lymph nodes obtained from ovalbumin immunized BALB/c mice or non-immunized mice were examined to confirm antigen specificity.

Immunoperoxidase labeling for light microscopic observation of the distribution of follicular dendnritic cells (FDCs) or Tcells

We performed immunohistochemical labeling to examine the distribution of FDCs or Tcells at the light microscopic level. Cryostat sections mounted on adhesive coated glass slides were fixed in absolute acetone for 10 min at room temperature and rinsed with PBS. The sections were then incubated in 5 mM periodate solution for 20 min to block endogenous peroxidase activity. Samples were then incubated in 5% BSA to inhibit non-specific antibody binding for

at least one hour. Sections were then incubated overnight at 4℃ with a rat monoclonal antibody specific for murine FDCs (FDC-M2[15] ; kind gift from Dr. Marie H. Kosco-Vilbois, Immunology Department, Serono Pharmaceutical Research Institute, Geneva, Switzerland) or specific murine Tcells (Thy1.2 ; 53-2.1, #BD-553000, BD Pharmingen, San Diego, CA). The sections were washed in PBS and incubated with a biotinylated mouse-anti rat IgG heavy and light chain (Jackson ImmunoResearch Laboratories) for one hour. The sections were then washed in PBS and incubated with ABC (Vectastain Elite ABC Kit, Vector Laboratories) for one hour. After washing in PBS and Tris-HCl buffer sections were submerged into 0.03% DAB solution with 0.01% H_2O_2 followed by counterstaining with 0.5% methyl green.

As a specificity control, isotype-matched non-specific antibodies were used as primary antibodies. Additionally, sections were examined for reactivity with secondary antibodies and endogenous peroxidase activity.

Peanut agglutinin (PNA) labeling for detection of germinal centers

GCs were visualized using the PNA binding capacity of GC B-cells with horseradish peroxidase conjugated PNA as described previously[25]. Briefly, cryostat sections were fixed in 0.5% glutaraldehyde for 30 min, washed in PBS, and incubated in 5% BSA for 30 min to block non-specific PNA binding. After a brief washing, the sections were incubated in biotinylated PNA (L6135, Sigma Chemical, St. Louis, MO) overnight at 4℃, washed in PBS and incubated with peroxidase-conjugated streptavidin (UltraTech-HRP Streptavidin-Biotin Universal Detection System ; Immunotech, Marseille, France) for 60 min. The peroxidase activity was visualized with DAB, and sections were counterstained with methyl green.

Morphometric analysis of apoptosis

To quantitatively examine apoptosis, the number of macrophages ingesting anti-ssDNA antibody-positive bodies within GCs was counted by light microscopy using a × 40 objective. The mean number of positive macrophages counted from each GC of a random cut surface from each lymph node was calculated as the apoptotic activity.

In vivo *inhibition of apoptosis*

The following apoptosis inhibitors were injected intraperitoneally one day before secondary immunization to examine the role of apoptosis on the GC reaction and specific antibody production : $ZnCl_2$, a general endonuclease inhibitor[16] (20 mg/kg ; Junsei Chemical, Tokyo, Japan) and a synthesized peptide, Z-VAD-FMK, a specific caspase 3 inhibitor[17] (30 mg/kg ; Peptide Institute, Osaka, Japan). The next day, the treated mice were subcutaneously injected into the footpads and behind the nape of neck with SRBC. Ten weeks later, animals were boosted with the same antigen by the same route of immunization to elicit the secondary immune response. On the 7th day after the boost, the draining lymph nodes were obtained and designated as day 7 lymph nodes. Animals not receiving a secondary boost were designated as day 0 lymph nodes. The experiments were performed in triplicate.

Statistical analysis

Significant differences were detected using a Mann-Whitney U-test.

RESULTS

Histologic and histochemical features of GCs during the secondary immune response.

To better understand the role of apoptosis during the GC response, we first had to establish the histologic and histochemical characteristics of GC during the secondary immune response. Lymph nodes from SRBC-injected mice were harvested at days 0, 3, and 11 after secondary immunization, and tissue samples were stained with hematoxylin & eosin (Figs. 1a, 2a, and 3a). Additionally, specific labeling with PNA as well as immunohistochemical staining with anti-SRBC, anti-ssDNA, FDC-M2, and Thy1.2 were examined (Figs. 1b-f, 2b-f, and 3b-f).

