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The Avian Chb6 Alloantigen Triggers Apoptosis in a Mammalian Cell Line

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Many developing B lymphocytes are deleted by apoptosis. However, the mechanism signaling their demise remains poorly understood. Like mammals, chicken B cells are selected during their development; >95% of the cells in the bursa of Fabricius die without entering the secondary immune system. The molecule chB6 (Bu-1) has been used as a marker to identify B cells in the chicken. ChB6 is a type I transmembrane glycoprotein whose function is enigmatic. We have provided evidence that chB6 can induce a rapid form of cell death exhibiting characteristics of apoptosis. Here we further examine cell death induced by chB6 in a transfected mouse cell line. ChB6 is shown to cause apoptosis in this cell line as detected by a TUNEL assay for DNA fragmentation. This apoptosis is subject to regulation by signals from growth factor or by Bcl-xL. Furthermore, we show that Ab binding to chB6 leads to cleavage of caspase 8, caspase 3, and poly(ADP ribose) polymerase. Overall, these data support the hypothesis that chB6 is a novel death receptor on avian B cells.

The regulation of cell death is critical for the immune system, both to eliminate cells at the end of an effective immune response and to prevent the emergence of autoreactive cells from the primary organs. The elimination of potentially self-reactive cells is observed during the development of both T and B lymphocyte populations (1). In the thymus, defective T cells are deleted during thymic education. Developing B cells in the bone marrow that fail to express functional Ig or prove to be self-reactive are eliminated by apoptosis. Although it is clear that potentially autoreactive cells are deleted in their development before interaction with exogenous Ag and that this deletion occurs via apoptosis, there is keen interest in the actual mechanism initiating this deletion (2). Certainly surface Ig can play a role in this process, but in some circumstances stimulation via Ig can lead to anergy or receptor editing rather than deletion (2). In vivo, it appears that a close interaction with self-Ag on the surface of stromal cells is needed to effect deletion, raising the possibility that other molecules may be involved.

In avian species B lymphocytes develop in the bursa of Fabricius, a primary lymphoid organ required for diversification of Ig genes (3). The bursa is seeded by a wave of precursor B cells between days 8 and 14 of embryogenesis (4). These precursors have already rearranged Ig gene segments at both the heavy and light chain loci (5–8), but since there are few functional Ig genes, the Ig expressed on these immigrant B cells has little diversity. In the bursa these precursor B cells form lymphoid follicles; two to four precursor cells initiate each follicle and proliferate to form 3 × 10^5 cells by 5–7 wk of age (9). As these cells proliferate, a process of somatic gene conversion diversifies the rearranged Ig gene segments by copying portions of unused gene segments onto the functionally rearranged Ig (10, 11). Despite the proliferation of B cells within the bursa, it is estimated that <5% of these cells will leave the bursa and enter the secondary organs (12). This is probably analogous to the large number of cells lost during mammalian B cell development (13). Presumably, these cells fail a selection event either because gene conversion generates a nonfunctional Ig gene or because the Ig produced interacts with self-Ags. Lymphocytes from the bursa are markedly susceptible to apoptosis when the normal microenvironment of the bursa is disrupted (14). Phorbol esters can prolong the lives of these cells in vitro (15, 16). This suggests that within the bursa, B lymphocytes are subject to a dynamic set of signals regulating their development and viability. It would appear that there is a default pathway to apoptosis that is negatively regulated by signals from a protein kinase C isoform, hence the delayed apoptosis in the presence of phorbol ester.

The bursa thus represents a novel system in which to analyze signals regulating B cell development during this selection process. However, relatively little work has been forthcoming, and there are few markers to identify distinct stages of B lymphocyte development in the bursa. One widely used marker of avian B cells is the chB6 alloantigen (formerly called Bu-1) (17–19). There are currently three recognized forms of the chB6 alloantigen (20). ChB6 is a novel, highly glycosylated, protein expressed on B lymphocytes and a subset of macrophages (18, 20). We have previously reported that chB6 can initiate a rapid form of cell death when bound by an anti-chB6 Ab (21). Here we present further characterization of cell death triggered by chB6 in a transfected mammalian cell line. We elected to perform these experiments in mammalian cells because of a paucity of reagents proven to detect avian homologs of apoptosis-related proteins.

