Follicular Dendritic Cells Inhibit Apoptosis in Human B Lymphocytes by a Rapid and Irreversible Blockade of Preexisting Endonuclease

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Summary

During germinal center reactions, a minority of B lymphocytes are selected after successful binding to follicular dendritic cells (FDCs). The majority of the B cells, however, die by apoptosis. One of the characteristics of apoptosis is rapid fragmentation of DNA by an endogenous endonuclease. The regulation of apoptosis and endonuclease activity in germinal center (GC) B cells is largely unknown. In this study we have investigated the induction and inhibition of endonuclease activity in GC B cells. We also investigated the role of FDCs, surface Ig (sIg), sIgM, CD21, CD22, CD40, and intracellular Zn^{2+} in the regulation of endonuclease activity. We have found that DNA fragmentation in GC B cells is caused by a preexisting endonuclease very similar to NUC-18 (an 18-kD endonuclease identified in rat thymocytes). Endonuclease activity in GC B cells appears to be rapidly and irreversibly blocked after interaction with FDCs, but not after cross-linkage of sIg, sIgM, CD21, CD22, or CD40. Addition of soluble CD40–human IgM fusion protein (sCD40) to FDC–B cell cultures also did not interfere with FDC-mediated B cell rescue. Chelation of intracellular Zn^{2+} during FDC-B cell cultures resulted in abrogated B cell rescue. These data suggest that FDCs inhibit apoptosis in GC B cells by a rapid inactivation of preexisting endonuclease using a mechanism distinct from CD40 ligation.

Apoptosis is a type of cell death characterized by chromatin condensation, membrane blebbing, and fragmentation of chromatin into multiple nucleosome-sized units owing to the action of an endonuclease (1–3). Apoptosis occurs in developmentally regulated cell death in the embryo (4, 5), in deletion of autoreactive T cell clones during thymic maturation (6, 7), in senescence of neutrophil polymorphs (8), following removal of specific growth factors or addition of physiologic hormones (9–12), and in several stages of B lymphocyte development (reviewed in references 13 and 14). Mature B cells can undergo apoptosis in the lymphoid follicles during germinal center (GC)[1] reactions. B cells (centroblasts) in the dark zone of GCs will hypermutate their V region genes, thus generating variant B cell receptors (BCRs). If the progeny of centroblasts (centrocytes) enter the light zone of GCs, they will die by apoptosis unless they are rescued by binding to follicular dendritic cells (FDCs) (15). This is the presumed basis for affinity maturation during immune responses.

Much of the physiology of lymphocyte apoptosis was studied in cell lines and thymocytes. Protein kinase C (PKC) activation appears to protect certain T cell hybridoma lines from steroid-induced apoptosis (16) and apoptosis in endothelial cells (17). However, it has also been reported that PKC activation can induce apoptosis in thymocytes (18). In many cell types apoptosis requires de novo protein and RNA synthesis (1, 19–22), arguing for the existence of so-called "death proteins." In contrast, certain other cell lines have been shown to undergo increased apoptosis when macromolecular synthesis is inhibited (21, 23–27), suggesting that the endonuclease is already present but is inactive because of the presence of labile "protective or inhibitory proteins." Although several rescue signals for GC B cells have been described (15, 28–31), the mechanisms regulating apoptosis and endonuclease activity in GC B cells remain unknown.

In this study, we have investigated the induction and inhibition of endonuclease activity in GC B cells and the influence of FDCs, soluble CD40 (sCD40), intracellular Zn^{2+}, and cross-linkage of sIg, sIgM, CD21, CD22, and CD40 on endonuclease activity. Optimal conditions for endonuclease ac-

[1] *Abbreviations used in this paper:* ACT-D, actinomycin-D; AET, 2-aminoethylthiouronium bromide; ATA, aurintricarboxylic acid; BCR, B cell antigen receptor; CD40L, CD40 ligand; CHX, cycloheximide; FDC, follicular dendritic cell; FLCL, FDC-like cell line; GC, germinal center; HD, high density; LD, low density; PB, peripheral blood; PKC, protein kinase C; sCD40, soluble CD40–human Ig fusion protein; sIg, surface Ig; TPEN, (*N,N,N',N'*-tetrakis [2-pyridyl-methyl] ethylenediamine), a Zn^{2+} chelator.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/06/1985/11 $2.00
Volume 181 June 1995 1985–1995

tivity in GC B cell nuclei were determined. We will present evidence that protein synthesis is not necessary for the activation of apoptosis in GC B cells. It will be shown that even the blocking of de novo protein synthesis results in increased apoptosis, suggesting that protein synthesis is part of an apoptosis-inhibiting process in GC B lymphocytes. Moreover, we will demonstrate that FDCs may give their major rescuing signal through a mechanism distinct from the interaction between CD40 and CD40 ligand (CD40L) and that intracellular Zn^{2+} has an important role in FDC-mediated rescue of GC B cells.

Materials and Methods

Isolation of FDCs. FDCs were isolated from tonsils as described by Parmentier et al. (32). In brief, freshly obtained tonsils were cut into small pieces and incubated twice for 30 min at 37°C under continuous shaking in 30 ml of IMDM plus 90 μg/ml gentamicin (IMDM/g) containing 200 U/ml collagenase type IV (Worthington Biochem Corp., Freehold, NJ) and 10 U/ml DNase I (Boehringer Mannheim GmbH, Mannheim, FRG). Next, the cell suspensions were cooled on ice, washed, and subjected to 1-*g* sedimentation (30 min, 0°C) in discontinuous BSA gradients consisting of layers of 1.5, 2.5, and 5% BSA in HBSS. Cells at the interfaces between 2.5 and 5% BSA were collected, washed, and layered on Percoll gradients (Pharmacia Diagnostics AB, Uppsala, Sweden), consisting of layers with densities of 1,070, 1,060, and 1,030 mg/ml, and centrifuged for 20 min at 1,200 *g*. Cells with densities of <1,060 mg/ml were collected. These FDC-enriched cell suspensions were used for further experiments and contained 5–20% DRC-1$^+$ (Dakopatts, Glostrup, Denmark) cells.

B Cell Isolation from Tonsils and Peripheral Blood. Purified tonsillar B lymphocytes were isolated according to the method described by Falkoff et al. (33). Briefly, tonsillar cell suspensions were depleted of T cells by rosetting with SRBCs treated with 2-aminoethylisothiouronium bromide (AET; Sigma Immunochemicals, St. Louis, MO). The rosetted cells were removed by centrifugation on Lymphoprep (1,077 mg/ml; Nycomed, Oslo, Norway). The final cell population contained >98% CD20$^+$ cells (B cells) and <4% CD3$^+$ cells (T cells).