In lymph nodes at day 0, PNA labeling revealed abortive or underdeveloped GCs (Fig. 1b). As the secondary immune response proceeded, GCs expanded such that fully developed GCs were observed on day 7 (Fig. 2b) and some regression had occurred by day 11 (Fig. 3b). We next used a fluorescently labeled rabbit antiserum specific for SRBC to examine antigen localization in the developing GC response. Strong reticular labeling was seen in the GC light zone especially at day 3 (Figs. 2c and 3c) while no specific GC staining was seen at day 0, in animals not receiving a second immunization (Fig. 1c). We next used anti-ssDNA and peroxidase to detect apoptotic bodies. Tingible body macrophages (TBMs) ingesting apoptotic bodies were seen in the dark zone of GCs. Some anti-ssDNA reactive cells were seen at day 0 (Fig. 1d), and this number decreased by day 3 (Fig. 2d). However, the number of ssDNA-positive cells increased dramatically by day 11 (Fig. 3d). Finally, we examined the *in situ* distribution of FDCs and Tcells by immunohistochemical labeling using FDC-M2 or Thy1.2, respectively. The reticular meshwork of FDCs was evenly distributed throughout the GC while a limited number of Tcells were scattered within the GC light zone at days 0 and 3 (Figs. 1e, 1f, 2e, and 2f). As GC development

