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Bromelain Modulates T Cell and B Cell Immune Responses *in Vitro* and *in Vivo*

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The ability to modulate immune responses is a major aim of many vaccine and immunotherapeutic development programs. Bromelain, a mixture of cysteine proteases, modulates immunological responses and has been proposed to be of clinical use. However, the identity of the immune cells affected by bromelain and the specific cellular functions that are altered remain poorly understood. To address these shortcomings in our knowledge, we have used both *in vitro* and *in vivo* immunological assays to study the effects of bromelain. We found that bromelain enhanced T cell receptor (TCR) and anti-CD28-mediated T cell proliferation in splenocyte cultures by increasing the costimulatory activity of accessory cell populations. However, despite increased T cell proliferation, bromelain concomitantly decreased IL-2 production in splenocyte cultures. Additionally, bromelain did not affect TCR and CD28-induced proliferation of highly purified $CD4^+$ T cells, but did inhibit IL-2 production by these cells. *In vivo,* bromelain enhanced T-cell-dependent, Ag-specific, B cell antibody responses. Again, bromelain induced a concomitant decrease in splenic IL-2 mRNA accumulation in immunized mice. Together, these data show that bromelain can simultaneously enhance and inhibit T cell responses *in vitro* and *in vivo* via a stimulatory action on accessory cells and a direct inhibitory action on T cells. This work provides important insights into the immunomodulatory activity of bromelain and has important implications for the use of exogenous cysteine proteases as vaccine adjuvants or immunomodulatory agents.

Key Words: **T cells; B cells; bromelain; cysteine proteases; IL-2; immunomodulation.**

INTRODUCTION

The ability to modulate T cell activation has important implications for conditions where T cells are either inappropriately or suboptimally activated, such as may occur in autoimmune or infectious disease, respectively. Efficient T cell activation requires a primary signal delivered through the Ag-specific T cell receptor (TCR)[1] and a second, costimulatory signal. CD28 has been identified as a principal source of costimulatory signal for naive T cells and is required for IL-2 production and proliferation. Ligands for CD28 include CD80 (B7-1) and CD86 (B7-2) found on dendritic cells, macrophages, and activated B and T cells. CD80 and CD86 are also ligands for CTLA4, a negative regulatory receptor found on both $CD4^+$ and $CD8^+$ T cells (1, 2). Recently, additional members of this receptor–ligand family have been discovered, including ICOS (3, 4) and B7-related protein 1 (4), that appear to have regulatory roles during an adaptive immune response.

Bromelain is a proteolytic enzyme extract obtained from pineapple stems (*Ananus comosus*) that modulates T cell responses. Bromelain increases CD2-mediated T cell activation (5), enhances antigen-independent binding of T cells to splenocytes, and increases IFN-γ-dependent TNFα, IL-1α, and IL-6 production (6) in human peripheral blood mononuclear cells (PBMCs). The mechanisms by which bromelain elicits these stimulatory effects remain unclear. Studies show that bromelain removes specific cell surface receptors, including CD44, CD45RA, CD6, CD7, and CD8, while increasing surface expression of MHC class I (5) and the beta(2)-integrins CD11a–c (7). These data suggest that bromelain's effects might simply be caused by a nonspecific proteolytic degradative action at cell surfaces to alter receptor–ligand interactions. In addition to bromelain's stimulatory action on T cells, bromelain also inhibits T cell responses (8, 9). Targoni *et al.* (9) show that an enzyme mixture containing bromelain inhibited murine experimental allergic encephalomyelitis (EAE), a model of T-cell-mediated autoimmune disease. This effect was attributed to proteolytic cleavage of the accessory molecules CD4, CD44, and CD80 that mediate T cell and APC interactions (9). We have

[1] Abbreviations used: SRBC, sheep red blood cells; PFC, plaque forming cells.

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also shown that bromelain inhibits T cell responses, but it has a more complex mode of action than simply removing cell surface receptor sites. We showed that bromelain blocked combined phorbol ester and ionophore (surface-receptor-independent) induced activation of extracellular regulated kinase (ERK)-2 and $p21^{Ras}$ in T cell hybridomas. In association with decreased ERK-2 and $p21^{Ras}$ activation, bromelain inhibited IL-2, IL-4, and IFN-γ production (8). These data suggest that in addition to altering receptor–ligand interactions, bromelain also has a selective and more specific action on T cells.

