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Stress Renders T Cell Blasts Sensitive to Killing by Activated Syngeneic NK Cells

Brian A. Rabinovich,*† John Shannon,* Ruey-Chyi Su,* and Richard G. Miller2*†

Exposure of primary T cell blasts to stress in the forms of heat, hydrogen peroxide, or high-density growth conditions resulted in a state of enhanced susceptibility to killing by syngeneic IL-2-activated NK cells or lymphokine-activated killer cells, but not to killing by CTL. Cytotoxicity was perforin mediated and was not due to decreased target expression of total MHC class I. The levels of stress used had little effect on cell viability. For thermal stress, sensitization increased with temperature, required a minimum exposure time, and disappeared when cells were given a long enough recovery time. Our data support a model that predicts that activated NK cells play a role in the immunosurveillance of nontransformed stressed cells in normal animals. The Journal of Immunology, 2000, 165: 2390–2397.

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E xposure to environmental stress is an inevitable event in the life span of a cell. Physiological stressors include heat, reactive oxygen species, and inflammatory cytokines. Under harsh conditions, exposure to stress may lead to structural damage such as protein denaturation and DNA fragmentation. Cells have adapted powerful and complex responses to stress that include stress protein synthesis (2) and growth arrest (3–10). Cells have adapted powerful and complex responses to stress that include stress protein synthesis (2) and growth arrest (11, 12). The goal of the cell is either to repair damage that could potentially lead to neoplastic transformation or undergo apoptosis.

In the field of tumor biology, a large literature exists suggesting that localized and systemic exposure to stressors such as heat shock and radiation lead to tumor cell death. In many cases, the same magnitude of stress administered directly in vitro was insufficient to induce tumor cell death. For example, Burd and colleagues (13) implanted BALB/c mice with syngeneic colon 26 tumor cells and observed that significant tumor cell death occurred only in those animals that were subsequently administered whole body hyperthermia (WBH)3 at 39.8°C. Yamada and colleagues (14) observed that, following implantation of a squamous cell carcinoma cell line into C3H mice, localized hyperthermia of the tumor-infiltrated region resulted in extensive destruction of the tumor. In both cases, the magnitude of heat stress used was not lethal to tumor cells if administered in vitro. The degree of heat shock required to induce cell death in vitro has been studied. Exposure of tumor cell lines to temperatures <42°C in vitro leads to a dose-dependent cell cycle arrest but is nonlethal (15, 16).

The observation that in vivo exposure of tumor cells to nonlethal forms of stress results in tumor cell death has led to the hypothesis that the immune system may actively eliminate stressed tumor cells. In reports by Multhoff and colleagues (16–20) and Scott and colleagues (21), tumor cells exposed to nonlethal levels of heat, arsenite, and alkyl-lysophospholipids (antitumor agent) were actively lysed by allogeneic, IL-2-activated NK cells (LAK) in vitro. Furthermore, Burd and colleagues (13) observed that the antitumor effect of WBH was lost if NK cells were depleted before WBH with antiasialo GM1.

NK cells play a major role in the innate immune response (22–24). Although their precise role in the immune system remains partly unclear, NK cells survey tissues for infected or otherwise abnormal cells (24). Temporally, their response precedes T and B cell-mediated immune responses (24). Thus, NK cells provide immediate protection from danger and shape the ensuing adaptive immune response via the secretion of soluble factors, including cytokines (24). NK cells express both activation and inhibitory receptors that interact with ligands on target cells. For the most part, NK cells do not kill syngeneic target cells. However, NK cells can be induced to kill normally resistant cells either through the down-regulation of an inhibitory ligand or the up-regulation of an activation ligand on the target cell. Inhibitory receptors expressed by mouse NK cells include the Ly-49 family of receptors that interact with MHC class I molecules (22, 23). Additionally, MHC class I molecules capable of accepting peptide (peptide-receptive MHC) are also recognized by specific NK inhibitory receptors (25, 26). The rat NKRP1 activation receptor was the first NK activation receptor described (27). In B6 mice, almost all NK cells express NKRPIC (28), a homologue of NKRP1 (29), which has been shown to function as an activation receptor (30, 31). To date, NK activation ligands recognized by the NKRP1 family of NK activation receptors have not been identified. NK cells, then, receive both positive and negative signals when interacting with their target. The final lytic response mediated by an NK cell is predicted by the “teeter-totter” model of NK cell activation (26), whereby the sum of positive and negative signals determines whether the NK cell is activated or not. The mechanism(s) by which stress enhances the susceptibility of tumor targets to NK-mediated lysis in the studies described above (16–21) must have involved either the up-regulation of an activation ligand or the down-regulation of an inhibitory ligand, or both, on stressed cells.

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3 Abbreviations used in this paper: WBH, whole body hyperthermia; BFA, brefeldin A; CFSE, carboxyfluorescein-succinyl-ester; CM, complete media; DJIC5(5), 1,1′,3,3′,3′′-hexamethylindocarbocyanine iodide; hsp, heat shock protein; LAK, lymphokine-activated killer; MICA, MHC class I-related molecule-A; mIL-2, murine IL-2; NWNA, nylon wool nonadherent; PKO, perforin knockout mice.