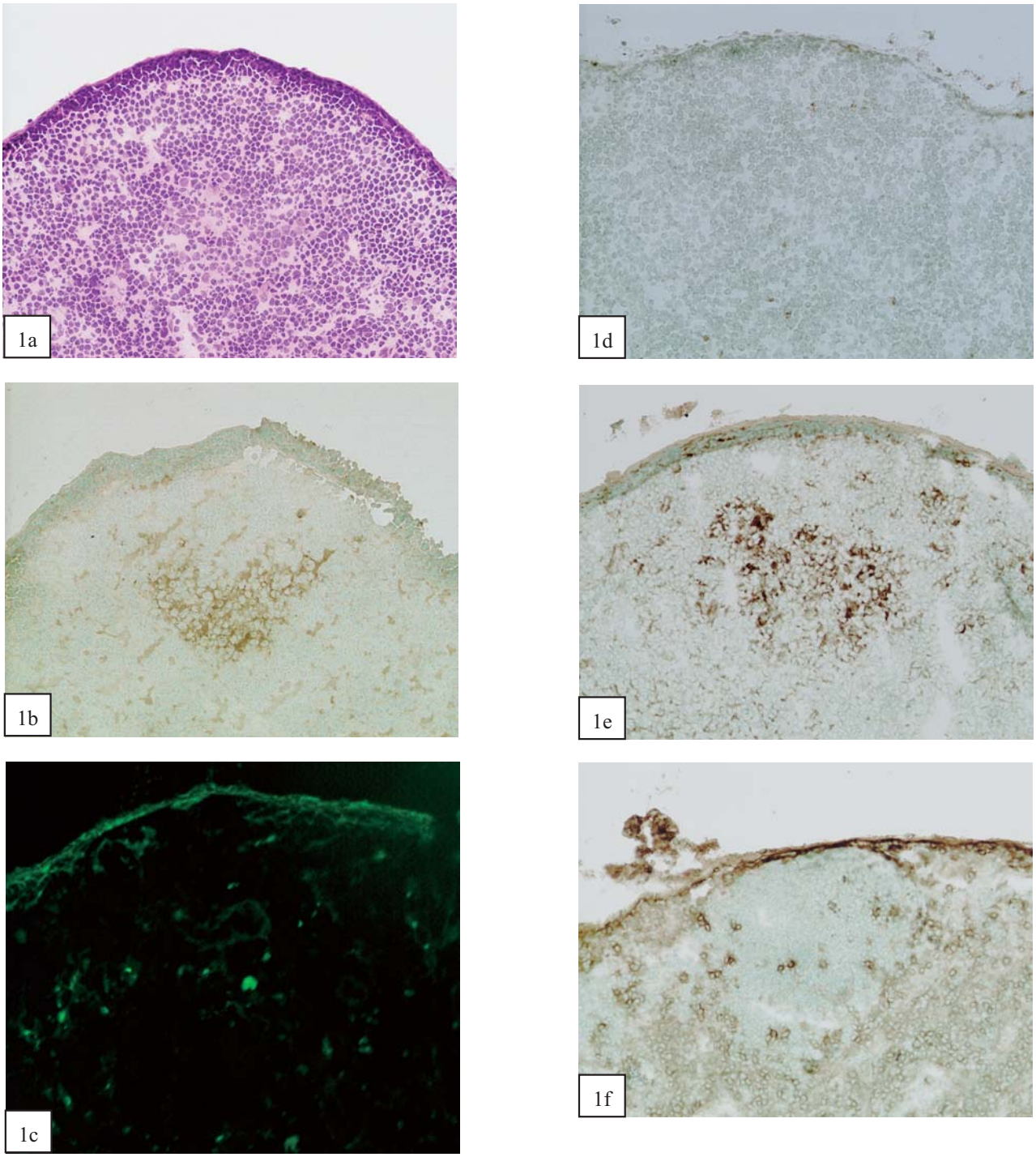


Fig. 1. Light micrographs illustrating conventional histology, peanut agglutinin (PNA) labeling, antigen localization, apoptotic activity, and distribution of follicular dendritic cells (FDC) or T cells in the draining lymph nodes of BALB/ c mouse 10 weeks after primary SRBC injection (day 0). An underdeveloped germinal center (GC) was observed as shown in Fig. 1a and it was labeled by PNA in Fig. 1b. No significant antigenic localization was seen (Fig. 1c). A few scattered tingible body macrophages containing apoptotic bodies were seen (Fig. 1d). Additionally, a reticular meshwork of FDCs was distributed in the center of GC (Fig. 1e), and Tcells were scattered throughout the GC (Fig. 1f). Fig. 1a : H&E, Fig. 1b : horseradish peroxidase-PNA labeling, Figs. 1c, 1d, 1e, and 1f : indirect immunofluorescent labeling with anti-SRBC antibody, anti-ssDNA antibody, FDC-M2, and Thy1. 2, respectively. Figs. 1a- 1f ; × 200.