Low density (LD) and high density (HD) B cell fractions were obtained according to the method of Koopman et al. (34). Briefly, B cells were centrifugated (15 min, 1,200 *g*, 4°C) on a Percoll gradient, consisting of four density layers (1,077, 1,067, 1,056, and 1,043 mg/ml). Cells at the 1,043/1,056 interface (LD B cells) and at the 1,067/1,077 interface (HD B cells) were used. LD B cell fractions consisted mainly of GC B cells (~70% CD38$^+$; ~20% sIgD$^+$ and CD39$^+$), and HD B cell fractions were enriched for mantle zone B cells (50% sIgD$^+$ and CD39$^+$; 40% CD38$^+$).

Purified GC B cells were obtained by incubating the LD B cell fraction with antibodies against sIgD (JA11; Oxoid, London, UK) and CD39 (AC2; Immunotech, Luminy, France) followed by depletion of the labeled cells using sheep anti-mouse Ig–coated Dynabeads (Dynal, Oslo, Norway). Purified GC B cell fractions consisted of >95% CD38$^+$ cells and <5% CD39$^+$ and sIgD$^+$ cells.

Peripheral blood (PB) B cells were isolated from heparinized buffy coat cells. Freshly obtained buffy coats were diluted 1:1 with HBSS followed by density centrifugation using Lymphoprep (1,077 mg/ml) to separate RBCs and granulocytes from PBMCs. PBMCs were treated with 100 mM leucine-O-methylester (Sigma) for 40 min at room temperature and pH 7.4. T cells were depleted by rosetting with AET-treated SRBCs followed by centrifugation (30 min, room temperature, 1,000 *g*) on a Ficoll gradient consisting of three densities (1,065, 1,075, and 1,085 mg/ml). Cells with densities >1,065 mg/ml and <1,085 mg/ml were harvested. The final cell populations contained 70–90% CD21$^+$ cells and 5–30% CD3$^+$ cells.

Cell Cultures. B cells (10^6/ml) were cultured for 4–48 h in 24-wells culture plates (Costar Corp., Cambridge, MA) in IMDM/g plus 10% FCS in the presence or absence of several different agents: cycloheximide (CHX; 50 μM; Sigma), actinomycin-D (ACT-D; 10 μg/ml; Sigma), the endonuclease inhibitor $ZnCl_2$ (5 mM; Merck, Darmstadt, FRG), and several different antibodies (all at 5 μg/ml) (Table 1). B cells were incubated for 30 min at 0°C with these antibodies, followed by an additional incubation with goat anti-mouse (IgG+A+M) antibodies (5 μg/ml; Zymed Labs, Inc., S. San Francisco, CA) in order to cross-link the first antibodies.

FDC-enriched cell suspensions were cultured in IMDM/g plus 10% FCS in the presence or absence of 500 ng/ml sCD40 (36), at 10^6 cells per ml for 4–48 h, or the Zn^{2+} chelator *N,N,N',N'*-tetrakis(2-pyridyl-methyl)ethylenediamine (TPEN; 10 μM; Sigma) (39), at 2–4 × 10^6 cells per ml for 16 h. To investigate endonuclease activity, FDC–B cell clusters and single cells were separated by 1-*g* sedimentation in IMDM/g plus 30% FCS (30 min, 0°C). Clusters were harvested from the pellet and contained 90–95% CD20$^+$ B cells and 5–10% FDCs. Single B cells were harvested from the interface. Apoptosis in FDC–B cell clusters was studied in six-well culture plates (Costar Corp.). Cultured cells were stained in the well for 15 min with Hoechst 33342 (Sigma), centrifuged for 3 min at 200 *g*, and covered with a coverglass (24 × 24 mm), and excess culture medium was removed. The cell cultures were examined directly in their culture wells by fluorescence microscopy as previously described (15), using a Leitz Orthoplan microscope (E. Leitz, Wetzlar, FRG) with Ploem-Opak illumination. An NPL 50×/1.00 oil immersion objective was routinely used.

DNA Fragmentation Assay of Isolated Nuclei. Cell nuclei were isolated according to Nieto and López-Rivas (40). Briefly, 2 × 10^6 cells per incubation were spun down and disrupted with 1.5 mM $MgCl_2$ (Merck) (30 min, 0°C). Nuclei were washed with 1.5 mM $MgCl_2$ and resuspended in 100 μl of TSN buffer (10 mM Tris, 200 mM sucrose, 60 mM NaCl, pH 7.5). For DNA fragmentation assays, isolated nuclei were incubated in Eppendorf vials for 4 h at 0 or 37°C in the presence or absence of Ca^{2+} (5 mM) and Mg^{2+} (10 mM). Optimum conditions for endonuclease activity were determined by performing fragmentation assays in TSN buffer at pH 6–9.5, with varying concentrations of Ca^{2+} and Mg^{2+} and in the presence or absence of Zn^{2+} (100 μM), Na^+ (80 or 800 mM), aurintricarboxilic acid (ATA; 300 μM; Sigma), EGTA (5 mM; Sigma), or EDTA (5 mM; Merck).

DNA fragmentation was determined using DNA electrophoresis. Nuclei were lysed with 500 μl of TTE buffer (10 mM Tris-HCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.6), and fragmented DNA was separated from intact chromatin by centrifugation for 10 min at 4°C and 14,000 *g*. The supernatant, containing the fragmented DNA, was collected carefully and placed in an Eppendorf vial. The intact chromatin in the pellet was resuspended in 500 μl of TTE buffer. 100 μl of 5 M NaCl (0°C) was added followed by vigorous vortexing. Subsequently, 700 μl of isopropanol (−20°C) was added, and after vortexing, the vials were placed overnight at −20°C to precipitate DNA. Next, the vials were centrifuged for 10 min at 4°C and 14,000 *g*, the supernatants were discarded, and the pellets were washed carefully with 70% ethanol at −20°C. After centrifugation, the supernatants were discarded and the pellets were dried. DNA was dissolved in 20–50 μl of TBE buffer (9 mM Tris, 9 mM boric acid, 2 mM EDTA, pH 8). The DNA concentra-