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To date, nonlethal hyperthermia has been observed to sensitize tumor cells to LAK-mediated lysis. Whether nontransformed cells are also sensitized by nonlethal forms of stress has not been investigated. In the current study, we have used a murine system to investigate the phenomenon of stress-induced sensitivity to LAK cells using nontransformed primary T cell blasts as targets and purified syngeneic LAK cells as effectors. We observed that nonlethal forms of stress render T cell blasts sensitive to LAK cell-mediated lysis. The potential significance of this result is discussed.

Materials and Methods

Animals

C57BL/6 (B6), and (C57BL/6 × BALB/c)F1 (F1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Perforin knockout mice (PKO) engineered on the B6 background were generously provided by Dr. H. Hengartner (Zurich, Switzerland) (32). Mice were kept in a specific pathogen-free animal colony in the Ontario Cancer Institute. In most experiments, 5- to 7-wk-old male mice were used (although either sex gave similar results).

Class I MHC-binding peptides

A K\(^d\)-restricted epitope of chicken OVA, SIINFEKL (OVA\(_{257-264}\)) (33), was prepared by the Ontario Cancer Institute Biotechnology Laboratory, by using an Applied Biosystems Peptide Synthesizer (Applied Biosystems, Foster City, CA). OVA peptide is a natural ligand for K\(^d\) and binds with high affinity (33, 34).

mAbs and flow cytometry

Hybridoma 25D1.16 (anti-K\(^b\)-OVAp complex) was the generous gift of Dr. R. Germain (National Institutes of Health, Bethesda, MD) (35). Hybridomas PK136/HH191 (NK1.1), TIB 207 (rat anti-mouse CD4), TIB 205 (rat anti-mouse CD8), and 2.4G2 (rat anti-mouse FcγRIII–α) were obtained from the American Type Culture Collection (Manassas, VA). mAbs were purified from culture supernatants by combined protein A (Sigma, St. Louis, MO) and Gammabind Plus (Pharmacia, Piscataway, NJ) chromatography. Annexin-V FITC, PE-conjugated anti-NK1.1 (PK136), FITC-conjugated anti-d\(^4\), biotinylated anti-K\(^d\), PE-conjugated K\(^d\), FITC-conjugated D\(^d\), PE-conjugated anti-B220, and FITC-conjugated anti-TCR\(\alpha\)β were purchased from Pharmingen (San Diego, CA). Brefeldin A (BFA), streptavidin PE, and 7-aminocytocin D were purchased from Sigma. DiIC\(_1\)(5) was purchased from Molecular Probes (Eugene, OR). Labeling of purified Abs with FITC was performed as described (36). To test for peptide-receptive K\(^e\) expression on Con A blasts, the procedure of Su and colleagues was used (25, 26). Briefly, Con A-activated lymphoblasts were preincubated with OVA peptide (1 \(\mu\)g/ml) for 45 min at room temperature to fill peptide-receptive K\(^e\) molecules, washed free of unbound peptide, incubated with NMS and 24G2 supernatant to block nonspecific staining, and then stained for K\(^e\)-OVAp peptide complexes using FITC-labeled 25D1.16 mAb (35). PE-conjugated mAbs specific for K\(^d\) and K\(^e\) and FITC-conjugated mAbs specific for D\(^d\) and D\(^d\) (Pharmingen) were used to address overall MHCI class I expression on Con A blasts. Flow cytometric acquisition and analysis were performed using a Becton Dickinson FACScaliber and CellQuest software (Becton Dickinson, Mountain View, CA).

Preparation of nylon wool nonadherent (NWNA) splenocytes

Spleens were pressed through a wire mesh with a disposable syringe plunger into complete medium (CM), which consisted of a-MEM (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% FCS (Life Technologies), 50 \(\mu\)M 2-ME, and 10 mM HEPES buffer. Released cells were washed once, underlaid with 4 ml lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada), and centrifuged at 2500 rpm for 20 min to remove erythrocytes and nonviable leukocytes. Leukocytes were isolated from the interface, washed twice, resuspended in 5 ml CM, and then loaded onto a nylon wool column (1.2 g nylon wool in 20-ml syringes; autoclaved, pregelatinized with 30 ml warm CM containing 1% FCS). After a 75-min incubation at 37°C, the nonadherent cells were eluted from the column with CM containing 1% FCS, washed one time, and resuspended in CM.

In vivo priming of NK with poly(I:C)

Poly(I:C) (Sigma) was diluted in PBS at a concentration of 100 \(\mu\)g per 300 \(\mu\)l. Nude mice were injected i.p. with 300 \(\mu\)l poly(I:C) on days 0 and 1. Primed animals were sacrificed on day 3, spleens collected, and NK isolated via nylon wool depletion, as described above.