Despite the recent interest in bromelain as an immunomodulator, it is not clear which specific cell population is affected by bromelain since many studies are conducted using crude cell preparations such as PBMCs or semi-purified T cells (5, 6, 10). Furthermore, since bromelain, a natural plant extract, both stimulates and inhibits immune responses, it is not clear whether these opposing effects reflect differences in the source of bromelain used and its composition or differences in the experimental systems used in independent studies. Therefore, in this study we sought to characterize both the stimulatory and the inhibitory effects of bromelain *in vitro* and *in vivo* and studied its effect on both mixed and highly purified cell populations. We found that bromelain enhances TCR-mediated T cell proliferation in whole splenocyte cultures, but does not affect the proliferation of purified $CD4^+$ or $CD8^+$ T cells. The increased T cell proliferation induced by bromelain-treated splenocytes was not caused by a direct effect of bromelain on T cells, but instead, via an effect on accessory cell populations. Despite the increase in T cell proliferation, bromelain simultaneously inhibited TCR-mediated IL-2 production by both splenocyte cultures and purified $CD4^+$ T cells. *In vivo*, bromelain increased Ag-specific B cell antibody responses, while simultaneously blocking IL-2 mRNA accumulation. Together, these data suggest that bromelain can simultaneously enhance and inhibit T cell responses *in vitro* and *in vivo* via a stimulatory action on APC and a direct inhibitory action on T cells.

MATERIALS AND METHODS

Antibodies

Anti-CD28 mAb (PV-1) (11), anti-CD3ϵ-chain mAb (145-2C11) (12), and anti-TNP mAb (isotype control for PV-1 and 145-2C11) were used in cell culture. The following mAbs were used for FACs analysis: anti-CD4 mAb (Sigma, Dorset, UK), anti-CD8 mAb (Sigma), anti-CD25 mAb (Sigma), anti-CD45RB mAb (Sigma), anti-CD44 mAb (BD Pharmingen, San Diego, CA), anti-CD80 mAb (1610A1) (13), anti-CD86 mAb (GL-1) (American Type Culture Collection (ATCC), Bethesda, MD), anti-MHC class II mAb (TIB120) (ATCC), and control IgG2a (AFRC, MAC4; isotype control for 1610A1, GL-1 and TIB120) (European Collection of Animal Cell Cultures, ECACC, Porton Down, UK).

Animals and Reagents

Female BALB/c mice (6–8 weeks of age; Tuck and Co., Essex, UK) housed under conventional conditions were used in all experiments. SRBCs were from TCS Biologicals (Buckingham, UK) and guinea pig complement was from SeraLab (Loughborough, UK). Crude bromelain (E.C. 3.4.22.32; specific activity 1541 nmol/min/mg) was purchased from Hong Mao Biochemicals (Rayong, Thailand) and trypsin (porcine pancreas, E.C. 3.4.21.4; specific activity 5270 nmol/min/mg) was from Sigma. Specific activity of proteases was determined by monitoring the release of *p*-nitroaniline from the peptide-*p*-nitroanilide (pNA) substrate Z-Arg-Arg-pNA (Bachem, Saffron Walden, UK) (14). Bromelain and trypsin were diluted in phosphate-buffered saline (0.1 M, pH 7.4; PBS) or 0.9% (w/v) NaCl for *in vitro* and *in vivo* uses, respectively. Other reagents were purchased from Sigma.

Cell Preparation

Spleens from at least three mice were pooled and passed through a 20-μm sieve. Erythrocytes were then lysed at 5×10^7 cells/ml in lysing buffer (140 mM NH_4Cl, 17 mM Tris, pH 7.2) at room temperature for 5 min. Lysis was terminated by the addition of RPMI 1640 (Gibco BRL, Paisley, UK) and washing the cells (splenocytes) three times. Splenocytes were then isolated over Histopaque 1083 (1700 rpm, 15 min, room temperature; Sigma), washed twice in RPMI 1640, and resuspended in tissue culture medium (TCM; RPMI 1640, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin; Gibco BRL).