Materials and Methods

Cell lines

The murine FL5.12 cell line was the parental line used in transfection studies. The FL5.12 line was transfected with pcDNA-3 vector (Invitrogen, San Diego, CA) containing either chB6.1 (accession no. X92866) or chB6.2 (accession no. 92867) cDNA (20). Single-cell clones were isolated.
by limiting dilution. Individual clones expressing either chB6.1 or chB6.2 were then superimposed with the plasmids pGKHygro and pSFFV-Bcl-xL in a 1:10 ratio. Transfectants were isolated by resistance to hygromycin B in the pGKHygro plasmid. Single-cell colonies were isolated by limiting dilution and overexpression of Bcl-xL confirmed by Western blotting. A control FL5 cell line overexpressing Bcl-xL was provided by Dr. L. Boise (University of Miami, Miami, FL). WEHI 3B cells were grown as a source of IL-3. The human Jurkat T cell line was also used to confirm the size of activated caspases on Western blot.

Cell culture
FL5.12-based cell lines were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Life Technologies), 10% WEHI culture supernatant as a source of IL-3, 10 mM HEPES (pH 7.4), antibiotic/antimycotic, t-glutamine, and 50 μM 2-ME. Jurkat cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FBS, antibiotic/antimycotic, and t-glutamine. WEHI 3B cells were cultured in IMDM supplemented with 10% FBS, antibiotic/antimycotic, and t-glutamine. All media were filter-sterilized and stored at 4°C. All cell lines were incubated at 37°C in 5% CO2 and passed approximately every 2 days.

Cell death assays
Trypan blue exclusion. FL5.12-based cell lines were plated at a concentration of 10⁴ cells/ml in a 24-well microtiter plate in either FL5 culture media or RPMI supplemented with 10% NBS. Cells were cultured for 15 h at 37°C in 3% CO2. At 15 h, 20-µl samples were taken from each well and scored for viability based on ability to exclude the dye trypan blue. Following counts, cells were treated with either 1 µl 21-1A4 (anti-chB6.1, asics) or 1 µl FUS-11G2 (anti-chB6.2, asics) (22). Samples were removed at intervals, and viability was determined as described above. All cultures were performed in triplicate.

TUNEL assay. FL5.12-based cell lines were treated as described above. Approximately 10⁴ cells/sample were spun onto glass slides in a Cytospin 2 cytocentrifuge (Shandon, Pittsburgh, PA) for 5 min at 1000 rpm. A TUNEL assay to determine fragmentation of DNA was performed to detect apoptotic cells (In Situ Cell Death Detection kit; Roche Biochemical, Indianapolis, IN). Briefly, cells were fixed in a solution of 4% paraformaldehyde in PBS for 30 min at room temperature. Slides were rinsed three times in PBS. Endogenous peroxidases were dissolved by incubating slides in a solution of 0.3% H2O2 in methanol for 30 min at room temperature. Slides were rinsed twice in PBS. Slides were then incubated in peroxidase conjugated with 0.1% sodium citrate and 0.1% Triton-X in diH2O for 2 min on ice. TUNEL reaction solution containing TdT and fluorescein-labeled dUTPs was applied, and slides were incubated in a humidified chamber for 1 h at room temperature. Negative controls from each sample were incubated with only fluorescein-labeled dUTPs. Slides were rinsed three times in PBS. Slides were incubated in substrate solution of 4-chloronaphthol/3,3′-diaminobenzidine (Pierce, Rockford, IL) in stable peroxide buffer for 15 min at room temperature. Slides were rinsed three times PBS. Slides were incubated in substrate solution of 4-chloronaphthol/3,3′-diaminobenzidine (Pierce, Rockford, IL) in stable peroxide buffer for 15 min at room temperature. Slides were rinsed three times PBS in PBS. Slides were visualized by microscopy at ×40 magnification.