T cell depletion by magnetic bead separation

NWNA splenocytes were prepared as described. To deplete CD4\(^+\) and CD8\(^+\) T cells, the cells were suspended in rat anti-mouse CD4 and rat anti-mouse CD8 (Becton Dickinson) mAbs (10 \(\mu\)g/ml) at 10 \(\times\) 10\(^6\) cells/ml. The mixture was rocked gently for 1 h at 4°C. Excess Abs were removed by washing the cell pellet twice with CM containing 1% FCS. The cell pellet was resuspended at 10 \(\times\) 10\(^6\)/ml and sheep anti-rat Dynabeads (Dynal, Oslo, Norway) were added to the cell suspension at a ratio of 3:1 (bead:cell). The mixture was rocked gently for 1 h at 4°C. At the end of the incubation, the immunomagnetic complex was removed by magnetic separation. The unbound fraction was collected, washed, and resuspended in CM. Efficiency of T cell depletion and nylon wool depletion of adherent cells was confirmed by staining the unbound fraction with FITC anti-TCR\(\alpha\)β and PE anti-B220 mAbs and analyzed via flow cytometry on a FACScaliber. Depletion of T cells was routinely 85–95%.

Preparation of LAK cells

The method used for producing LAK cells was virtually identical with that previously described (31). Briefly, 2 \(\times\) 10\(^6\) NWNA spleen cells (1–1.5 ml) were cultured at 37°C, 7% CO\(_2\)/air atmosphere for 2–3 days in 5 ml CM, containing 500 U/ml mouse rIL-2 in six-well flat-bottom plates. LAK cells were generated from PKO mice using a similar procedure, but including a negative bead sort for CD4\(^+\) and CD8\(^+\) T cells, as previously described. The purity of LAK cell cultures was confirmed by staining harvested cells with FITC anti-TCR\(\alpha\)β and PE anti-NK1.1 mAbs and analyzed via flow cytometry on a FACScaliber. NK1.1\(^+\) cells generally represented >95% of harvested cells. Mouse rIL-2 was obtained as a supernant from the mouse IL-2 cDNA-transfected cell line X63Ag8-653, kindly provided by Dr. H. Karasuyama (University of Tokyo, Tokyo, Japan) (37).

Target cell generation

Target cells consisted of F1, or B6 Con A (Con A) T cell blasts produced by incubating 7.5 \(\times\) 10\(^5\) splenocytes for 60 or 75 h in 10 ml CM supplemented with 2 \(\mu\)g/ml Con A in 50-ml flasks incubated upright at 37°C, 7% CO\(_2\)/air atmosphere. After 60 or 75 h, T cell blasts were either left at 37°C (unstressed), heated (described below), or treated with hydrogen peroxide (described below). T cell blasts were then harvested, washed, and cultured at 37°C at 2 U/ml murine IL-2 (milliliter) for various periods of time that corresponded to the recovery period. Cells were subsequently harvested and incubated for 2 min in 200 mM \(\alpha\)-methylmannoside/\(\alpha\)-methylglucoside (in CM), washed, and labeled with 0.4 mM of sodium \(^{51}\)Cr-chromate (DuPont Chemicals, Mississauga, Ontario, Canada). Typically, 5 \(\times\) 10\(^5\) cells were labeled with \(^{51}\)Cr for 90 min at 37°C in a total volume of 200 \(\mu\)l consisting of 100 \(\mu\)l FCS and 100 \(\mu\)l sodium \(^{51}\)Cr-chromate (normal saline buffer). Following radioactive labeling, cells were washed three times with CM containing 1% FCS to remove dead cells and nonincorporated Na\(^{51}\)CrO\(_4\) in the media.

Stress protocols

Stressors consisted of either hyperthermia, oxidation, or high-density growth conditions. For hyperthermia and oxidation, maximum stress levels used decreased the overall viability of exposed cells by <15%. Viability was addressed using trypan blue exclusion and flow cytometric analysis of DiIC\(_1\)(5) and annexin-V staining. Typically, hyperthermia was imposed in a controlled programmable waterbath (VWR Scientific, Toronto, Ontario, Canada) at temperatures ranging from 40°C to 41°C for a duration of 1–4 h. Oxidation was imposed via the addition of hydrogen peroxide (Sigma) to cultures of T cell blasts using concentrations ranging from 10 mM to 300 \(\mu\)M. High-density growth conditions were imposed by initiating spleenocyte cultures at a cell density of 3 \(\times\) 10\(^4\) cells in 10 ml CM supplemented with 2 \(\mu\)g/ml Con A in 50-ml flasks and otherwise treated as previously described.

Cytotoxicity assay

Methods for measuring lytic activity were identical with those previously described (31). Briefly, \(^{51}\)Cr-labeled T cell blasts were plated together with LAK cell effectors in 96-well V-bottom microtiter plates. LAK were added

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in 100 μl aliquots in CM, followed by the addition of targets (10^5 cells/well also in 100 μl aliquots) to achieve a final volume of 200 μl/well. Specified lysis was calculated as described in the cytotoxicity assay section. In each value represents the mean ± SE of five replicates. E is the experimental mean of 51Cr released; S, the amount of 51Cr released when the target cells were cultured in medium alone; and T, the total amount of 51Cr released in the presence of 2% acetic acid.