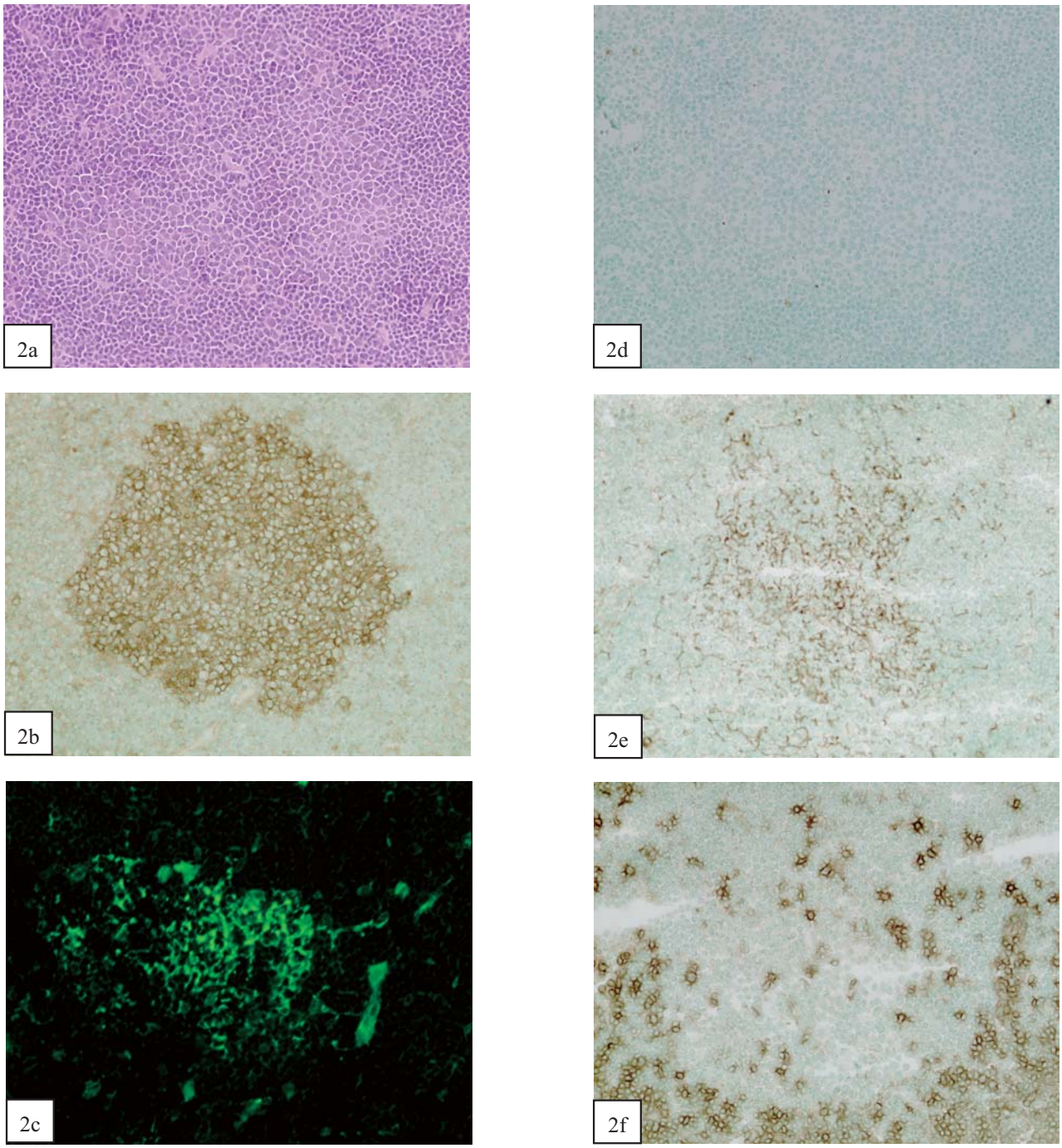


Fig. 2. Light micrographs illustrating conventional histology, peanut agglutinin (PNA) labeling, antigen localization, apoptotic activity, and distribution of follicular dendritic cell (FDC) or T cells in the draining lymph node of actively immunized BALB/ c mouse 3 days after secondary immunization (day 3). A developing germinal center (GC) was distinctly labeled with PNA (Figs. 2a and 2b). Injected antigen was detected throughout the GC (Fig. 2c), and decreased apoptosis was also seen (Fig. 2d). Increased numbers of FDCs and T cells were seen within the GC (Figs. 2e and 2f). Fig. 2a : H&E, Fig. 2b : horseradish peroxidase-PNA labeling, Figs. 2c, 2d, 2e, and 2f : indirect immunofluorescent labeling with anti-SRBC antibody, anti-ssDNA antibody, FDC-M2, and Thy1. 2, respectively. Figs. 2a- 2f ; × 200.