Table 1. *Antibodies Used*

Antibody	Marker/isotype	Source
OKT3	CD3/IgG2a	Ortho, Raritan, NJ
BU32	CD21/IgG1	Binding Site, Birmingham, UK
To-15	CD22/IgG2b	Dakopatts, Glostrup, Denmark
S2C6	CD40/IgG1	Gift from Dr. S. Paulie (Dept. of Immunology, The Arrhenius Laboratories, Stockholm University, Stockholm, Sweden) (35)
G28-5	CD40/IgG1	Gift from Dr. J. Ledbetter (Oncogen Corp., Seattle, WA) (36)
EA-5	CD40/IgG1	Gift from Dr. T. LeBien (University of Minnesota, Dept. of Laboratory Medicine and Pathology, Medical School, Minneapolis, MN) (37)
14G7	CD40/IgM	Gift from Dr. R. van Lier (Dept. of Autoimmune Diseases, CLB, Amsterdam, The Netherlands)
5D12	CD40/IgG2b	Our laboratory (38)
3C6	CD40/IgG2b	Our laboratory (38)
4103	Goat anti-human Ig	Tago, Inc., Burlingame, CA
Immuno beads	Rabbit anti-human IgM	Bio Rad Laboratories, Richmond, CA

tion was measured by spectrophotometry at 260 nm. Electrophoresis of the fractions containing fragmented DNA (supernatant fractions after lysis and centrifugation) was done for 2 h at 3 V/cm in 1.5% agarose gels supplemented with ethidium bromide. DNA was visualized using UV light.

Determination of Apoptosis Using Cytospin Centrifugation. Cytospin preparations were made immediately after culture using a Shandon cytospin centrifuge (Shandon Inc., Pittsburgh, PA), stained with May–Grünwald Giemsa (Merck) and enumerated for the percentage of apoptotic cells. At least 300 cells were counted in each preparation.

Results

Apoptosis Is a Feature of GC B Cells but Not of Resting B Cells. Upon culture at 37°C, LD B cell fractions from human tonsils showed rapid apoptosis. Apoptotic cells could be detected even within 3 h of culture, and their numbers increased further to reach 50–60% after 16 h (Fig. 1 *a*). Furthermore, in purified GC B cells, ~60% of the cells were apoptotic after 16 h (data not shown). High density B cell fractions from human tonsils also showed some apoptosis upon culture in vitro, but the number of apoptotic cells was profoundly lower as compared with that of the LD B cells (Fig. 1 *b*). Also, after an initial rise in apoptotic cell numbers, no further increase was seen after 6 h of culture. By contrast, PB B lymphocytes did not show any apoptosis when cultured in vitro for as long as 96 h (≤1%).

Apoptosis in Isolated LD B Cells Cannot Be Blocked by Protein Synthesis Inhibitors. To determine whether de novo protein synthesis is required in LD B cells for entering apoptosis, LD B cells were cultured in the presence of the protein synthesis inhibitors CHX or ACT-D. Addition of these inhibitors did not result in decreased apoptosis in LD B cells, but rather in increased apoptosis (Fig. 1 *a*), indicating that endonuclease must have already been present in the cells and is under control of one or more regulatory proteins with a high turnover rate. Apoptosis in tonsillar B lymphocytes could be completely blocked by the addition of the endonuclease inhibitors Zn^{2+} (5 mM) (Fig. 1 *a*) and ATA (200 μM) (data not shown). PB B cells did not enter apoptosis even after culture in the presence of CHX for 96 h (<1%), suggesting that endonuclease is totally absent in these B cells.

Endonuclease Activity Is Present in Nuclei of LD B Cells and Purified GC B Cells but Not in Nuclei of HD B Cells, PB B Cells, or the EBV-transformed B Cell Line JY. Because addition of CHX or ACT-D did not inhibit but rather enhanced apoptosis, it can be hypothesized that endonuclease is already present in tonsillar B cells. To address this suggestion, nuclei from LD B cells, purified GC B cells, HD B cells, PB B cells, and an EBV-transformed B cell line (JY) were isolated and DNA fragmentation was studied. Isolated nuclei from GC B cells and LD B cells showed high endonuclease activity, as demonstrated by the characteristic ladder pattern in the gels. In contrast, no endonuclease activity could be observed in nuclei from PB B cells and the JY cell line (Fig. 2). In HD B cell nuclei no or very slight DNA fragmentation was seen.

Optimum Conditions for Endonuclease Activity in Tonsil B Cells. To determine optimum conditions for endonuclease activity, isolated nuclei from B cells were incubated in TSN buffers at pH 6–9.5 in the presence or absence of Ca^{2+} and Mg^{2+}. DNA gel electrophoresis showed that endonuclease in B cells has its maximum activity at pH 7.5–8 and in the presence of 5 mM Ca^{2+} and 10 mM Mg^{2+} (Fig. 3). En-

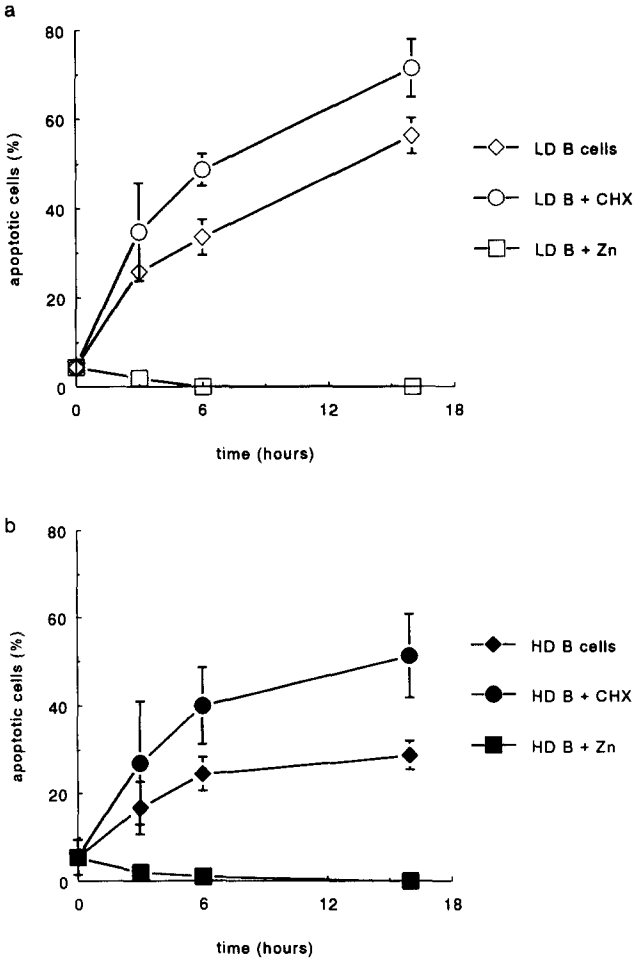

Figure 1. Apoptosis of LD (*a*) and HD (*b*) B cells after 3, 6, or 16 h of culture in the presence or absence of 50 μM CHX or 5 mM Zn^{2+}, enumerated in cytospin preparations stained with May–Grünwald Giemsa. Data are represented as mean percentage of apoptotic cells ± SD from four experiments using different donors. (*a*) Apoptosis in LD B cell–enriched cell populations. No addition (○), CHX (◇), or Zn^{2+} (□). (*b*) Apoptosis in HD tonsillar B cells (>1,065 mg/ml). No addition (●), CHX (◆), or Zn^{2+} (■).

