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Immunology

Generation of a fusion partner to sample the repertoire of splenic B cells destined for apoptosis

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ABSTRACT **B cells proliferate and diversify in germinal centers in response to antigen. Only a small percentage of these B cells will emerge to form the serum antibody response. Other B cells making lower affinity antibodies, acquiring nonsense mutations, or expressing autoreactivity as a result of somatic mutation undergo an apoptotic cell death and are not efficiently sampled in current analyses of B-cell hybridomas. We have demonstrated that expression of *bcl-2* in the NSO myeloma fusion partner leads to a higher yield of viable hybridomas, with a selective increase in hybridomas from B cells that produce autoantibodies and are seldom recovered when spleen cells from non-autoimmune mice are fused to the conventional NSO cell line. Using this fusion partner, we have generated hybridomas from anti-DNA antibody-producing transgenic B cells that are anergic *in vivo* and destined for apoptosis. These studies provide a strategy to sample the repertoire of B cells that arise *in vivo* but are not selected to contribute to the expressed antibody response. Furthermore, they demonstrate that restricted expression of *bcl-2* in B cells contributes to the maintenance of self-tolerance in secondary lymphoid organs.**

Upon exposure to antigen, B cells proliferate within germinal centers (GCs) of secondary lymphoid organs (1). In GCs, somatic mutation of immunoglobulin genes occurs and leads to the generation of clonally related B cells with diversified antigen receptors. Within this population, antigen selection results in affinity maturation of the humoral response (2–6). As pathogenic autoantibodies are encoded by somatically mutated immunoglobulin genes, it is probable that autoantigenic specificities are acquired in GCs (7–9). These autoreactive cells, if not regulated, may represent the pathogenic B cells of autoimmune disease.

During the maturation of the humoral response, through interaction with antigen and T cells, there is increased expression of several proteins that contribute to B-cell proliferation and activation. Regulated Bcl-2 expression has previously been demonstrated to play an essential role in determining the repertoire of surviving B cells (10, 11). Transgenic mice constitutively expressing Bcl-2 in B cells display elevated autoantibody levels (12), demonstrating a resultant defect in B-cell repertoire selection. In addition, mating of these mice to transgenic mice producing autoreactive B cells demonstrated that *bcl-2* expression inhibited autoantigen-induced deletion of autoreactive B cells that had escaped from the bone marrow and enhanced the incidence of autoimmune disease (13).

Hybridoma technology has been invaluable in studying the development of the B-cell immunoglobulin repertoire and in characterizing the expressed antibody response to particular antigens (14). However, hybridomas can be triggered to undergo apoptosis through signaling that is believed to be representative of pathways of apoptosis that occur *in vivo* (15, 16). This suggested to us that splenic cells that are programed for cell death, such as isolated GC B cells, may complete this suicidal pathway even as newly created hybridomas and consequently bias the analysis of the immune response. We, therefore, transfected the NSO mouse myeloma fusion partner with the cDNA for *bcl-2* in an effort to obtain hybridomas that would provide a more complete understanding of the B-cell repertoire.

MATERIALS AND METHODS

Transfection of NSO Cell Line. An 850-bp fragment was isolated from pbluebcl-2 (17) by digestion with *Not* I and *Apa* I and inserted into the cloning site of pRC/CMV (Invitrogen) using standard cloning protocols. A 1-kb *Xba* I fragment encompassing the immunoglobulin heavy chain enhancer was inserted into the *Bgl* II site (18). The construct was then transfected into NSO^{wt} by electroporation at 600 V, 25 μF, ∞ and Ω, and selection was carried out in 2 mg of geneticin per ml. In addition, NSO^{wt} was also transfected with pRC/CMV vector alone. Expression of *bcl-2* mRNA was determined by Northern analysis of cellular RNA probed with the 850-bp *bcl-2* fragment. An ethidium bromide-stained gel was visualized to quantify RNA loading.

Assay for Apoptosis. NSO^{wt}, $NSO^{pRC/CMV}$, and NSO^{bcl-2} were incubated with 1 μM staurosporine for 18 hr prior to DNA extraction. The cells were pelleted and resuspended in cell lysis buffer (5 mM Tris·HCl, pH 7.5/20 mM EDTA/0.5% Triton X-100) for 20 min on ice. After centrifugation, the supernatant was treated with RNase A (100 μg/ml) and proteinase K (200 μg/ml). The solution was phenol and phenol/chloroform extracted. The DNA was precipitated in sodium acetate and ethanol and analyzed on a 1% agarose gel.