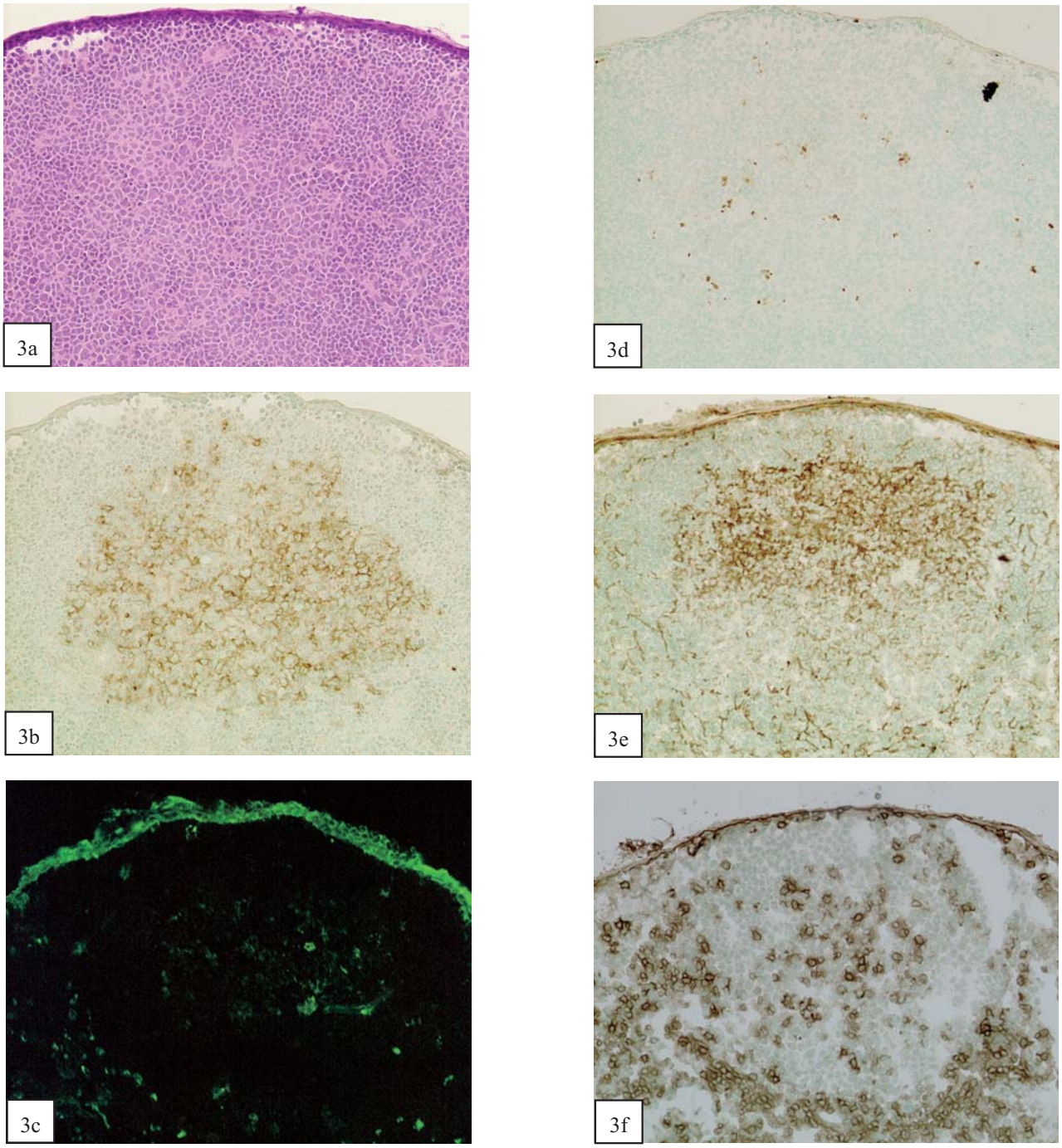


Fig. 3. Light micrographs illustrating conventional histology, peanut agglutinin (PNA) labeling, antigen localization, apoptotic activity, and distribution of follicular dendritic cell (FDC) or T cells in the draining lymph node of actively immunized BALB/ c mouse 11 days after secondary immunization (day 11). A well-developed germinal center (GC) that strongly labeled with PNA was seen (Figs. 3a and 3b). Decreased antigen levels were seen throughout the GC compared with day 3 GCs (Fig. 3c). Increased numbers of tingible body macrophages containing apoptotic bodies were distributed in the GC (Fig. 3d). A meshwork of FDCs and T cells were seen in GC light zone (Figs. 3e and 3f). Fig. 3a : H&E, Fig. 3b : horseradish peroxidase-PNA labeling, Figs. 3c, 3d, 3e, and 3f : indirect immunofluorescent labeling with anti-SRBC antibody, anti-ssDNA antibody, FDC-M2 and Thy1. 2, respectively. Figs. 3a- 3f ; × 200.

proceeded, however, the FDC meshwork localized to the apical area of the GC (Fig. 3e), corresponding to the light zone, and greater numbers of T cells were also found there (Fig. 3f).

Histologic and histochemical features of GCs in passively immunized mice

The mice examined were directly immunized with SRBC in the footpad or the nape of the neck (Figs. 1, 2, and 3). We next examined the kinetics of the secondary immune response for animals passively immunized. Passive immunization was achieved by first injecting mice with anti-SRBC antiserum followed subcutaneous injection of SRBC one day later. Lymph nodes were then harvested at different time points and GCs were examined histologically and histochemically by hematoxylin & eosin staining, PNA labeling, and immunohistochemical labeling using anti-ssDNA.

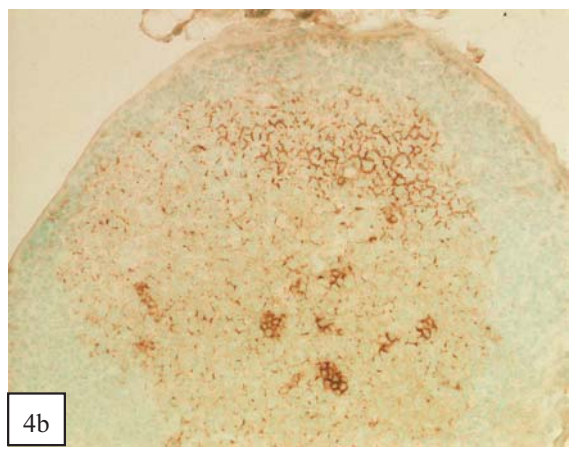
Following passive immunization, we observed fully developed GCs by day 7, and the structure of these GCs did not change by day 15 (Figs. 4a and 4b). Additionally, on day 15 several TBMs ingesting apoptotic bodies were observed in the dark zone of the GCs (Fig. 4c).

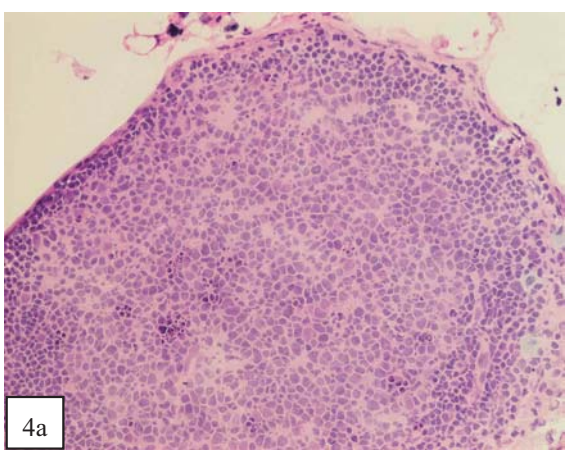
Kinetic analysis of the apoptotic activity in GCs of the draining lymph nodes of actively immunized mice

Having examined the extent of apoptosis in GCs during the early phases of the secondary immune response, we next examined the kinetics of apoptosis in GCs 5 to 10 weeks after the primary immunization by counting the number of ssDNA-containing TBM in each GC. The number of TBM containing apoptotic bodies decreased gradually with time after the primary immunization, suggesting that apoptosis was less prevalent (Fig. 5). We next examined apoptosis in GCs after secondary immunizations administered 5 or 10 weeks after the primary immunization, and, as shown in Figs. 6 and 7, respectively, apoptosis decreased early after antigenic boost. After several more days, however, apoptosis increased in both immunization groups, peaking at day 15 and day 11 for the 5-week and 10-week groups, respectively. After these times, apoptosis gradually declined.