donuclease activity could be blocked completely by the addition of Zn^{2+} (100 μM), ATA, NaCl, EGTA, or EDTA to the incubation medium (Fig. 4). Addition of 5 mM Mn^{2+} did not result in activation, but rather in inhibition of endonuclease activity. These data indicate the similarity of the B cell endonuclease with NUC-18 (41).

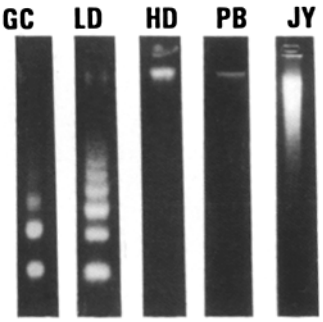

Figure 2. Endonuclease activity in isolated nuclei from GC B cells (*GC*), LD B cells (*LD*) HD B cells (*HD*), PB B cells (*PB*), JY cells (*JY*). Endonuclease activity is visualized by DNA gel electrophoresis of low molecular weight DNA isolated from the various cell types

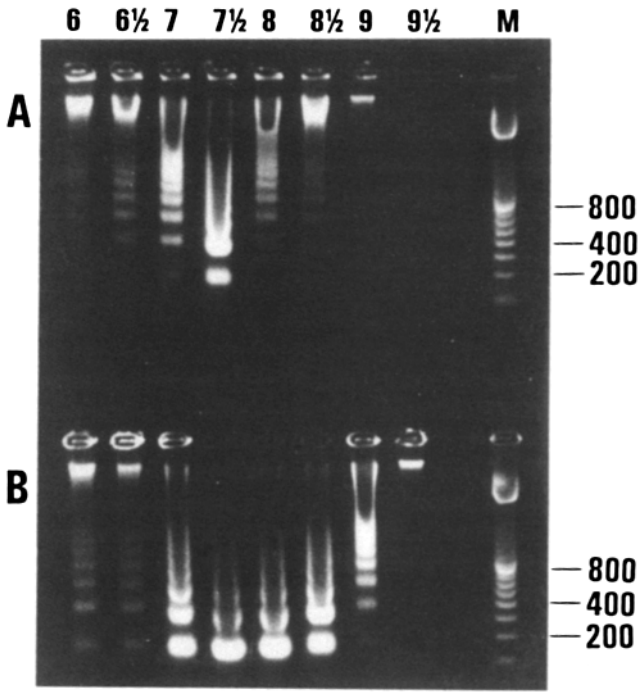

Figure 3. pH optimum for endonuclease activity in tonsillar B cell nuclei in the absence (*A*) or presence (*B*) of Ca^{2+} (5 mM) and Mg^{2+} (10 mM). pH values are indicated above the gel. *M*, 100-bp marker.

FDCs and FDC-like Cell Lines Totally Inhibit Endonuclease Activity in GC B Cells by a Mechanism Dependent on Cell–Cell Contact. From previous experiments (15), it is known that FDCs can prevent GC B cells from entering apoptosis by a mechanism dependent on cell–cell contact. To investigate whether endonuclease activity in isolated GC B cell nuclei is altered after interaction with FDCs, GC B and FDCs cells were cultured for 4–120 h. Subsequently, single and clustered cells were separated, the nuclei were isolated, and endonuclease activity was determined. DNA gel electrophoresis showed that already after 4 h of interaction, endonuclease activity in the nuclei of GC B cells isolated from clusters was totally absent and remained absent even after 5 d of culture. In contrast, nuclei from cells that had remained single retained fully active endonuclease (Fig. 5).

To test whether the EBV-transformed and cloned FDC-like cells (FLCLs) that we have previously described (42) were also capable of turning the endonuclease off, the same experiment was performed using FLCLs. Like FDCs, FLCLs were able to rescue B cells from apoptosis in vitro. In addition, it appeared that interaction of LD B cells with FLCLs for 16 h also led to a total loss of endonuclease activity in the clustered B cells (Fig. 6). It is likely that inhibition of endonuclease activity by FLCLs would take <16 h, but although binding of B cells to FLCLs was already in progress after 4 h of culture, culture periods <16 h did not yield sufficient clustered B cells to perform endonuclease assays.

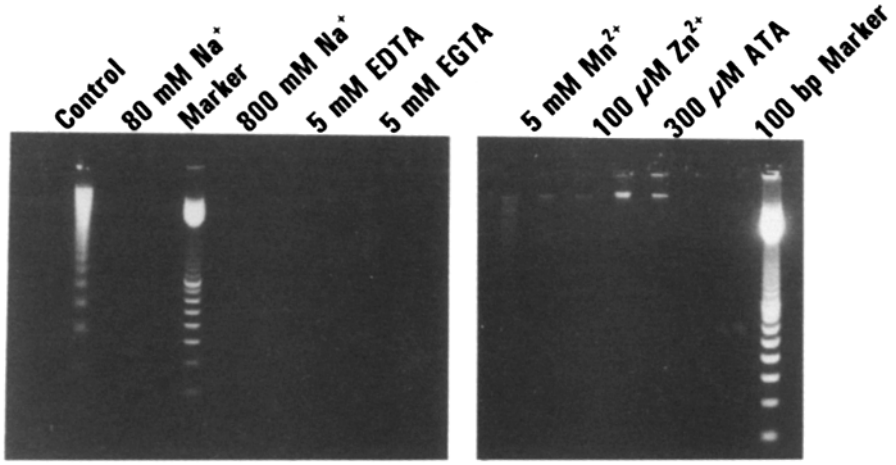

Figure 4. Inhibition of endonuclease activity in tonsillar B cell nuclei by different agents, as indicated above the gels. Endonuclease activity assays were done without (left lane of each pair) or with the addition of Ca^{2+} and Mg^{2+} (right lane of each pair).