Western blot analyses
Bcl-xL expression. FL5.12-based cell lines were spun down for 5 min at 1,200 rpm in a Beckman TJ-6 centrifuge (Beckman, Palo Alto, CA). Pellets were washed in ice-cold 1/5 PBS and resuspended in 5 ml lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton-X, 0.5% deoxycholate, and a protease inhibitor mixture (Complete Mini Protease inhibitor; Roche Biochemical). Samples were incubated on ice for 10 min, then transferred to microcentrifuge tubes. Cellular and nuclear debris was removed by spinning lysates for 30 min at 14,000 rpm in a tabletop microcentrifuge. A bichinonic acid protein assay (Pierce) was performed to determine the protein concentration of whole cell lysates.

Volumes of lysates from each cell line corresponding to 25 μg protein were placed in microcentrifuge tubes, and equal amounts of Laemmli sample buffer were added. Lysates were boiled for 10 min. Samples were electrophoresed through a 15% SDS-PAGE gel at 200 V for 1 h. Molecular weight markers (5 μl; Bio-Rad, Hercules, CA) were included on these gels. Proteins were then transferred to supported nitrocellulose (Micron Separations, Westborough, MA) membranes for 1 h at 100 mA.

Membranes were blocked overnight in 5% milk protein in PBS at 4°C. Membranes were washed twice in TBS-Tween. Membranes were probed with anti-Bcl-xL primary Ab (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/1000 in blocking solution for 1 h. Membranes were washed twice in TBS-Tween. Membranes were then incubated with anti-rabbit IgG-HPF-conjugated secondary Ab (Roche Biochemical) diluted 1/300 in blocking solution for 1 h. Membranes were washed twice in TBS-Tween. Bands were visualized by addition of substrate solution containing 4-chloronaphthol/3,3′-diaminobenzidine (Pierce) in stable peroxide substrate buffer.

Caspar and poly(ADP ribose) polymerase (PARP) cleavage. FL5.12-based cell lines were plated at concentrations of 10⁶ cells/ml in 24-well microtiter plates in either FL5 culture medium or RPMI supplemented with 10% NBS and incubated at 37°C for 15 h. Cells were treated with 1 µl 21-1A4 (anti-chB6.1) or 1 µl FUS-11G2 (anti-chB6.2) (22) and incubated for 1 h. Cells were washed in PBS and resuspended in 100 µl lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton-X, 1% deoxycholate, and protease inhibitor mixture (Roche). Samples were incubated on ice for 10 min and transferred to microcentrifuge tubes. Cellular and nuclear debris was removed by spinning lysates for 30 min at 14,000 rpm in a tabletop microcentrifuge.

Volumes of lysates equaling 10⁴ cells were placed into microcentrifuge tubes, and equal amounts of Laemmli sample buffer were added. Samples were boiled for 10 min and electrophoresed through 15% SDS-PAGE gels at 200 V for 1 h. Molecular weight markers (5 μl; Bio-Rad) were included in these gels. Proteins were transferred to supported nitrocellulose membranes for overnight incubation in blocking solution at 4°C.

Membranes were blocked overnight in blocking solution containing 0.5% BSA and 0.1% Tween 20 at 4°C. Membranes were washed twice in TBS-Tween. Membranes were probed overnight with either anti-caspase 3 primary Ab (Santa Cruz Biotechnology) diluted 1/100 in blocking solution, anti-caspase 8 primary Ab (Santa Cruz Biotechnology) diluted 1/100 in blocking solution, or anti-PARP Ab (New England Biolabs, Beverly, MA) diluted 1/250 in blocking solution. Membranes were washed twice in TBS-Tween. Membranes were incubated with anti-goat IgG HRP-conjugated secondary Ab (Roche Biochemical) diluted 1/500 in blocking solution for ~3 h at 4°C. Membranes were washed twice in TBS-Tween. Bands were visualized by addition of substrate solution containing 4-chloronaphthol/3,3′-diaminobenzidine substrate in stable peroxide buffer.

