Brief Definitive Report

ACTIVATION PRIMES HUMAN B LYMPHOCYTES TO RESPOND TO HEAT SHOCK

By NEIL L. SPECTOR,* ARNOLD S. FREEDMAN,* GORDON FREEMAN,* JEFFREY SEGIL,* JAMES F. WHITMAN,* WILLIAM J. WELCH,‡ AND LEE M. NADLER*

*From the *Division of Tumor Immunology, Dana-Farber Cancer Institute and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115; and the ‡Departments of Physiology and Medicine, University of California–San Francisco, San Francisco, California 94110*

When confronted with heat or other stresses, pro- and eukaryotes preferentially synthesize proteins termed the heat shock or stress proteins (hsps) (1, 2). Although the function(s) of these proteins has long been a matter of speculation, recent evidence suggests that hsps and in particular the 70-kD hsp family (hsp70) are important in protecting cells from the adverse effects of noxious stimuli (3–5). Of these proteins, hsp70 is the most phylogenetically conserved and extensively studied. Most mammalian cells express two predominant forms of hsp70, an abundant constitutive member (hsp73) and a highly stress-inducible member (hsp72) (6). Previous studies have shown a strong correlation between the induction and accumulation of hsp70 and the ability of cells to survive a lethal heat-shock event, a phenomenon referred to as thermotolerance (7–9).

To date, most of the in vitro models of thermotolerance use a mild heat treatment to induce hsp70 expression, before exposing cells to an otherwise lethal heat shock event. Unfortunately, both the biological correlate of the sublethal heat treatment and the physiologic significance of increased hsp70 expression are still unknown. Activation of human B lymphocytes via the physiologic antigen receptor provided a novel system with which to study the role of hsps since in the intact host these events occur in the context of fever and inflammation. In the study to be described below we demonstrate that triggering B cells via the antigen receptor induces hsp70 expression and is accompanied by the induction of thermotolerance without the need for a prior sublethal heat treatment.

Materials and Methods

B Cell Preparations. After obtaining appropriate Human Protection Committee validation and informed consent, human splenic mononuclear cells were isolated as previously described (10).

In Vitro B Cell Stimulation. 10^6 cells/ml were cultured at 37°C in RPMI 1640 media (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated FCS, 2 mM glutamine,

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1 mM sodium pyruvate, and 5 μg/ml gentamycin in tissue culture flasks (Corning Glass Works, Corning, NY) supplemented with either *Staphylococcus aureus* Cowan Strain I (SAC) (Calbiochem-Behring Corp., San Diego, CA) or affinity-purified rabbit anti-human Ig coupled to Affi-Gel beads (anti-μ beads) (Bio-Rad Laboratories, Richmond, CA) (10).

Heat Shock. Cells at 37°C were harvested and transferred to RPMI 1640 media prewarmed to either 45° or 50°C. For the survival studies, cells were heat shocked for 30, 60, or 120 min and then returned to 37°C for 4–6 h after which survival was determined by trypan blue exclusion.

Metabolic Radiolabeling. Before labeling, cells were harvested and washed twice in methionine and cysteine-free media (Gibco Laboratories) and then radiolabeled with 10 μCi/ml of both L-[^{35}S]methionine and L-[^{35}S]cysteine (Amersham Corp., Arlington Heights, IL) for 1 h. The cells were then washed three times with PBS and lysed in a buffer containing 0.5% (vol/vol) Brij and 0.5% NP-40, 20 mM Tris-HCl, pH 7.8, 150 mM NaCl and 1 mM PMSF.

SDS-PAGE and Immunoblotting. Protein concentrations in cell lysates were determined by the Bradford method (11), and equal amounts of protein were resolved on a 10% SDS-polyacrylamide gel under reducing conditions (12) and visualized by autoradiography. Immunoblot analysis was performed using a mAb (N27) under conditions as previously described (3).

Preparation of RNA and Northern Blot Analysis. Total cellular RNA was purified by the guanidium thiocyanate/CsCl method (13). Equal amounts and intactness of RNA were verified by ethidium bromide staining (data not shown). Equal amounts of total cellular RNA were run on a 1.3% agarose gel and transferred onto a Zeta-Probe membrane (Bio-Rad Laboratories). Hybridization was performed using a ^{32}P-labeled cDNA probe for the human hsp70 gene (kindly provided by Dr. Richard Morimoto, Northwestern University, Evanston, IL) under previously described conditions (14, 15).