FDCs and B cells interact via several membrane markers, among which are interactions through the adhesion molecules CD54 (ICAM-1) and CD109 (VCAM-1) and their counterparts CD11a/CD18 (LFA-1) and CD49d/CD29 (VLA-4), respectively; interactions between immune complexes (iccosomes) on FDCs, BCR, and CD21 on B lymphocytes; and probable interaction between CD22 on B cells and CD75 on FDCs (unpublished observations). Through several of these interactions, it is known that they can postpone apoptosis in B cells to some extent (28, 34, 43). To investigate whether one of these pairs of ligand–receptor interactions between FDCs and B cells may contribute to the described inhibition, GC B cells were preincubated and cultured with antibodies against CD21, CD22, or sIg. After 16 h, cells were harvested, and cytospin preparations of whole cells as well as endonuclease activity assays with isolated nuclei were performed. Cross-linkage of CD21 or CD22 with specific mAbs did not lead to inhibition of apoptosis in whole cells even after additional cross-linkage with goat anti-mouse antibodies. Additionally, endonuclease activity was not affected (Fig. 7). Cross-linkage of antigen receptors resulted in 30–50% inhibition of apoptosis (Table 2) but not in inhibition of endonuclease activity in isolated nuclei (Fig. 7).

Cross-Linkage of CD40 by Antibodies Does Inhibit Apoptosis of LD and Purified GC B Cells, but Does Not Inhibit Endonuclease Activity in Nuclei of LD and GC B Cells. As other groups have reported that cross-linkage of CD40 on B cells in vitro prevents those B cells from entering apoptosis (28, 29), we decided to investigate whether signaling via CD40 would also influence endonuclease activity in nuclei of GC B cells. Therefore, LD B cells and GC B cells were preincubated and cultured with anti-CD40 or -CD3 antibodies. After 16 h, both cytospin centrifugation and endonuclease activity assays were performed. From the cytospin preparations it appeared that cross-linkage of CD40 on B cells resulted in inhibition of apoptosis of B cells ranging from 40 to 90% (Table 3). Incubation with anti-CD3 antibodies did not result in inhibition of apoptosis. By contrast, gel electrophoresis of DNA after endonuclease activity assays showed that cross-linkage of CD40 on B cells for 4 or 16 h did not inhibit endonuclease activity in the nuclei of these cells (Fig. 8). To investigate whether CD40–CD40L bridging contributes to the en-

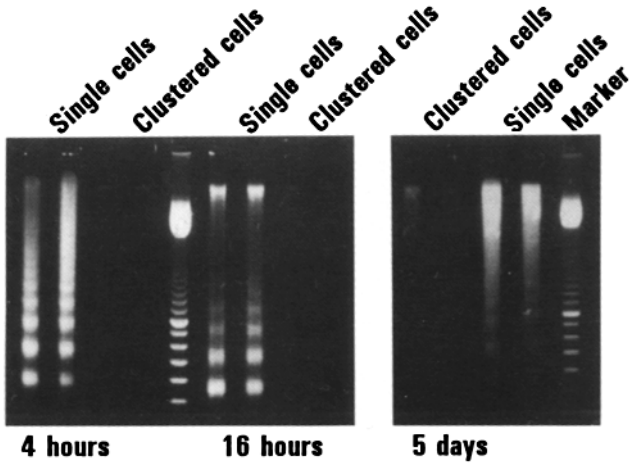

Figure 5. Influence of interaction with FDCs on endonuclease activity in GC B cells. Endonuclease activity assays (see Materials and Methods) show that 4 h after FDC–B cell binding no endonuclease activity is present in clustered B cells. Endonuclease activity assays were performed in the absence (left lane of each pair) or presence (right lane of each pair) of TPEN. DNA was isolated from nuclei of single cells (*Single cells*) or from clustered B cells (*Clustered cells*) after 4, 16, or 120 h of culture with FDCs.

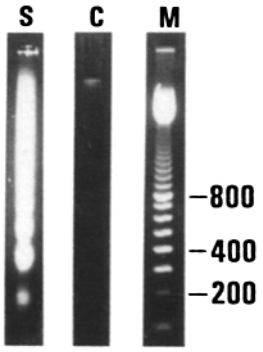

Figure 6. Influence of interaction with FLCLs on endonuclease activity in LD B cells showing that endonuclease activity is no longer present in nuclei isolated from clustered cells. DNA was from single (*S*) or clustered (*C*) cell nuclei. *M*, 100-bp marker.

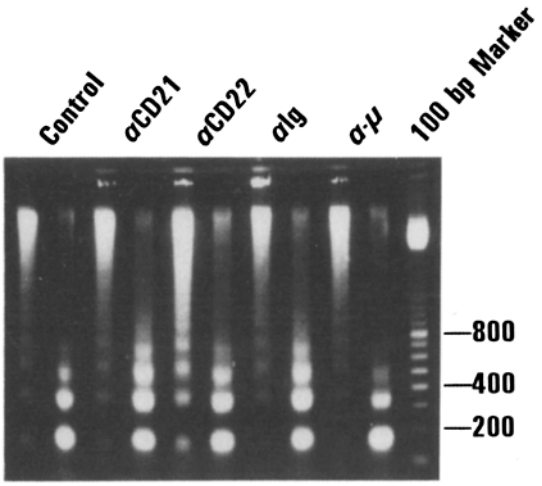

Figure 7. Cross-linkage of CD21, CD22, or the BCR on GC B cells does not inhibit endonuclease activity. For each pair of lanes, the left lane represents no stimulus and the right represents stimulus with Ca^{2+} and Mg^{2+}. Endonuclease activity was visualized by DNA gel electrophoresis of low molecular weight DNA isolated from cells cultured without antibodies (*Control*) or with antibodies, as indicated above the gel.

donuclease inhibition induced by FDCs, FDC-enriched cell suspensions were cultured in the presence of sCD40. After 16 h, clusters and single cells were isolated, and endonuclease activity assays were performed. Gel electrophoresis showed that addition of sCD40 to FDC–B cell cultures could not prevent the inhibition of endonuclease activity in clustered B cell nuclei (Fig. 9).