**CTL generation**

Alloreactive CTL generation was achieved as commonly performed. Briefly, 1 × 10^6 splenocytes derived from F1 or C3H (responder cells) were cocultured with 2 × 10^5 C3H or F1 stimulator cells, respectively. Stimulator cells were exposed to 2000 rad of γ-irradiation before their addition to culture, the latter performed in 10 ml CM in 50-ml flasks incubated upright at 37°C, 7% CO2/air atmosphere for 4–5 days. Following incubation, CTL were washed once and underlaid with 4 ml lymphocyte-M (Cedarlane Laboratories) and centrifuged at 2500 rpm for 20 min to remove nonviable leukocytes. CTL were then resuspended in CM, counted, and used equivalently to LAK as effectors in cytotoxicity assays, as previously described.

**Cold target competition assay**

Stressed F1, radiolabeled Con A lymphoblasts were tested as targets against F1 LAK cells, as described in the cytotoxicity assay, except that unlabeled F1 Con A lymphoblasts, either stressed or un-stressed, were included in the well at 0-, 1-, 3-, or 5-fold multiplicities of the labeled targets, as indicated. Cold and hot targets were premixed before the addition of effector cells (i.e., LAK cells). A 4-h 51Cr release assay was performed in 96-well V-bottom microtiter plates, and specific 51Cr release was measured. Specific lysis was calculated as described in the cytotoxicity assay section.

**Flow cytometric conjugation assay**

Effector LAK cells were labeled with 0.03 μM carboxyfluorescein-succinyl-ester (CFSE; Molecular Probes). T cells blasts (stressed or control) were labeled with 50% PKH26 (Sigma), according to manufacturer specifications. Labeled LAK and targets were then mixed in a final volume of 0.5 ml CM in 6-ml polystyrene FACS tubes (Becton Dickinson) at E:T ratios of 1:1 or 3:1. Target cell number was kept constant at 2 × 10^5 cells. Cell mixtures were spun at 1000 rpm for 3 min and immediately incubated at 37°C for 30 min. Following incubation, tubes were placed on ice, and cell pellets were carefully disrupted via gentle mixing and immediately run through a FACScaliber. Conjugates were identified as those events that were both PKH26^+ (FL2) and CFSE^- (FL1).

All experiments performed in this study were reproduced at least twice and some as many as 18 times.

**Results**

**Primary T cell blasts exposed to thermal stress demonstrate an enhanced sensitivity to lysis by MHC-matched IL-2-activated NK cells**

We have examined the impact of several forms of stress on the sensitivity of normal nontransformed cells to LAK cell-mediated lysis. Con A-activated F1 (C57BL/6 × BALB/c) splenocytes were used as a source of normal target cells (T cell blasts). At the initiation of culture, splenocytes were seeded at 7.5 × 10^5 cells/ml in medium containing Con A (2 μg/ml). Under these conditions, T cells were observed to proliferate and reach a maximum cell density at ~60 h into culture. At this time point, B cells had largely disappeared, whereas T cells constituted >90% of the cells (data not shown). We examined the impact of thermal stress on LAK cell lysis of cells derived from cultures (described above) 60 h after initiation by exposing them to temperatures up to 41°C for periods of 1–4 h. Following stress, Con A blasts were cultured at 37°C in medium containing 2 U/ml IL-2 for various periods of time (recovery period), after which the cells were used as targets in standard 4-h chromium release assays. Fully syngeneic F1 LAK cells were used as effectors. In most cases, Con A blasts were stressed, labeled with Na2Cr2O7, and then immediately used as targets so that the time required for labeling (on average about 2.5 h) constituted the recovery period.

F1 T cell blasts stressed at 41°C for 2 h were rendered far more susceptible to lysis by LAK cells than un-stressed controls, as evidenced by enhanced killing of the stressed target vs the control, a difference that titrated with the E:T ratio (Fig. 1A).

We examined whether thermal stress alone in the absence of LAK effectors had a major impact on the viability of the stressed cells. In all cases, thermal stress was observed to have some cyto-toxic effect. Viability was assessed both by trypan blue exclusion and by flow cytometric analysis of mitochondrial membrane potential and surface expression of phosphatidylserine via DiIC5(5) and annexin-V staining. The latter two were used as indications of apoptosis. Those cells that were annexin-V^- and DiIC5(5)^+ were considered viable. Exposure at 41°C resulted in less than a 15% decrease in cellular viability (data not shown). In all data reported in this study, we have taken the maximum acceptable magnitude of stress as that which decreases the overall viability by no more than

![Figure 1](http://www.jimmunol.org/)
15%. Irrespective of the stress imposed, following chromium labeling and washing, viability was always >90%, as determined by trypan blue exclusion. At no time was stress observed to increase percentage spontaneous release values by >10% of total release (and usually less).

Effect of exposure to other forms of stress on susceptibility to LAK-mediated lysis

We examined the effect of a major change in culture conditions of T cell blasts on sensitivity to LAK-mediated lysis. Con A-activated splenocyte cultures were initially seeded at a 4-fold higher population density (3 × 10^6 cells/ml, high-density growth conditions). These cells were observed to be highly sensitive to LAK-mediated lysis compared with Con A blasts generated under standard conditions (Fig. 1B).