Fusions with Primary Spleen Cells. Spleen cell suspensions were prepared at 4°C. The splenocytes were divided into two equal populations and fused with either the NSO^{wt} or NSO^{bcl-2} myeloma partner according to standard procedures (19). Wells were scored for hybridoma growth after 10 days. All BALB/c mice used in this study were 6–8 wk of age. For antigen-activated splenic fusion, BALB/c mice were immunized intraperitoneally with 100 μg of keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant. One month later, the mice were injected intravenously with 25 μg of phosphocholine coupled to KLH (PC-KLH) in 0.9% NaCl solution and 12 days later were injected with 100 μg PC-KLH. Immunized mice were sacrificed on days 2, 5, 7, and 9 following the final immunization. The transgenic mice utilized in this study were 6–8 wk of age (20).

RESULTS

***bcl-2* Expression Rescues the NSO Fusion Partner from Apoptosis.** There are several lines of data to suggest that the NSO fusion partner does not form stable hybridomas with all

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Abbreviations: GC, germinal center; KLH, keyhole limpet hemocyanin; PC-KLH, phosphocholine coupled to KLH; dsDNA, double-stranded DNA.

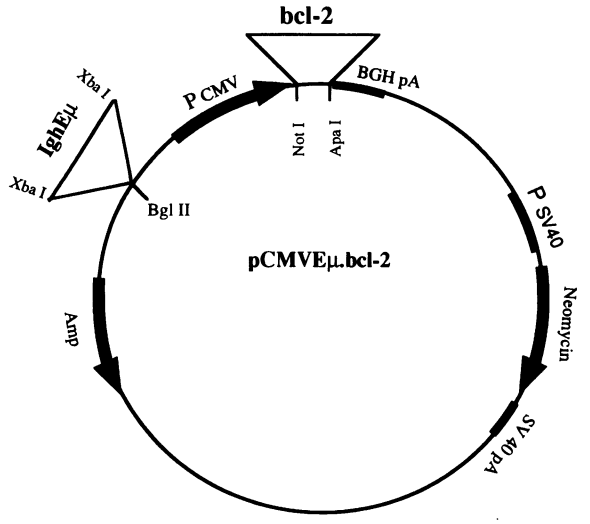

FIG. 1. *bcl-2* construct prepared as described in the text. *bcl-2* expression is regulated by a constitutive cytomegalovirus promoter (P_{CMV}) under the influence of the immunoglobulin heavy chain enhancer. Resistance to geneticin is provided by the bacterial neomycin-resistance gene, which is under a simian virus 40 (SV40) promoter.

B cells but rather generates hybridomas from antigen-activated B cells that have been positively selected in GCs (5). Based on extensive data supporting a role for *bcl-2* in resistance to apoptosis and specifically in the selective rescue of antigen-specific GC B cells (21–26), and our concern that the NSO fusion partner was sampling only the positively selected B-cell population, we transfected NSO^{wt} cells with a human *bcl-2* cDNA under a constitutive cytomegalovirus promoter and immunoglobulin heavy chain enhancer (Fig. 1). The transfected cells, NSO^{bcl-2}, express *bcl-2*, as determined by Northern analysis, while untransfected cells do not (Fig. 2). To determine whether *bcl-2* expression could rescue NSO^{bcl-2} from signals that mediate apoptosis, we treated both NSO^{wt} and NSO^{bcl-2} with staurosporine, a protein kinase inhibitor and known inducer of apoptosis (27). Following treatment with staurosporine, NSO^{wt} displays the morphological and biochemical changes characteristic of apoptosis. NSO^{bcl-2}, in contrast, displays increased resistance to apoptosis, while NSO^{wt} transfected with vector alone, $NSO^{pRC/CMV}$,

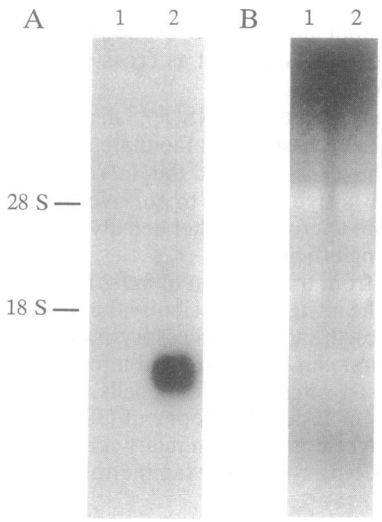

FIG. 2. Northern analysis of cellular RNA to determine *bcl-2* expression. Lanes: 1, NSO^{wt}; 2, NSO^{bcl-2}. (*A*) RNA probed with an 850-bp *Not* I/*Apa* I *bcl-2* fragment. (*B*) Ethidium bromide-stained gel prior to transfer onto nitrocellulose.