Densitometry was performed on a chemiluminescent imager (Alpha Innotech, San Leandro, CA), and data were analyzed using Chemi-Imager 4.4 software (Alpha Innotech). Briefly, molecular weight markers were identified, and a standard curve was used to best identify the appropriate sized bands. Lysates of Jurkat T cells were included on blots to aid in identification of uncleaved caspases. In the case of caspase 3, an inhibitor peptide (Santa Cruz Biotechnology) was used in a series of experiments to specifically identify bands representing full-length and cleaved products of caspase 3. An overlay was then drawn over the bands of interest. An irrelevant area of the nitrocellulose blot was used to correct for background. Using this overlay, the densitometer reported intensities of the bands marked relative to background in arbitrary units. The same overlay was used for all blots probed with a particular Ab.

Results
Previous work demonstrated that chB6 can initiate a rapid form of cell death with some characteristics of apoptosis (21). In avian cells chB6 triggers apoptosis rapidly, leading to DNA degradation within 1 h (P. E. Funk, manuscript in preparation). This suggests that chB6 could be an unusual type of death receptor. Unfortunately, the limited availability of reagents to detect apoptosis-related proteins in avian cells makes exploring signal transduction through chB6 difficult. Therefore, we used a murine cell line to examine the mechanism of chB6-initiated cell death. The murine FL5.12 cell line was characterized as a pro-B lymphocyte and is dependent on exogenous IL-3 for its growth and survival (23). Numerous studies have used FL5 cells in the study of apoptosis, including the recent discovery that apoptosis due to IL-3 withdrawal is dependent on production of a secreted lipocalin that induces apoptosis via binding to receptors on the cells (24). The FL5.12 cell line represents an amenable experimental system in which to analyze the events in chB6-induced cell death. Previous studies demonstrated that chB6 can mediate a death response, as assessed by the loss of ability to exclude a vital dye in transfected

Abbreviation used in this paper: PARP, poly(ADP ribose) polymerase.
FL5.12 cells when cocultured with anti-chB6 Ab (21). We used this same culture protocol to determine whether this cell death was apoptotic using the TUNEL assay to detect DNA degradation. Clonal FL5.12 cell lines stably transfected with either chB6.1 or chB6.2 cDNA were cultured in the presence or the absence of IL-3 for 16 h. Anti-chB6.1 or anti-chB6.2 ascites was added for the last hour of culture. Samples were withdrawn, spun onto glass slides, and stained via the TUNEL method (Fig. 1). In agreement with previous results, anti-chB6 Abs cause cell death only when the cells have been deprived of IL-3 for 15 h before exposure to anti-chB6 Ab, as measured by the failure to exclude vital dye (Fig. 2, A and C) (21). This staining with vital dye is followed by DNA degradation detected by TUNEL assay (Fig. 1). Forty-seven percent of the cells stain TUNEL positive when deprived of IL-3 and exposed to anti-chB6. This is nearly a 3-fold increase in TUNEL staining cells over cultures simply deprived of IL-3 or deprived of IL-3 and exposed to control ascites with an Ab to another allele of chB6 and nearly a 20-fold increase over cultures containing IL-3 even in the presence of anti-chB6 Ab. Control TUNEL reactions in the absence of TdT enzyme yielded no staining in any culture condition (data not shown).

From this we can conclude that chB6, when triggered by Ab, dramatically increases the number of cells undergoing apoptosis when FL5.12 transfectants are deprived of IL-3. These cells will eventually undergo apoptosis due to withdrawal of growth factor, and some apoptotic cells are detectable in these cultures. The increase in apoptotic cells in the absence of IL-3 and presence of anti-chB6 are attributed to the death response initiated by transfection chB6 when triggered by allele-specific Ab. Importantly, this experiment also demonstrates that chB6 ligation causes the same result, apoptosis, in both chicken and mammalian cells within a similar time frame, arguing that there is a highly conserved pathway of chB6 action.