Results

Crosslinkage of sIg Induces hsp70 Expression. Highly purified unactivated human splenic B lymphocytes were stimulated through crosslinkage of sIg with SAC or anti-μ beads. Using radiolabeling of proteins as an index of metabolic activity, maximal synthetic activity after activation coincided with the onset of cellular proliferation at 36–40 h (Fig. 1 *a*, lane *3*). Although at control temperatures (37°C) unstimulated and 72-h activated B cells were morphologically and phenotypically distinct, by 72 h after activation the cells had returned to a relatively low synthetic state similar to the unactivated cells (Fig. 1 *a*, lane *4*). To specifically examine hsp70 expression after activation, cells at various times after stimulation were examined by immunoblotting (Fig. 1 *b*). While unstimulated cells exhibited a weak signal for hsp73, the relative amount increased significantly by 48 h after stimulation (Fig. 1 *b*). In contrast, the 72-kD protein that was not detected in unactivated cells, was induced by 48–72 h after activation (Fig. 1 *b*). Moreover, hsp induction was selective in that expression of the low molecular weight hsp (hsp28) did not change after activation (data not shown).

B Cell Activation via sIg Leads to the Development of Thermotolerance. To determine if the induction of hsp70 after B cell activation is functionally significant, we took highly purified unactivated cells and those cultured in the presence of mitogenic concentrations of anti-μ beads for 72 h and incubated them at various temperatures above 37°C (control). At control temperatures both the unactivated and activated cells were 100% viable. In contrast, at temperatures between 45 and 50°C significant differences existed in cell survival when comparing resting and activated cells. Fig. 2 illustrates the results of five independent experiments (using five different spleens) in which activated B cells demonstrated a significant twofold survival advantage after hyperthermia as compared with unactivated cells.

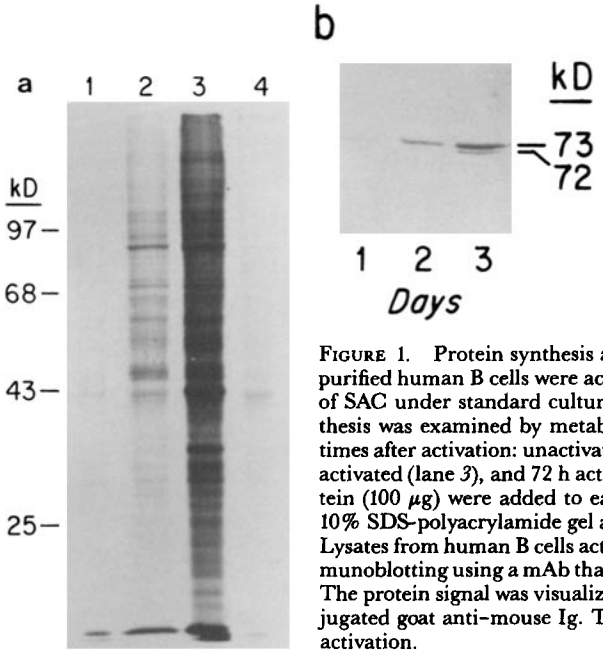

FIGURE 1. Protein synthesis after B lymphocyte activation. Highly purified human B cells were activated with mitogenic concentrations of SAC under standard culture conditions (37°C). (*a*) Protein synthesis was examined by metabolically radiolabeling cells at various times after activation: unactivated (lane *1*), 4 h activated (lane *2*), 40 h activated (lane *3*), and 72 h activated (lane *4*). Equal amounts of protein (100 μg) were added to each lane. Proteins were resolved on a 10% SDS-polyacrylamide gel and visualized by autoradiography. (*b*) Lysates from human B cells activated with SAC were analyzed by immunoblotting using a mAb that recognizes both hsp72 and hsp73 (3). The protein signal was visualized with a horseradish peroxidase–conjugated goat anti-mouse Ig. The lane numbers represent days after activation.

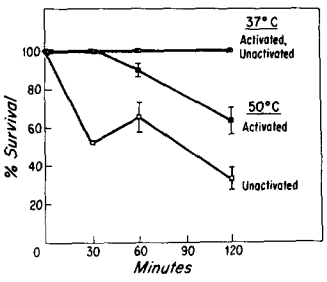

FIGURE 2. Influence of activational state on survival of B cells confronted with heat shock. Equal numbers of unactivated and 72-h SAC-activated human B cells were exposed to hyperthermia (50°C) for either 30, 60, or 120 min. After heating, the cells were returned to 37°C for 4–6 h and their viability was assessed by trypan blue exclusion. Unactivated cell are represented by the open symbols and activated cells by closed symbols. 37°C controls (○), 50°C (□). The results plotted are the mean values of experiments using five different spleens. The differences in survival between unactivated and activated cells at each of the 50°C time points has a statistical significance of $p < 0.005$ (paired Student's *t*-test).