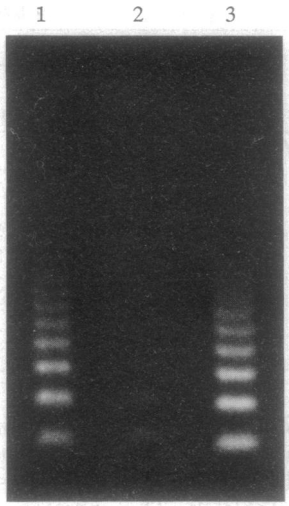

FIG. 3. Gel electrophoresis of DNA extracted from cell lines following treatment with 1 μM staurosporine. The DNA "ladder" composed of 180- to 200-bp internucleosomal fragments is a characteristic feature of apoptosis (28). Lanes: 1, NSO^{wt}; 2, NSO^{bcl-2}; 3, $NSO^{pRC/CMV}$.

behaves like untransfected cells (Fig. 3). In addition, NSO^{wt} is also more susceptible than NSO^{bcl-2} to apoptosis induced by nutrient depletion, high-density cell growth, and aminopterin (data not shown).

NSO^{bcl-2} Fusions Show an Increased Representation of IgG-Secreting B Cells. We next attempted to determine whether the susceptibility of NSO^{wt} to apoptosis could bias the repertoire of hybridomas obtained from spleen cell fusions. Initial fusions performed with spleen cells from unimmunized BALB/c mice revealed an increased yield of hybridomas with NSO^{bcl-2} as compared to NSO^{wt} (Table 1).

To determine whether this simply reflected an overall increase in fusion efficiency or was specific to a particular B-cell population, we performed fusions with spleen cells from immunized mice. Following secondary immunization with PC-KLH, NSO^{bcl-2} demonstrated a 2- to 5-fold increase in hybridoma production as compared to NSO^{wt} (Table 1).

Table 1. Hybridoma yields from splenic fusions

Mouse	Fusion partner	Wells with hybridomas	
		No.	%
Naive BALB/c			
1	NSO^{wt}	108/480	22
	NSO^{bcl-2}	286/480	60
2	NSO^{wt}	106/480	22
	NSO^{bcl-2}	229/480	48
3	NSO^{wt}	121/864	14
	NSO^{bcl-2}	300/797	38
Immunized BALB/c			
Day 2	NSO^{wt}	39/720	5
	NSO^{bcl-2}	96/346	28
Day 5	NSO^{wt}	60/900	6
	NSO^{bcl-2}	117/900	13
Day 7	NSO^{wt}	55/1015	5
	NSO^{bcl-2}	159/1015	16
Day 9	NSO^{wt}	21/120	17
	NSO^{bcl-2}	55/60	92
Transgenic			
1	NSO^{wt}	132/480	28
	NSO^{bcl-2}	291/480	61
2	NSO^{wt}	95/960	10
	NSO^{bcl-2}	173/689	25

Table 2. Isotype and antigenic specificity of hybridomas from immunized mice

Experiment	Fusion partner	IgM+ hybridomas Total	%	PC-KLH specific	%	IgG+ hybridomas Total	%	PC-KLH specific	%
Immunized BALB/c									
Day 2	NSO^{wt}	3/30	10	2/3	66	19/30	63	16/19	84
	NSO^{bcl-2}	5/72	7	5/5	100	62/72	86	58/62	94
Day 5	NSO^{wt}	22/58	38	10/22	45	15/58	26	9/15	60
	NSO^{bcl-2}	18/83	22	10/18	56	38/83	46	20/38	53
Day 7	NSO^{wt}	6/38	16	3/6	50	24/38	63	12/24	50
	NSO^{bcl-2}	24/120	20	11/24	46	83/120	69	53/83	64
Total	NSO^{wt}	31/126	25	15/31	48	58/126	46	37/58	64
	NSO^{bcl-2}	47/275	17	26/47	55	183/275	67	131/183	72

Interestingly, among the hybridomas secreting immunoglobulin, there was a significant increase in the percentage of IgG-producing clones (67% vs. 46%, $P < 0.005$) in the NSO^{bcl-2} fusion (Table 2). This increase occurred in the absence of any significant increase in the percentage of hybridomas secreting IgM. The data are consistent with NSO^{bcl-2} leading to a preferential representation of antigen-activated B cells. In three additional fusions performed with primary PC-KLH-immunized BALB/c spleens, a similar difference ($P < 0.005$) was observed in the percentage of IgG clones obtained with either NSO^{bcl-2} (213/412, 52%) or NSO^{wt} (105/328, 32%). Again, there was no appreciable difference in the percentages of IgM-secreting hybridomas (22% vs. 24%, respectively).