Chelation of Intracellular Zn^{2+} Enhances Apoptosis in LD B Cells and Abrogates the FDC-dependent Rescue from Apoptosis of LD B Cells. Recently, McCabe et al. (39) and Treves et al. (44) reported that chelation of intracellular Zn^{2+} by a metal ion chelator (TPEN) with high affinity for Zn^{2+} triggered apoptosis in thymocytes and PB lymphocytes. Because it is known that Zn^{2+} is a potent direct endonuclease inhibitor, we decided to investigate whether Zn^{2+} might also have

Table 2. *Inhibition of Apoptosis in Purified GC B Cells after Cross-linkage of Different Membrane Molecules*

Molecule	Exp. 1	Exp. 2
CD21	0	0
CD22	0	0
sIg	27	29
sIgM	35	46
CD40 (14G7)	50	37

GC B cells were cultured for 16 h in the presence of different mAbs. Antibodies against CD21, CD22, and CD40 were cross-linked with goat anti-mouse Ig. Percent inhibition of apoptosis was determined as (percent apoptosis in control − percent apoptosis in test)/(percent apoptosis in control). Percent apoptosis in control incubations (goat anti-mouse Ig only) was 50–60%.

Table 3. *Inhibition of Apoptosis in LD B Cells by Anti-CD40 Antibodies*

mAb	Exp. 1	Exp. 2
S2C6	81	81
G28-5	42	54
EA-5	65	92
14G7	62	85
3C6	85	65
5D12	69	62

LD B cells were cultured for 16 h in the presence of different anti-CD40 mAbs. Anti-CD40 antibodies were cross-linked with goat anti-mouse Ig. Percent inhibition of apoptosis was determined as (percent apoptosis in control − percent apoptosis in test)/(percent apoptosis in control). Percent apoptosis in control incubations (goat anti-mouse Ig only) was 50–60%.

effects on apoptosis in B cells. Therefore, isolated B cells were cultured for 24 h in the presence of TPEN, and apoptosis was estimated in cytospin preparations stained with May–Grünwald Giemsa. Addition of TPEN resulted in a dose-dependent increase in apoptosis of tonsillar B cells (Fig. 10). Because Zn^{2+} chelation increased apoptosis in B cells, we investigated whether intracellular Zn^{2+} is involved in FDC-mediated rescue of GC B cells from apoptosis. FDC-enriched cell suspensions were cultured in the presence of 10 μM TPEN, stained with Hoechst 33342, and examined by fluorescence microscopy. Addition of TPEN to FDC–B cell cultures resulted in a dramatic decrease in FDC-mediated rescue of GC B cells (Fig. 11; Table 4), suggesting that intracellular Zn^{2+} plays a key function in FDC-mediated rescue of LD B cells. To determine whether the observed rapid inhibition of endonuclease by FDCs could be caused by a direct action of Zn^{2+} on endonuclease in GC B cell nuclei, the endonuclease activity assay with B cells isolated from FDC clusters was done in the presence of 10 μM TPEN. If endonuclease activity in the nuclei of clustered B cells was inhibited by a direct action of Zn^{2+} on the endonuclease, chelation of Zn^{2+} with TPEN during endonuclease activity assays should negate the inhibition of endonuclease observed after FDC–B cell interaction. However, addition of TPEN did not restore endonuclease activity in nuclei of clustered B cells (Fig. 5).

Discussion

Apoptosis is a common form of cell death characterized by extensive chromatin cleavage into oligosomal length fragments. The DNA is fragmented by the action of an endogenous endonuclease. In this study we have investigated characteristics and activation/inhibition mechanisms of endonuclease of GC B cells. GC B cells rapidly enter apoptosis upon culture in vitro (Fig. 1 *a*). Addition of protein synthesis inhibitors did not result in a decrease in the number of apoptotic

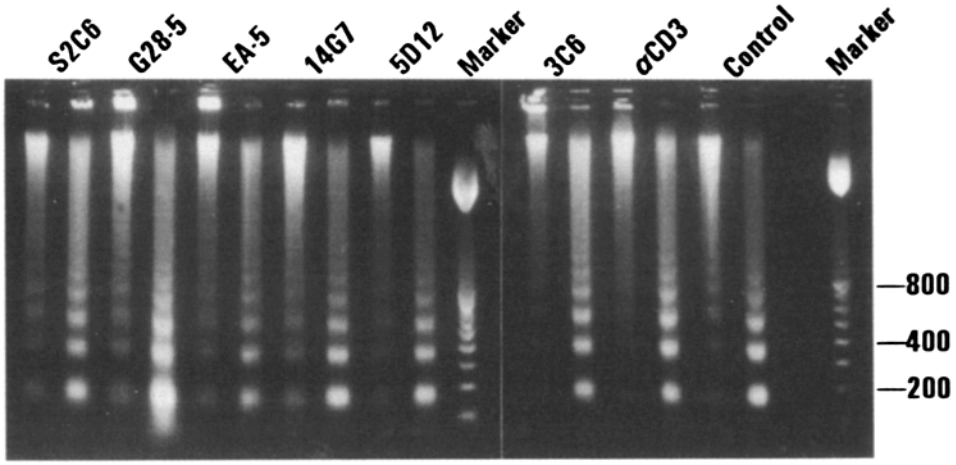

Figure 8. Cross-linkage of CD40 on LD B cells for 16 h does not lead to rapid inhibition of endonuclease activity. For each pair of lanes, the left lane represents no stimulus and the right represents stimulus with Ca^{2+} and Mg^{2+}. Endonuclease activity was visualized by DNA gel electrophoresis of low molecular weight DNA isolated from cells cultured without antibodies (*Control*) or in the presence of anti-CD40 or -CD3, as indicated above the gels.

cells; an increase of the number of apoptotic cells was even detected (Fig. 1 *a*). This suggests that endonuclease is already present in GC B cells and is under the control of one or more inhibitory proteins with a high turnover rate, since increased apoptosis by CHX or ACT-D was seen already after a few hours. These results evince that apoptosis in GC B cells is induced via the so-called "release mechanism" (13, 23, 45, 46). In this model, the death machinery is already present, but its action is held under control by inhibitory proteins with short half-lives. The presence of a preformed death machinery is found only in GC B cells and not in resting B lymphocytes, as PB and HD B cells did not enter apoptosis when cultured in the presence of CHX or ACT-D. In our experiments with enriched mantle zone B cells, we often found some endonuclease activity as well as effects of CHX and ACT-D. This was more evident in cytospin preparations than in DNA fragmentation assays. However, as HD B cell