Activation Primes B Cells to Respond to Heat Shock by Increasing the Expression of hsp70 mRNA. To better understand why activated cells preferentially survive hyperthermia, we compared the heat shock response in resting and activated B cells looking for either qualitative or quantitative differences. Using Northern blot analysis to assess the response of B cells to heat at the molecular level, we observed that hsp70 mRNA (2.6-kb band) transiently increased in unactivated cells after hyperthermia, but rapidly returned to baseline levels within 2–4 h (Fig. 3). In sharp contrast, both the 4- and 72-h activated cells exhibited significantly higher levels of hsp70 mRNA after heat shock (Fig. 3). Moreover, the activated cells continued to express increased levels of hsp70 mRNA for as long as 7 h.

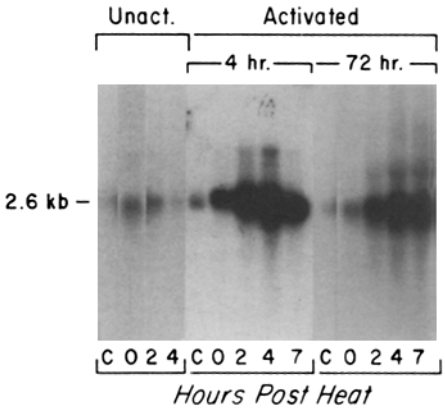

FIGURE 3. Steady-state levels of hsp70 mRNA after heat shock. Unstimulated and 4-h and 72-h SAC-activated B cells were exposed to a sublethal heat shock event (45°C for 30 min) and then returned to 37°C after which total cellular RNA was isolated at various time points after heating. Equal amounts of RNA (2.5 μg) were loaded onto each lane and verified by ethidium bromide staining (data not shown). RNA was isolated either immediately after heating (lane *0*), 2 h (lane *2*), 4 h (lane *4*), and 7 h (lane *7*). The controls (lane *C*) were maintained at 37°C.

Discussion

Activation of human B lymphocytes in vitro provided a novel system with which to examine the biologic relevance of the heat shock response during an immune response. Our results indicate the following: (*a*) concomitant with triggering B cells by crosslinking sIg there is a selective increase in the levels of hsp72/73, (*b*) activated cells exhibited higher degrees of thermotolerance when compared with resting cells as determined by cell survival, and (*c*) activated cells are more capable of responding to stress due to their markedly increased expression of hsp70.

To date our understanding of how immune cells function in the context of the noxious stimuli associated with both infection and inflammation is unknown. We provide evidence that the activation of human B cells results in the selective induction of hsp 72/73. Considering the evidence that hsp 70 appears to be essential in enabling cells to survive lethal stresses, we hypothesized that its increased expression after activation would protect B cells from noxious environmental stimuli (e.g., heat). To confirm this hypothesis, we have demonstrated that activated B cells survived lethal stimuli significantly better than their unactivated counterparts. These observations could not be accounted for by survival differences inherent to the two B cell populations since at control temperatures both resting and activated cells exhibited 100% survival.

In attempting to explain why activated cells preferentially survive cytotoxic events, we found that unactivated B cells were unable to significantly induce hsp70 in response to stress. This is a novel observation in that traditionally it has been assumed that mature cells maintain the ability to mount an effective heat-shock response. As seen in Fig. 3, significant quantitative differences in hsp70 mRNA levels between the unstimulated and stimulated cells were observed after heat shock. These observations can not be attributed to differences in the proliferative state between the two B cell populations, since both the resting and 72-h activated cells exhibited almost identical protein synthetic patterns (Fig. 1 *a*, lanes *1* and *4*). This is the first demonstration that activation is an important priming event enabling B cells to respond to noxious stimuli in their environment.

Our observations are consistent with the notion that activation of B cells through crosslinkage of sIg is a "biologic equivalent" of sublethal heat exposure in that it is

capable of inducing thermotolerance. In terms of the possible physiologic relevance of these observations, we propose that it is the activation process itself that is vital in protecting B cells exposed to stressful environmental stimuli. These observations shed a completely new light on the importance of B cell activation.

Summary

Crosslinkage of the B cell antigen receptor by anti-μ beads or SAC results in the selective induction of hsp70. We have observed that activated cells, having enhanced expression of hsp70, survive lethal stimuli much better than their unactivated counterparts. These results are in accordance with the proposal that hsp70 is essential for cells to survive lethal environmental stresses. Moreover, the activation event itself primes B cells thereby enabling them to increase the expression of both hsp70 mRNA and protein. This is the first demonstration that triggering of B cells via crosslinkage of sIg is accompanied by the induction of thermotolerance without the need for a prior sublethal heat treatment.

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