The effect of administration of apoptosis inhibitors

The accumulation of ssDNA in TBM is an indirect measure of apoptosis, and we wished to confirm the occurrence of apoptosis in GC using the well-characterized apoptosis inhibitor Z-VAD-FMK. Indeed, treatment of immunized animals with Z-VAD-FMK significantly decreased the accumulation





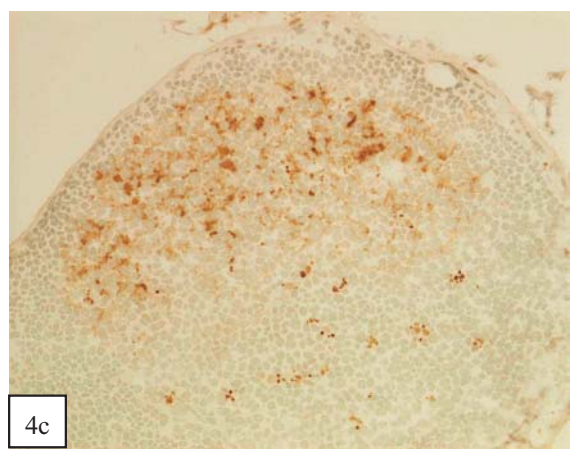


Fig. 4. Light micrographs illustrating conventional histology, peanut agglutinin (PNA) labeling, and apoptotic activity in the draining lymph node of passively immunized BALB/ c mouse 15 days after secondary immunization. A well-developed germinal center (GC) with strong PNA staining was seen (Figs. 4a and 4b). Several tingible body macrophages containing apoptotic bodies were seen in the dark zone of the GC, and a speckled deposition of immune complexes distributed in the light zone of the GC was also seen (Fig 4c). Fig. 4a : H&E, Fig. 4b : horseradish peroxidase-PNA labeling, Figs. 4c : indirect immunoperoxidase labeling with anti-ssDNA antibody. Figs. 4a- 4c ; × 200.

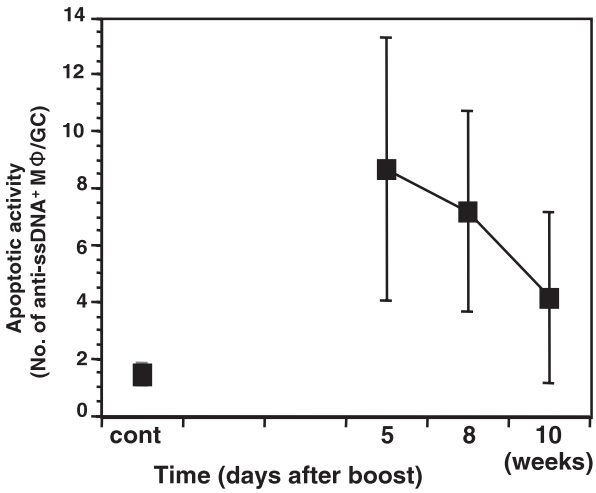


Fig. 5. Kinetics of apoptotic activity in germinal centers of draining lymph nodes after the primary immunization.

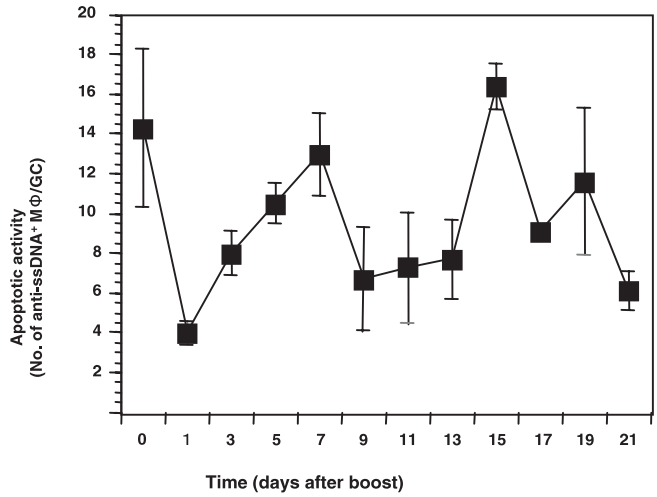


Fig. 6. Kinetics of apoptotic activity in the draining lymph nodes of mice undergoing a secondary immunization 5 weeks after primary immunization.