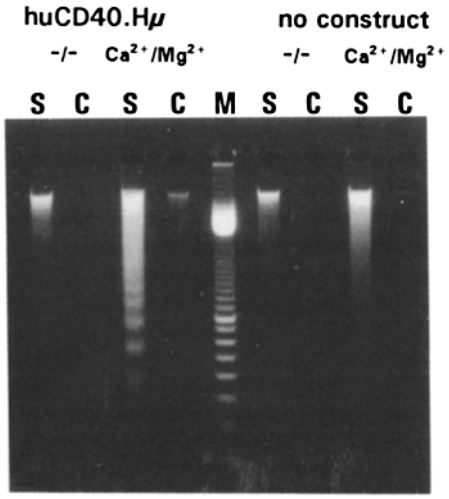

Figure 9. Addition of sCD40 (*huCD40.Hμ*) to FDC–B cell cultures does not inhibit FDC-mediated rescue of GC B cells. Endonuclease activity assays were done with or without addition of Ca^{2+} and Mg^{2+}, as indicated above the gel. DNA was isolated from nuclei of single (*S*) or clustered (*C*) cells. *M*, 100-bp marker.

fractions may be contaminated with GC B cells, it cannot be excluded that the observed endonuclease activity is caused by the presence of GC B lymphocytes. Furthermore, at the level of cell nuclei, we could easily demonstrate the presence of preformed endonuclease in isolated nuclei of GC B cells (Fig. 2). Additionally, the supposed absence of endonuclease in nuclei of HD B cells, PB B cells, and JY cells was demonstrated. The optimum conditions for endonuclease activity in GC B cells were found to be pH 7.5 with 5 mM Ca^{2+} and 10 mM Mg^{2+} (Fig. 3). The activity was completely blocked by the addition of Zn^{2+} (250 μM), ATA (300 μM), NaCl (80–800 mM), 5 mM EDTA, or 5 mM EGTA (Fig. 4). Addition of 5 mM Mn^{2+} did not activate endonuclease. A recent review by Peitsch et al. (47) and comparison of our data with results from other studies (41, 48–54) indicate that only the NUC-18 endonuclease meets the conditions found for the endonuclease of GC B lymphocytes. NUC-18 is a 18-kD nuclease, identified, purified, and characterized from apoptotic rat thymocytes (41). Whether DNA fragmentation in GC B cells is due to the action of NUC-18 or a NUC-18–like protein is currently unclear.

It is believed that GC B lymphocytes are rescued from apoptosis in vivo by interaction with FDCs. In experiments in vitro it was shown that FDCs (15) and EBV-transformed FLCLs (42) can prevent GC B cells from death by apoptosis. In addition, it was also shown that cross-linkage of CD40, sIg, CD21, CD38, LFA-1, or VLA-4 and the addition of certain growth factors could inhibit or postpone apoptosis in GC B cells (28, 29, 34, 43, 55). In our experiments, interaction with FDCs for as little as 4 h resulted in a complete inhibition of endonuclease activity in GC B cells (Fig. 5). This was also found with EBV-infected and cloned FLCLs (Fig. 6).

It is currently unknown whether the rescue from apoptosis by FDCs or FLCLs in vitro is an antigen-dependent process. We have shown previously that apoptosis in LD B cells is postponed for a few days by adherence to plastic-immobilized ICAM-1 and VCAM-1 and that cross-linkage of the antigen receptor in this system has a synergistic effect on the delay of apoptosis (34). On freshly isolated FDCs,

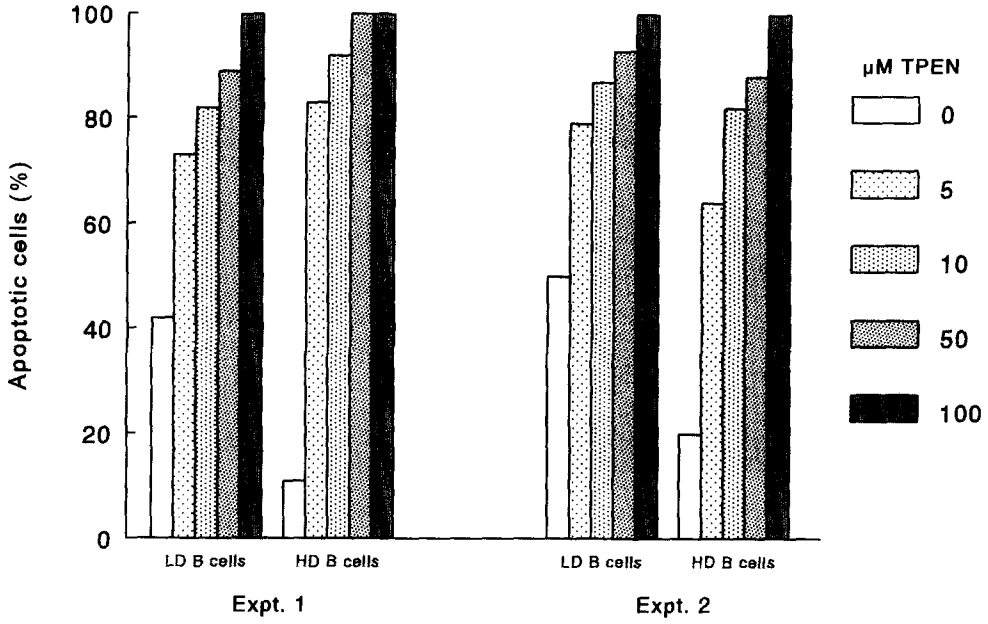

Figure 10. Percentage of apoptotic tonsillar LD and HD B cells after 16 h of culture in the presence of different concentrations of the Zn^{2+} chelator TPEN, determined in cytospin preparations stained with May–Grünwald Giemsa.

antigens in the form of immune complexes may still be present, as IgM is readily detected. Therefore, it is possible that B cell adherence to freshly isolated FDCs and their subsequent rescue from apoptosis is antigen dependent. For the binding of B cells to FLCLs and the FLCL-mediated rescue from apoptosis, it is difficult to believe that this is an antigen-dependent process, because after 6–8 mo in vitro, these FLCLs presumably no longer carry antigens. However, as several authors have shown that upon cross-linkage of antigen receptors on B cells the high avidity state of LFA-1 is induced very quickly (56, 57). It may be possible that the B lymphocytes used were already in a preactivated state as the result of antigens encountered in vivo and thus already express the high avidity state of LFA-1. This might bypass an antigen-dependent step in vitro.