Apoptosis is closely regulated by a number of pro- and anti-apoptotic factors; prominent among these are members of the bcl-2 gene family (25, 26). Murine Bcl-xL is a potent inhibitor of apoptosis due to IL-3 withdrawal in FL5.12 cells (27). As chB6 can trigger apoptosis in FL5.12 cells, we considered whether cell death initiated by chB6 could be inhibited by Bcl-xL. Murine IL-3-dependent cell lines transfected with either chB6.1 or chB6.2 were supertransfected with murine Bcl-xL, and clonal lines were obtained by limiting dilution. Overexpression of Bcl-xL in these lines was confirmed by Western blotting (Fig. 2A). These cell lines were deprived of growth factor for 15 h and were treated with either anti-chB6.1 or anti-chB6.2. Samples were removed at the indicated intervals, viability was determined based on ability to exclude...
trypan blue (Fig. 2, B and C), and apoptosis was confirmed by TUNEL assay (Fig. 3). Cell lines transfected with control vector exhibited a rapid decline in the number of viable cells within 1 h of administration of anti-chB6 Ab, with the majority of the cells dead by the end of the 24-h time course. Within 1 h of administration of anti-chB6 Ab, viable cell numbers were decreased by half. The majority of this cell death was apoptotic, as ~50% cells deprived of IL-3 and treated with anti-chB6 exhibited positive TUNEL staining. These findings are comparable to experiments using cell lines expressing either chB6.1 or chB6.2 alone. However, cell lines overexpressing the anti-apoptotic protein Bcl-xL, when deprived of IL-3 and treated with anti-chB6 Ab, survive as well as cells maintained in IL-3 over a 24-h time span. Cells overexpressing Bcl-xL, even when deprived of IL-3 and exposed to anti-chB6 Ab, do not exhibit significant TUNEL staining. From these experiments we conclude that Bcl-xL can inhibit the apoptotic signal initiated by chB6 in the absence of IL-3. Therefore, in these cells the apoptotic cell death caused by either growth factor withdrawal or chB6 can be inhibited by Bcl-xL.

The caspase family of proteases mediates several intracellular events that occur during apoptosis (28, 29). Caspases exist as proenzymes and are activated upon cleavage of the pro-domain. Since our data indicate that chB6 directly triggers apoptosis, potentially acting as a death receptor, we considered that both initiator and effector caspases would become activated as part of this pathway. We examined this by culturing transfected FL5.12 cell lines with allele-specific Ab to chB6 in the presence and the absence of IL-3. Cell lysates from the cultures were subjected to Western blot analysis (Figs. 4–6) for detection of active caspases 8 and 3 and inactivated PARP, a substrate of activated caspase 3. We then used densitometry to determine the relative amounts of inactive vs active caspases. In these experiments caspase 8 was always activated in the presence of anti-chB6 Abs, even when cells were incubated with IL-3 and in cell lines overexpressing Bcl-xL (Fig. 4). This suggests that chB6 is more closely coupled to caspase 8 and that chB6 initiates an apoptotic response via activation of caspase 8. Nevertheless, in the presence of IL-3 or due to the overexpression of Bcl-xL, this apoptotic signal is not propagated into an apoptotic response. Inhibition of apoptosis by growth factor or Bcl-xL does not involve inhibition of caspase 8 activation.

In contrast, caspase 3 activation was only detected in cells deprived of IL-3 and cultured with anti-chB6 Abs (Fig. 5). Little caspase 3 activation was seen in cells cultured with IL-3 or overexpressing Bcl-xL. From this we conclude that chB6 ligation does cause activation of caspase 3, but this activation is inhibited by survival signals derived either from the IL-3R or by the overexpression of Bcl-xL. This is mirrored in experiments examining the
inactivation of PARP. Inactivation of PARP facilitates degradation of DNA, a hallmark event of apoptosis (25). To date, studies have shown PARP cleavage to occur as a result of active caspase 3, but not other caspases. Therefore, PARP cleavage is another way to assess the activity of caspase 3 within a cell. In this study PARP cleavage followed the same pattern as caspase 3 cleavage (Fig. 6). Lysates from FL5.12 cell lines exhibited a marked decrease in the amount of active, uncleaved PARP when treated with anti-chB6 Abs in the absence of IL-3. However, continued culture in the presence of IL-3 or the overexpression of Bcl-xL resulted in only a modest increase in the amount of cleaved PARP, consistent with the low level of caspase 3 activation in these culture conditions. This strengthens our conclusion that chB6 ligation can trigger the generation of increased amounts of enzymatically active caspase 3, but this step of the apoptosis pathway is inhibited by survival signals from either the IL-3R or overexpression of Bcl-xL.