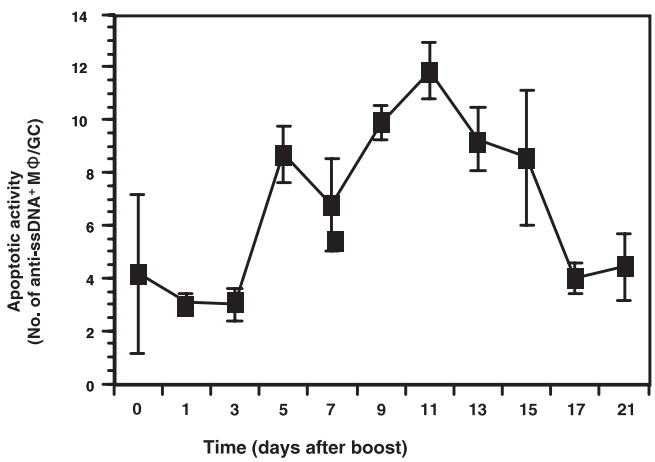


Fig. 7. Kinetics of apoptotic activity in the draining lymph nodes of mice undergoing a secondary immunization 10 weeks after primary immunization.

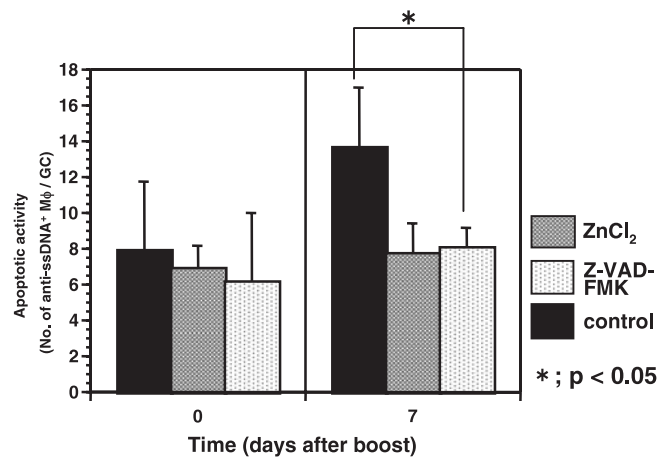


Fig. 8. Effect of apoptosis inhibitors on apoptotic body uptake in the draining lymph nodes actively immunized mice. $ZnCl_2$: SRBC actively immunized BALB/c mice injected with 20 mg/ kg $ZnCl_2$, intraperitoneally before the secondary immunization. Z-VAD-FMK : SRBC actively immunized BALB/c mice injected with 30 mg/kg Z-VAD-FMK, intraperitoneally before the secondary immunization. Control : SRBC actively immunized BALB/c mice without treatment.

of ssDNA positive TBM in the GC of day 7 lymph nodes compared to non-treated mice (Fig. 8).

Comparison of apoptotic activity between actively and passively immunized mice

We observed different kinetics of the GC response in actively and passively immunized animals (Figs. 1, 2, 3, 4, and data not shown). We next examined the differences in apoptosis between these animals. The peak apoptotic activity in actively immunized animals occurred at day 7, while peak apoptotic activity occurred at day 15 in passively immunized mice (Fig. 9).

DISCUSSION

During the GC response, rates of apoptosis can be divided into three distinct phases : 1) initial phase (within the first a couple of days), 2) reactive phase (from 5 days to 15 days after secondary immunization), and 3) late phase (after 15 days) as illustrated in Fig. 10.

During the initial phase of the GC reaction, GC apoptotic activity remains low, and, in some cases, may decrease. It is possible that newly generated GCs may arise within or nearby

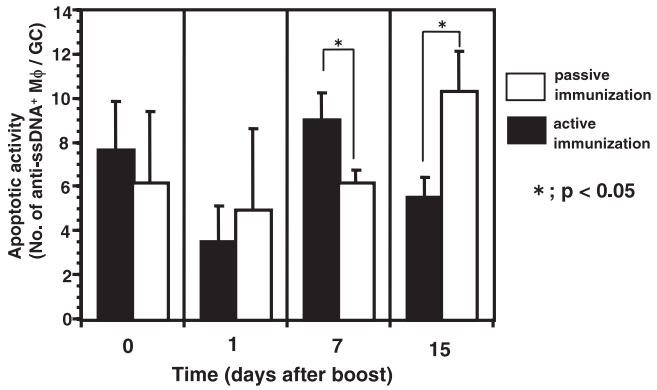


Fig. 9. Kinetics of apoptotic activity in the draining lymph node of passively immunized mice.

existing GCs, and this may explain the differences seen early in the GC reaction. This hypothesis is further supported by the observation that the greatest reduction in apoptosis was seen in mice boosted 5 weeks after the primary immunization. These animals retained a large number of GCs from the initial immunization that were undergoing more active apoptosis.