In line with data reported by others, we found that cross-linkage of CD40 on GC B cells for 4 or 16 h inhibits apoptosis in GC B cells (Table 3). Cross-linkage of CD40, however, did not lead to significant inhibition of endonuclease activity in GC B cell nuclei (Fig. 8). These results, together with the observation that addition of sCD40 to FDC–B cell cultures did not induce apoptosis in FDC–B cell clusters and failed to result in a reappearance of endonuclease activity in

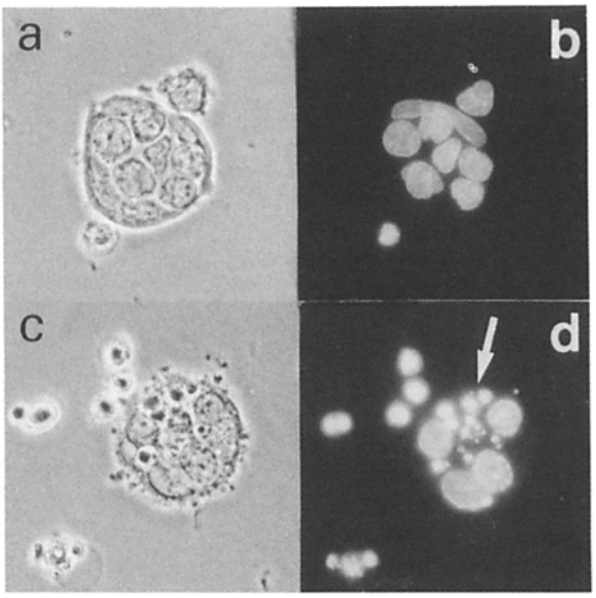

Figure 11. Photomicrograph of FDC–B cell clusters cultured in the absence or presence of the Zn^{2+} chelator TPEN (10 μM). Cultures were stained with Hoechst 33342. (*a*) Phase-contrast micrograph of an FDC–B cell cluster. (*b*) Fluorescence micrograph showing that all cells inside the cluster are viable (finely stained chromatin network). (*c*) Phase-contrast micrograph of an FDC–B cell cluster in the presence of TPEN. (*d*) Fluorescence micrograph showing several apoptotic cells inside the cluster (*arrow*). ×700.

Table 4. *Chelation of Zn^{2+} by TPEN Negates FDC-mediated Rescue of GC B Cells*

	Cells per cluster	% Apoptotic
Exp. 1		
Control	17 ± 5	0
TPEN	7 ± 2	85 ± 15
Exp. 2		
Control	6 ± 2	0
TPEN	7 ± 1	72 ± 8

FDC-enriched cell suspensions were cultured for 16 h in the absence or presence of the Zn^{2+} chelator TPEN at 10 μM. Data are represented as mean ± SD for five micrographs taken after 16 h of culture and staining for 15 min with Hoechst 33324; each micrograph depicts at least three FDC–B cell clusters. Only B cells in clusters were considered.

the nuclei of clustered cells (Fig. 9), suggest that FDCs may give the rescue signal to GC B cells by a mechanism distinct from CD40 ligation. As mentioned in Results, FDCs and B cells interact via several membrane markers, some of which can postpone or inhibit apoptosis in B cells to some extent (28, 34, 43). In our experiments only cross-linkage of the antigen receptor resulted in 30–50% inhibition of apoptosis in whole cells. In contrast, the activity of endonuclease in the nuclei of these cells remained, as with CD40 cross-linkage, uninhibited. It may therefore be possible that a combination of interactions between FDCs and B cells is responsible for the transmission and receipt of the rescue signal that leads to an irreversible blockade of endonuclease in GC B cells. However, it cannot be ruled out that FDCs down-regulate the endonuclease by an unknown mechanism. A study to investigate whether other B cell markers are involved in the interaction between B cells and FDCs or FLCLs is now in progress.

The finding that cross-linkage of CD40 and sIg on GC B cells results in a decrease of apoptotic cell death but does not inhibit endonuclease activity in isolated nuclei seems contradictory. An explanation may be that by cross-linking CD40 or sIg on GC B cells, the activation state of the cell is maintained on a "survival level" in such a way that the production of the supposed endonuclease inhibitor or other regulatory proteins is continued. The endonuclease itself may remain unaffected in the nucleus.

The observation that Zn^{2+} inhibits endonuclease activity in isolated nuclei and that chelation of intracellular Zn^{2+} leads to enhanced apoptosis of tonsillar B cells (Fig. 10) raises the possibility that the rapid abolishment of endonuclease activity after contact with FDCs might be regulated by a direct effect of intracellular Zn^{2+} on endonuclease. If in this system endonuclease is directly blocked by Zn^{2+}, addition of the Zn^{2+} chelator TPEN (39, 44) to the endonuclease assays should remove Zn^{2+} from the endonuclease, resulting in fragmentation of the DNA. Our results, however, showed that addition of TPEN does not lead to a reappearance of endonuclease activity (Fig. 5). It can be argued, therefore, that either Zn^{2+} does not directly inhibit endonuclease in the nuclei, the affinity of TPEN for Zn^{2+} (4×10^{15} M^{-1} [39]) is lower than the affinity of Zn^{2+} for endonuclease, or the endonuclease is removed from the GC B cell nuclei during interaction with FDCs. Nevertheless intracellular Zn^{2+} does play an important role during FDC–B cell contact because addition of TPEN to cultures of FDCs and B cells led to the appearance of apoptotic cells in established FDC–B cell clusters (Fig. 11; Table 4). If Zn^{2+} is not involved in a direct inhibition of the endonuclease, it may be necessary for transducing rescue signals, which is in line with reports from others (39, 44, 58–60).

In the current study we have shown that in contrast to interaction with FDCs or FLCLs, cross-linkage of CD40 on B lymphocytes does not inhibit endonuclease activity and that the presence of sCD40 during FDC–B cell interactions does not interfere with the FDC-mediated rescue. It can be argued, therefore, that the function of the CD40–CD40L interaction in vivo is not to rescue GC B cells from apoptosis, but rather to give signals to B cells for the induction of isotype switch and immunoglobulin production.

We wish to thank our colleagues in the department of Otorhinolaryngology of the Academic Medical Center, in the Onze Lieve Vrouwe Gasthuis Hospital, and in the Boven-IJ Hospital for providing us with tonsils; Drs. Paulie, Van Lier, Ledbetter, and LeBien for their generous gift of anti-CD40 antibodies; Dr. Lane (Institute for Immunology, Basel, Switzerland) for his generous gift of the soluble CD40 fusion protein; F. Stiekema for technical assistance; and J. Meenan for his critical reading of the manuscript.

This study was financially supported by Eurocetus B.V., The Netherlands, and The Netherlands Ministry of Economic affairs.

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Received for publication 10 August 1994 and in revised form 10 January 1995.

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