NSO^{bcl-2} Rescues an Autoreactive B-Cell Population. We then attempted to determine whether resistance of NSO^{bcl-2} to apoptosis may lead to a more complete representation of the peripheral B-cell repertoire, specifically leading to a more accurate evaluation of the extent of autoreactivity present in the periphery of non-autoimmune mice. To pursue this question, we performed fusions with spleen cells from transgenic mice in which a large percentage of peripheral B cells express the transgenic IgG2b heavy chain of an anti-double-stranded DNA (dsDNA) antibody. We have previously demonstrated that although these mice have low serum titers of IgG2b and express no anti-dsDNA activity in their serum, they nevertheless harbor in their spleen a population of anergic cells expressing transgene encoded anti-dsDNA antibody (20). Furthermore, there is a population of splenic B cells in these mice that undergoes apoptosis *in vivo* that is not seen in nontransgenic littermates (L. Spatz, personal communication). Many fusions of unstimulated splenic cells from these mice with NSO^{wt} have failed to yield hybridomas producing transgene encoded anti-dsDNA antibodies. To sample this autoreactive B-cell repertoire, we fused spleen cells from transgenic mice to NSO^{bcl-2} cells. We obtained, as expected, a greater yield of hybridomas with NSO^{bcl-2} cells (Table 1). In addition, however, a greater percentage of the surviving hybridomas in the NSO^{bcl-2} fusion were secreting IgG2b encoded by the transgene than in the fusion with NSO^{wt} cells (Table 3). More important, while none of 103 IgG2b-secreting hybridomas generated with NSO^{wt} produced an anti-dsDNA antibody, 16 of 248 IgG2b-expressing lines (6.5%) generated with NSO^{bcl-2} were DNA binding. Thus, the NSO^{bcl-2} fusion partner displayed the unique ability to form hybridomas with autoreactive B cells and enabled us to transform a population of anergic autoreactive B cells that are presumably programed for apoptosis.

Table 3. Fusions with IgG2b transgenic mice

Experiment	Fusion partner	Hybridomas secreting IgG2b No.	%
1	NSO^{wt}	23/94	25
	NSO^{bcl-2}	109/186	59
2	NSO^{wt}	10/70	14
	NSO^{bcl-2}	25/86	29

DISCUSSION

Though it has previously been possible to analyze self-reactive B cells present in various autoimmune models, NSO^{bcl-2} now permits an evaluation of the extent of autoreactivity existing in the naive and antigen-induced B-cell repertoire of non-autoimmune mouse strains. Data from many laboratories show that anti-dsDNA antibodies obtained from mice and humans with the autoimmune disease systemic lupus erythematosus are somatically mutated (8), suggesting that autoreactive B cells may routinely arise among GC B cells activated by foreign antigen. Studies of other pathogenic autoantibodies similarly show them to be somatically mutated. This study demonstrates that potentially autoreactive splenic B cells reside in peripheral lymphoid organs in a state that is inaccessible by fusion with NSO^{wt}. Restricted expression of *bcl-2 in vivo* has limited the repertoire of B-cell hybridomas. Furthermore, restricted *bcl-2* expression appears to be critical in preserving peripheral self-tolerance. NSO^{bcl-2} represents a powerful tool to capture and analyze both the B-cell response to nominal antigens as well as the tolerized B-cell repertoire, permitting a study of the generation and regulation of autoreactive B cells in non-autoimmune mouse strains. Preliminary experiments have been undertaken to analyze the NSO^{bcl-2} hybridomas from *in vivo* antigen-stimulated BALB/c mice for the presence of anti-dsDNA reactivity as well as cross-reactivity with both PC and dsDNA.

Hybridomas derived from NSO^{bcl-2} display features resulting from *bcl-2* expression that facilitate the analysis of such clones. We have found that the increased resistance to apoptosis allows hybridomas to survive longer despite nutrient depletion, to grow to higher density (29), and to achieve a higher cloning efficiency.

The results from transfection of *bcl-2* into the NSO fusion partner raise the intriguing possibility that the genes for other molecules (growth factors, accessory molecules, or growth factor receptors) can be transfected into a fusion partner to isolate previously inaccessible B cells, including those in various stages of B-cell development (30). *bcl-2* transfected into a T-cell hybridoma may similarly create a T-cell fusion partner that is better able to examine the T-cell repertoire.

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