During the reactive phase, apoptotic activity gradually increased and peaked. At this point in the GC reaction, wide spread cell proliferation with associated clonal selection and elimination of inappropriate clones is occurring. The GC reaction may directly contribute to early antibody production against exogenous antigens and improved antibody affinity. Tarlinton and Smith[18] observed AFCs in the bone marrow approximately 7 - 10 days after immunization, corresponding to the appearance of high-affinity antibody in the serum. The presence of widespread apoptosis in the GC at this time supports the role of the GC in specific antibody production and affinity maturation during the early period of a secondary immune response.

After the peak of GC apoptosis, apoptotic activity decreased in the late phase. A stable population of memory B-cells only becomes apparent after day 21 of the secondary immune response[18,19]. Thereafter, the memory population increases while the GC population decreases, although GCs remain up to 80 days post-immunization[19]. Similar information has been gleaned from analysis of the immune response by histology[20]. Thus, the late-phase GC reaction may facilitate the formation or maintenance of the memory cell population.

We calculated the number of macrophages with ingested apoptotic bodies (previously referred to tingible body macrophages, TBM) to determine the degree of apoptosis occurring within the GC. Apoptotic bodies are thought to be formed at relatively late stages of the apoptotic process and do not represent all apoptotic cells. Indeed, we recently used an electron microscopic adaptation of TUNEL (EM-TUNEL) to demonstrate that most germinal center B-cells located within

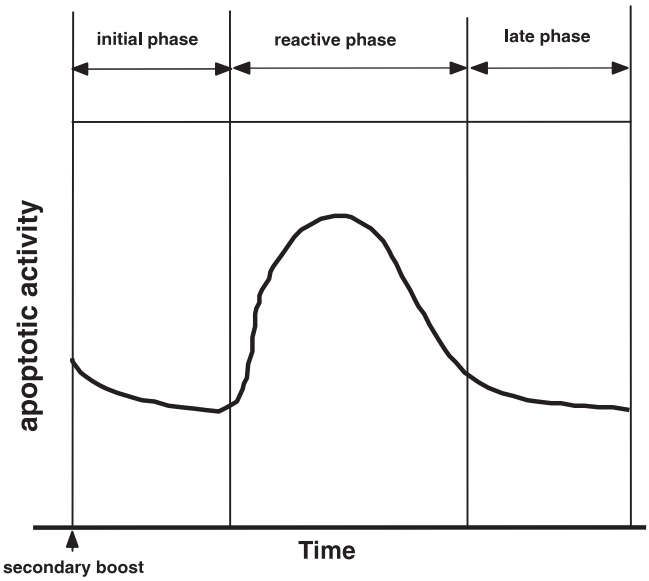


Fig. 10. Apoptosis occurs in three stages in the GC during the secondary immune response.

the light zone of GC are committed to apoptosis. Nevertheless, the number of TBM is a reliable indicator of apoptotic activity, and we confirmed the validity of TBM number as a measure of apoptosis using EM-TUNEL as a "gold standard" in a model of thymic apoptosis following glucocorticoid injection (unpublished data).

We also compared the extent and kinetics of apoptosis in the draining lymph nodes of the passively and actively immunized mice. In passively immunized mice, speckled deposits were observed in the light zone of the GCs, and these could represent FDCs containing SRBC-immune complexes that would cross-react with the anti-rabbit secondary reagent used to detect ssDNA. FDCs retain antigen in the form of immune complex and no memory B- or T-cells are involved in this reaction. Indeed, the apoptotic activity of GC cells did not increase even 7 days after secondary immunization. These observations suggest that antigen-antibody complexes retained by FDCs alone are unable to induce selection and affinity maturation in the GC and T-cell help is likely required for these processes. Indeed, the significance of the contribution of primed Tcells has been suggested by a mathematical kinetics model of the GC reaction[24].

Engagement of the B-cell antigen receptor, ligation of CD40, and/or expression of *bcl-XL* or *bcl-2*[22, 23, 25, 26] inhibit apoptosis during the GC reaction. In contrast to these antagonistic interactions, pro-apoptotic interactions or factors remain unclear. The present results will provide fundamental information for the establishment of novel experimental systems designed to elucidate the cellular dynamics and molecular mechanisms of the GC response *in vitro* and *in vivo*.

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