Discussion

ChB6 is an avian alloantigen that has been used extensively as a B-lineage cell marker (3, 16–20). However, little research has been performed to ascribe a physiological function to this unique molecule. Previous research demonstrated that chB6, when bound by Ab, triggers rapid cell death, exhibiting morphological features consistent with apoptosis (21). This suggested that chB6 could be a novel death receptor. The experiments described here provide additional evidence supporting this hypothesis by showing that
chB6 induces apoptosis in a transfected mammalian cell line and that this apoptosis is inhibited by overexpression of the anti-apoptotic protein Bcl-xL. Additionally, the data obtained connect chB6 killing activity to a conserved apoptotic pathway, showing that Ab binding to chB6 results in cleavage of a receptor-associated caspase, caspase 8, and a subsequent rise in the relative amount of the active form of caspase 3.

Death receptor-mediated apoptosis involves the activation of several members of the caspase family of proteases (28–31). For example, the death receptor Fas, when triggered by Ab or ligand, trimerizes to form a death effector domain that promotes the cleavage of pro-caspase 8 into active caspase 8. Active caspase 8 is the initiator caspase in the death receptor apoptosis pathway. Activation of caspase 8 via death receptor occurs even in transgenic animals overexpressing Bcl-xL (25, 32). In these animals the cells do not subsequently undergo apoptosis, demonstrating that activation of caspase 8 can be disengaged from the cell death machinery. Our findings demonstrate that chB6, when bound by Ab, can lead to the activation of caspase 8. This holds true even when Bcl-xL is overexpressed or cells are cultured in IL-3. This observation rules out the possibility that the death response observed was due solely to the lack of a survival signal being modulated by IL-3 and further supports the idea that chB6 directly mediated a death response. It is unclear from our data whether there is a direct link from caspase 8 activation to caspase 3 activation. The disengagement of this step in Bcl-xL-overexpressing cells suggests a mitochondria-dependent intermediate step.

The finding that chB6 leads to activation of caspase 3 would seem to be at odds with our previous report that cell death was not inhibited by the caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (21). It must be noted that in this earlier study cell death was assessed by permeability to vital dye, rather than DNA degradation. DNA degradation in apoptotic cells is dependent on the activation of caspase-activated DNase by the caspase-dependent cleavage of inhibitor of caspase-activated DNase (25). It is possible that membrane events proceed independently of DNA degradation; consequently, our earlier study may have judged cell death events that do not rely on caspase activation. It is also possible that zVAD does not inhibit avian caspases, since the earlier results were based on studies using avian cells. Experiments in the avian DT40 cell line yielded an ~50% drop in the number of TUNEL-positive cells when anti-chB6 Ab was given at the same time as benzoxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone, a caspase 3 inhibitor (P. E. Funk, manuscript in preparation), suggesting that caspases are activated by signals from chB6 in both mammalian and avian cells. We are pursuing further studies in avian cells to better understand the commonalities of death signals emanating from chB6.

The physical mechanism of chB6 death signaling is of fundamental interest. There is no apparent death domain in chB6, yet it mediates a signal that can lead to activation of caspase 8. It would seem unlikely that caspase 8 is recruited to chB6 via dimerization of death domain-containing proteins such as FADD. Examples of death domain-independent activation of caspase 8 have been reported (33–36). The mechanism of this activation has not been determined. ChB6 is normally present on DT40 B cells as a homodimer (M. Kharas, unpublished observation). It is possible that chB6 further multimerizes when bound by Ab or that aggregation of chB6 by Ab leads to the recruitment of other signaling proteins, conceivably including death domain-containing proteins. It is possible that chB6 recruits cytoplasmic proteins via charge interactions alone, since the cytoplasmic domain of chB6 is highly acidic, with a pKa of 4.5 (20, 21). ChB6 may complex with intracellular signal transducers and/or lead to phosphorylation of intracellular proteins, coupling events outside the cell with those occurring inside the cell. We are currently using mutagenesis to identify regions in chB6 essential to initiating the apoptotic signal.