We also examined the impact of exposure to hydrogen peroxide on LAK sensitivity of T cell blasts. T cell blasts exposed to 300 μM hydrogen peroxide for 1 h displayed an enhanced sensitivity to syngeneic LAK cells similar to that observed for thermal stress and high-density growth conditions (Fig. 1C).

Thermally stressed cells have the capacity to return to a nonsensitive state

We examined whether stressed T cells have the capacity to revert to a nonsensitized state. T cell blasts were stressed 60 h into culture by heating to 41°C for 2 h, followed by varied periods of recovery at 37°C in 2 U/ml mIL-2. As reported above, sensitization of T cell blasts to LAK cell-mediated lysis was observed as early as 2.5 h following stress (earlier data could not be collected because of the time limitations of chromium labeling). Sensitization persisted for at least 7 h; however, following a 24-h recovery period, a resistant phenotype reemerged (Fig. 1D).

Activated NK cells are better killers of stressed syngeneic targets

To investigate whether resting and activated NK were equally capable of preferential killing of stressed T cell blasts, we used NK cells isolated directly from the spleens of F1 nude mice as effector cells in the chromium release assay. F1 nude mice were either untreated or primed with poly(I:C) 3 days before the killing assay. Poly(I:C) is known to activate NK in vivo via an IFN-α-β-dependent mechanism (38, 39). Ex vivo NK from untreated and poly(I:C)-treated animals preferentially lysed stressed T cell blasts when compared with unstressed controls (Fig. 1E). The magnitude of killing when resting NK cells from untreated animals were used as effectors was <25% and 50% that observed when poly(I:C)-primed NK and LAK were used as effectors, respectively (even at the highest E:T tested) (Fig. 1, A and E).

LAK cell lysis of stressed T cell blasts is perforin mediated

To examine whether perforin is critical to the lysis of stressed cells by LAK, we used LAK cells derived from PKO mice (32, 40). The PKO strain used for this purpose was engineered on the C57BL/6 (B6) background. Therefore, we used B6 LAK as our source of perforin-competent LAK. LAK cells derived from PKO mice appeared indistinguishable from B6 LAK in IFN-γ, TNF-α production, IL-2 responsiveness, and NK1.1 expression (data not shown). T cell blasts derived from B6 animals were exposed to a model stress of high-density growth conditions and used as targets for LAK derived from B6 or PKO mice. LAK derived from B6 were observed to preferentially lyse stressed T cell blasts compared with control unstressed T cell blasts (Fig. 1F). Unlike LAK derived from B6 animals, PKO LAK were not observed to preferentially lyse stressed T cell blasts, and the level of killing of either target was very low (Fig. 1F).

Stress does not affect CTL-mediated killing

To address whether stress rendered T cell blasts more sensitive to lysis by effector cells capable of cytotoxicity in a nonspecific manner, we examined whether stressed cells were preferentially lysed by CTL specific for the MHC haplotype expressed by the target cell (F1, T cell blast). CTL raised from C3H mice against F1 (H2^a/b) were used as effectors and compared with LAK. Model stressors of 41°C for 4 h or high-density growth conditions were imposed on T cell blast target cells. LAK cells were observed to kill stressed cells preferentially (Fig. 2, C and D). However, C3H CTL primed against F1 (H2^a/b) did not preferentially kill stressed cells over control cells (Fig. 2, A and B). F1, CTL specific for C3H (H2^d) killed neither stressed nor control F1 target cells (data not shown).

Thermal sensitization is both time and temperature dependent

We addressed the effect of dose and exposure time to hyperthermia on the resultant sensitivity to LAK cell-mediated killing. T cell blasts treated at 37°C, 40°C, or 41°C for 2 h demonstrated a sensitivity to LAK cell-mediated killing that was directly proportional to the magnitude of stress exposure (Fig. 3A). Next, T cell blasts were exposed to a temperature of 41°C for 0, 1, 2, 3, or 4 h. As illustrated in Fig. 3B, thermal stress-induced sensitivity required greater than a 1-h exposure and plateaued after a 2-h exposure.

**Stressed T cell blasts form more conjugates with LAK than unstressed control cells**

Cold target inhibition experiments were performed using control nonradioactively labeled unstressed or stressed (41°C for 4 h) T cell blasts as competitors for Na^31CrO_4-labeled stressed cells in chromium release assays in which syngeneic LAK cells served as effectors.
sufficient to affect LAK activity (44), and was observed to be augmented by as much as 55% after a 3-h recovery period (data not shown).