Our experiments have shown that caspase 3 is activated when chB6 is bound by Ab. Subsequent cleavage and inactivation of PARP are also seen. Collectively, these findings indicate that a cell death signal sent via chB6 interacts with conserved intracellular apoptotic machinery. Three key events that occur in known receptor-mediated apoptosis pathways, activation of caspase 8, activation of caspase 3, and inactivation of PARP, are observed when mammalian cell lines transfected with chB6 are treated with allele-specific Ab. Even though chB6 activity occurs outside of its normal context of an avian cell in our experiments, we see events consistent with the Fas model of receptor-mediated death.

While our experiments use chB6 in a manner outside of its normal context of expression, developing chicken B cells in the bursa of Fabricius, our findings still offer clues to the physiologic function of endogenous chB6. Again, murine cell lines were used in our studies due to the lack of available reagents to study cell death in the chicken system. However, the end result of chB6 being bound by Ab remains the same in each experimental system, the appearance of apoptotic cells within 1 h of the addition of Ab (21). The physiologic regulation of chB6 activity by either growth factor or Bcl-xL overexpression further argues that these studies are examining a physiologic signal mediated by chB6. Weber (16) reported that chB6-initiated apoptosis can be prevented by coculture with 200 nM of the phorbol ester phorbol di-butyrate, further suggesting a physiologic death mechanism inhibited by protein kinase C isoforms rather than a membrane-associated event. Earlier results suggested the possibility that chB6 killed cells via a membrane insult rather than physiologic induction of apoptosis. We have found no evidence of plasma membrane damage in avian cells undergoing chB6-initiated apoptosis (P. E. Funk, manuscript in preparation).

Many questions remain about chB6 and its physiologic function. The location of chB6 on the cell surface and its transduction of a signal resulting in apoptosis are suggestive of a death receptor. Nevertheless, chB6 is present on the earliest identifiable B cell precursors, which should be protected from apoptosis (18, 19). Since chB6 is capable of mediating a death response, it may act in the selection process of developing B cells. This is consistent with its apparently accentuated activity on bursa-derived cells (16, 21). The endogenous ligand for chB6 remains unknown. However, we believe that such a ligand does exist. There may also be a ligand present in the bursal epithelium that modulates a survival signal to developing B cells. It is possible that the ligand for chB6 is responsible for eliciting a response to Ag. There may be different ligands that trigger this unique receptor to mediate different responses. Important questions regarding the function of chB6 remain. In addition, cells that have entered the peripheral immune system express chB6, but appear to be somewhat refractory to chB6-initiated apoptosis (21). Perhaps these cells have increased expression of anti-apoptotic genes. Cells expressing chB6, but developing outside the bursa, appear to be somewhat less susceptible to chB6-induced apoptosis than those in the bursa (16). Another possibility is that chB6 can deliver qualitatively different signals depending on input received from other receptors or based on binding of different ligands. The death receptor hypothesis implies the existence of a ligand(s), but none has been described for chB6.

Although there is no obvious homolog to chB6 based on nucleic acid or overall protein sequence, there must be an underlying conserved function enabling chB6 to trigger apoptosis in both avian and mammalian cells. We have also found that chB6 will trigger...
cell death in human Jurkat T cells (P. E. Funk, unpublished observation). We are currently testing other cell lines to determine whether chB6 can trigger apoptosis in a variety of contexts. However, the work presented here does suggest the possibility of a B cell-restricted death receptor. Our findings provide evidence for homology of function due to the consistent activity of chB6 in mammalian cell lines, the induction of cell death exhibiting characteristics of apoptosis.

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