We examined the effect of stress (41°C for 2 h) on cell surface expression of peptide-receptive MHC class I. Peptide-receptive MHC class I molecules are capable of binding high-affinity peptides that contain anchoring residues specific for motifs within the MHC class I groove (26). We chose Kα as our model MHC class I molecule and used a Kα-binding peptide (OVAp; SIINFEKL) (33, 34) to examine the expression of peptide-receptive Kα capable of binding OVAp. T cell blasts were pulsed by OVAp at several time points following stress. The cells were then stained for Kα-peptide complexes with a FITC-labeled mAb (25D1.16) recognizing the specific complex of Kα and OVAp (35) and analyzed via flow cytometry. BFA has been shown to inhibit protein processing and sequester proteins in the Golgi (45). Su and colleagues (26) demonstrated that treatment of Con A blasts with BFA resulted in a complete loss of surface expression of peptide-receptive Kα.

Therefore, we cultured Con A blasts in BFA for 8 h to obtain cells that served as a positive control for lack of peptide-receptive Kα expression (Fig. 4B). Unstressed T cell blasts expressed peptide-receptive Kα (Fig. 4A). Peptide-receptive Kα expression was markedly depressed immediately following stress (Fig. 4C), but recovered by 7 h poststress (Fig. 4D). Although peptide-receptive Kα expression recovered to unstressed levels after a 7-h recovery time, T cell blasts remained highly sensitive to LAK killing (Fig. 1D).

Discussion

In this study, primary T cell blasts were sensitized to killing by syngeneic LAK cells by nonlethal forms of stress, including hyperthermia, hydrogen peroxide, and high-density growth conditions. Previous studies have described a similar phenomenon using allogeneic human tumor cell lines as targets (16–21). The magnitude of stress used to induce a state of sensitivity in the cited studies, as in this study, was nonlethal in nature (16–21).

**Effect of stress on class I MHC**

Stress may affect sensitivity to LAK-mediated killing by depressing the expression of ligands for NK inhibitory receptors, MHC class I and/or peptide-receptive MHC class I (22, 23, 25, 26, 41–43). Con A blasts were examined via flow cytometry for the expression of Dκ, Kκ, Dα, and Kα at several time points following stress (heat, hydrogen peroxide, or high-density growth conditions). Immediately following stress, surface expression of total MHC class I was depressed by no more than 6%, a decrease insufficient to affect LAK activity (44), and was observed to be augmented by as much as 55% after a 3-h recovery period (data not shown).

We examined the effect of stress (41°C for 2 h) on cell surface expression of peptide-receptive MHC class I. Peptide-receptive MHC class I molecules are capable of binding high-affinity peptides that contain anchoring residues specific for motifs within the MHC class I groove (26). We chose Kα as our model MHC class I molecule and used a Kα-binding peptide (OVAp; SIINFEKL) (33, 34) to examine the expression of peptide-receptive Kα capable of binding OVAp. T cell blasts were pulsed by OVAp at several time points following stress. The cells were then stained for Kα-peptide complexes with a FITC-labeled mAb (25D1.16) recognizing the specific complex of Kα and OVAp (35) and analyzed via flow cytometry. BFA has been shown to inhibit protein processing and sequester proteins in the Golgi (45). Su and colleagues (26) demonstrated that treatment of Con A blasts with BFA resulted in a complete loss of surface expression of peptide-receptive Kα.

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**FIGURE 3.** Dose and time dependence and recovery from stress-induced sensitization. A, F1 T cell blasts (60 h of culture seeded at 7.5 × 10⁵ cells/ml) were heated at 37°C (□), 40°C (■), or 41°C (▲) for 2 h and then tested for sensitivity to syngeneic NK lysis at the indicated E:T ratios in standard 4-h chromium release assays. B, F1 T cell blasts, as in A, were exposed to 41°C for periods of 0, 1, 2, 3, and 4 h. Following exposure, cells were tested for sensitivity to syngeneic NK lysis at the indicated E:T ratios. C and D, F1 T cell blasts were subjected to heat stress at 41°C for 2.5 h (D) or unstressed (C), stained with PKH26 (red fluorescent dye), and mixed at a 1:1 E:T with CFSE (green fluorescent dye)-stained IL-2-activated F1 NK cells. Cells were allowed to form conjugates in a pellet at 37°C for 30 min and then analyzed via flow cytometry. Singlet NK were observed as single positive events in FL1. Singlet T cell targets were observed as single positive events in FL2. Conjugates between NK and T cell targets were identified as those events double positive for both green and red fluorescence.

**FIGURE 4.** Expression of peptide-receptive Kα molecules on the surface of T cell blasts exposed to stress. F1 T cell blasts were pulsed with a Kα-binding peptide (OVAp; SIINFEKL) for 45 min at 1 μg/ml. OVAp served to bind with high affinity to peptide-receptive Kα molecules. The Kα-OVAp complex was then labeled using 25D1.16 FITC. Flow cytometric data are presented as single-color histograms. A, Unstressed F1 T cell blasts. B, F1 T cell blasts treated with BFA (2.5 μg/ml) for 8 h (positive control for loss of peptide-receptive Kα). C, F1 T cell blasts exposed to 41°C for 2 h and immediately pulsed with OVAp (no recovery period). D, F1 T cell blasts exposed to 41°C for 2 h and pulsed with OVAp following a 7-h recovery period.
Thermal stress-induced sensitization was observed to be both dose and time dependent. Temperatures equal to or greater than 39°C were sufficient to sensitize targets. The amount of time exposed to heat shock required to achieve sensitization was >1 h and reached a plateau at 2 h. Exposure of the LAK effector cells to hyperthermia (41°C for 2.5 h) had no observable impact on their cytolytic capacity or preferential killing of stressed T cell blasts (data not shown). Stressed T cell blasts were able to revert to a LAK-resistant phenotype following a recovery period of >12 h at 37°C (Fig. 1D). Furthermore, stressed T cell blasts when cultured in IL-2 were able to divide and demonstrated similar growth kinetics to unstressed controls (data not shown). These data suggest strongly that the stress-induced LAK-sensitive phenotype is not permanent.

We were concerned whether exposure to stress merely invoked a state of nonspecific enhanced sensitivity to cells capable of cytotoxicity. CTL raised against determinants expressed by the T cell blast target cells did not preferentially lyse stressed cells (Fig. 2). This suggests strongly that stress does not induce an intrinsic nonspecific susceptibility of stressed cells to lysis and is consistent with a previous report indicating that CTL-mediated killing of stressed targets is unaffected or slightly inhibited when compared with the killing of unstressed cells (46).

The lytic outcome following LAK-target interaction is influenced at several levels. These include the adhesion between LAK and target (47), the net balance of activation and inhibitory signals (26) received by the LAK cells, and the sensitivity of the target cell to lytic factors released by the LAK (21, 48). In cold target inhibition studies, stressed cells were more effective cold target inhibitors of LAK-mediated lysis of stressed cells than unstressed control targets (data not shown). This is consistent with our conjugation assay results in which LAK-target conjugation frequency between LAK and stressed T cell blasts was nearly double that observed between LAK and the unstressed control (Fig. 3, C and D). LAK-mediated killing in association with no change in LAK to target conjugation frequency has been interpreted as resulting from down-regulation of an NK inhibitory ligand on the target cell (26), while increased conjugation frequency has been interpreted as resulting from up-regulation of an activation ligand on the target cell (31). According to this model, stress may induce the up-regulation of an activation ligand on T cell blasts that may be absent or expressed at lower levels before stress. Our observation that LAK, but not CTL, preferentially lysed stressed cells suggests that LAK cells specifically recognized the stress-induced alteration.

We wished to examine whether activation of NK cells with IL-2 was required to produce effector cells capable of preferential lysis of stressed T cell blasts. NK cells were isolated from the spleens of nude mice and used as effectors in chromium release assays without further activation. Mice were either untreated or given i.p. injections of poly(I:C) to activate NK cells. Poly(I:C) has been suggested to activate NK cells in an analogous fashion to many viruses (39). LAK may arise in inflammatory foci, in which local levels of inflammatory cytokines may be extremely high. Freshly isolated naive NK did preferentially lyse stressed T cell blasts, but the magnitude of killing was <25% that seen for LAK at even the highest E:T tested (Fig. 1, A and E). Freshly isolated NK from poly(I:C)-primed animals preferentially lysed stressed T cell blasts with a magnitude intermediate to that seen for resting NK and LAK (Fig. 1E). We interpret these data as suggesting that under normal conditions, naive NK have the capacity to preferentially recognize stressed cells. Under more restrictive conditions, such as that of an inflammatory focus, activated NK may more effectively lyse stressed cells. Intriguingly, such inflammatory foci often represent local pools of many stressors implicated by our studies as inducing sensitivity to activated NK-mediated lysis, including reactive oxygen species and (localized) hyperthermia. We speculate that under tightly controlled conditions, activated NK may function to limit immune responses by eliminating stressed T cells in such foci.

Enhanced conjugation frequency between LAK and stressed targets suggested that down-regulation of an NK inhibitory ligand on target cells as a result of stress was unlikely. In mice, NK inhibitory receptors belong to two major families, Ly-49 and CD94/NKG2, both of which recognize forms of MHC class I (22, 23, 41–43, 49). Blockade of Ly-49C on LAK effector cells with mAbs in the chromium release assay enhanced killing of both unstressed control and stressed T cell blasts (data not shown). Blockade of Ly-49A (specific for D^2) had no effect (data not shown). Because the blockade of Ly-49C affected the killing of both unstressed and stressed targets, it is unlikely that its ligand (K^b) is involved in the mechanism of stress-induced sensitization. Furthermore, enhanced killing of unstressed T cell blasts mediated by the blockade of signaling through Ly-49C was always observed to be <30% of the killing recorded for stressed T cell blasts (data not shown). This suggests that the effect of stress is far more robust than that mediated by the loss of signaling through any of the Ly-49 molecules we investigated and supports our model of up-regulation of an NK activation ligand on target cells exposed to stress.

We examined the effect of stress on the expression of total and peptide-receptive MHC class I. Peptide-receptive MHC class I contain an open peptide-binding groove and can accept high-affinity peptides (formerly termed empty MHC class I) (25, 26). These are relatively nonstable molecules and are recognized by NK inhibitory receptors such as Ly-49, which is specific for peptide-receptive K^b (but not K^b molecules containing peptide in their MHC class I groove) (25, 26). Following stress, a decrease in total MHC class I expression was not observed (data not shown), thereby eliminating a possible role for their loss in stress-induced sensitization. However, peptide-receptive MHC class I expression was observed to be completely ablated immediately following stress, but recovered within 7 h following stress (Fig. 4). Previous reports from our laboratory indicated that the recovery of peptide-receptive MHC class I requires ~90 min (26). We and others have previously observed that upon the loss of peptide-receptive MHC class I (26), syngenic cells are rendered highly sensitive to LAK cell-mediated lysis. It may be confidently predicted, then, that immediately following stress, stress-exposed cells would represent highly susceptible LAK targets. Moreover, notwithstanding the recovery of peptide-receptive MHC class I by 7 h following stress, stressed cells maintained a highly sensitive state to LAK-mediated lysis. This suggests that although the loss of peptide-receptive MHC class I molecules immediately poststress may contribute to stress-induced sensitization, it cannot explain enhanced sensitivity following longer periods of recovery at 37°C.

The nature of the putative stress-induced NK activation ligand remains unclear. One of the difficulties encountered in probing the nature of the ligand has been the general lack of knowledge pertaining to NK activation ligands. Although controversial, carbohydrate moieties are considered the most likely candidate. For example, Yagita et al. (50) observed that LAK cells lyse target cells treated with glycosyl-transferase inhibitors and accumulate high-mannose N-linked carbohydrate residues on their surface. Interestingly, hyperthermia has been reported to cause severe fragmentation of the Golgi apparatus (51), home to the glycosyl-transferases involved in building complex N-linked carbohydrate residues from high-mannose ones. It is tempting to speculate that stress sensitizes
T cell blasts to activated NK lysis through modification of glyco-sylation patterns. This hypothesis requires further investigation.

Multhoff and colleagues (17, 18) have implicated hsp70 as a possible stress-induced NK activation ligand expressed on some human tumor cells. The high degree of similarity between the crystal structure of hsp70 and MHC class I makes hsp70 an attractive ligand recognized by LAK cells (52, 53). Using mAbs specific for hsp70, Multhoff et al. (16, 18–20, 54) observed the inhibition of heat-induced LAK killing of several tumor cell lines including K562. Multhoff and Hightower suggest that the acidic pH of some tumor cell plasma membranes may induce a conformational change in hsp70 that facilitates membrane anchoring (55) in a similar fashion to that which has been described for diptheria toxin (55, 56). We examined the influence of Abs directed against hsp70 for their impact on the lysis of stressed T cell blasts and were unable to demonstrate any effect (data not shown). Nor, using flow cytometry, could we observe hsp70 on the cell surface (data not shown).

Another stress-induced NK activation ligand has recently been reported. Groh et al. (57) identified MICA, a stress-induced human class I like molecule on intestinal epithelia cells and some tumor cells. The receptor for MICA was identified as an orphan C-type lectin NK receptor, CD94/NKG2D (58). However, MICA expression has not been observed on T cells, and a homologue for MICA is not encoded in the murine genome (T. Spies, unpublished ob-servation). Therefore, a direct role for MICA in our system is not likely.

Another possibility is that stress does not up-regulate expression of an NK activation ligand, but induces the clustering of such on the plasma membrane. The clustering of molecules on the cell surface of BW5147 cells following transfection with ezrin, a cytoskeletal protein that mediates uropod formation, has been reported to sensitize the transfected targets to LAK killing (59). All the stressors used in our studies are known to induce the phophor-ylation of hsp27, which interacts with the actin cytoskeleton in-ducing cellular rigidity (60). Furthermore, oxidation of the plasma membrane has been reported to result in membrane blebs via an actin-dependent mechanism (61). However, treatment of stressed T cell blasts with cytochalasin B or methyl-β-cyclodextrin, which disrupts actin-mediated clusters and lipid rafts, respectively, did not alter their sensitivity to LAK-mediated lysis (data not shown).

Our data also provide insight into the variation pertaining to LAK-mediated killing often observed against supposedly LAK-resistant targets in the literature. This contrasts the relative con-sistency observed in killing when CTL are used as effectors against resistant targets. This variation in LAK-mediated background kill-ing is usually ignored by investigators because of an inability to explain it. This study provides a possible mechanism for the ob-served variation, suggesting it may result from a lack of control of environmental stress in study design.

In summary, our results demonstrate that exposure of normal nontransformed cells to nonlethal stress renders such cells sensi-tive to syngeneic activated NK-mediated lysis. The phenomenon is specific to NK1.1+ cells and is perforin mediated. Stress-induced sensitization cannot be explained by alterations in expression of MHC class I peptide complexes. Furthermore, the observed loss of peptide-receptive MHC class I expression may contribute to stress-induced sensitization, but only transiently. In those tissues to which activated NK cells have access, activated NK may be capable of killing stressed cells before neoplastic transformation can occur. Finally, activated NK cells may play an important role in the limiting of immune responses in the stressful environments of inflammatory foci.

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