$CD4^+$ T cells, $CD8^+$ T cells, and B cells were isolated from splenocytes by magnetic activated cell sorting using magnetic microbeads conjugated to anti-CD4 (L3T4), anti-CD8 (Ly-2), and anti-CD45R (B220) mAbs, respectively, according to the supplier's instructions (Miltenyi Biotec, Gladbach, Germany). This procedure yielded cells that were >98% pure, as assessed by flow cytometry. In some experiments, cells were irradiated (1500 rad) prior to culture.

Cell Culture

Cells were cultured in triplicate in 0.2 ml TCM in 96-well, flat-bottom, microculture plates (Nunc, Rosskilde, Denmark) at 1×10^5 cells per well, unless otherwise specified. Cells were stimulated with combinations of immobilized (plate-bound) anti-CD3ϵ (5 μg/ml) and soluble anti-CD28 mAbs (10 μg/ml). For im-

mobilized anti-CD3ϵ mAb presentation, mAb was diluted in PBS, added to microculture plates (50 μl), and incubated for 16 h at 4°C. Wells were then washed three times in PBS. Cultures were incubated at 37°C in humidified 5% (v/v) CO_2 for 36 h, pulsed overnight with 0.5 μCi of [^{3}H]TdR, harvested onto glass fiber filters, and counted. In some experiments, supernatant was collected from cultures after 24 h of incubation for IL-2 analysis.

FACS Analysis of Cell Surface Molecules

Cells (1×10^5) were incubated for 30 min on ice with 0.5 ml FACS blocking buffer (50% (v/v) horse serum (Sigma), 50% (v/v) FACS buffer (1% (v/v) horse serum in PBS, 0.1% (w/v) NaN_3). Cells were pelleted by centrifugation at 4°C and resuspended in 100 μl FACS buffer containing an appropriate FITC-conjugated mAb. Cells were incubated on ice for a further 30 min, washed once in FACS buffer, resuspended in 1% (w/v) paraformaldehyde (Sigma), and stored in the dark at 4°C until analysis. Cells were analyzed on a Becton–Dickinson FACScan flow cytometer (Becton–Dickinson, Mountain View, CA) by setting a gate on lymphocyte populations using forward and side scatter and plotting data on a log scale.

Measurement of IL-2

IL-2 activity from *in vitro* experiments was measured using the IL-2-dependent cell line CTL-L, as previously described (15). Units of cytokine activity were defined relative to standard curves with recombinant human IL-2 (Genzyme, Cambridge, MA). IL-2 mRNA accumulation in splenocytes *in vivo* was measured using a semi-quantitative RT-PCR assay as previously described (16).

Bromelain Treatment of Cells

Cells (1×10^7/ml) were incubated with gentle agitation at 37°C for 30 min in RPMI 1640 containing various concentrations of bromelain as indicated in the text and figure legends. Mock-treated cells were treated with an equal volume of PBS (diluent for bromelain). Following incubation, cells were washed three times in RPMI 1640 and then resuspended in TCM. The endotoxin content of diluted bromelain preparations used was less than 10 ng/ml as determined by a colormetric Limulus amebocyte lysate assay (17). In some experiments bromelain was heat-inactivated prior to addition to cells by incubating a 10 mg/ml solution at 65°C for 30 min.

Hemolytic Plaque Assay

Mice were administered bromelain (0 to 200 μg in 200 μl), proteolytically equivalent amounts of trypsin, heat-inactivated bromelain, or diluent (0.9% (w/v) NaCl; 200 μl) on days indicated in the figure legends. Mice were then immunized intraperitoneol with 100 μl SRBC (1×10^7 cells). Mice were killed by cervical dislocation 7 days after immunization, spleens were removed, and single cell splenocyte suspensions were prepared as described above. The number of B cells secreting antibodies specific for SRBC was determined using the Jerne hemolytic plaque-forming assay (18). Briefly, assays were performed in triplicate in 160 μl, consisting of 1×10^6 splenocytes, 6×10^6 SRBC, and 1:27 (v/v; diluted in RPMI 1640) guinea pig complement. The reaction mix was placed in a chamber created by joining two glass slides together with double-sided tape and then sealing them with wax. Samples were incubated at 37°C for 1 h, prior to counting plaque-forming cells (PFC; B cells secreting Ab specific for SRBC).

Data Analysis

In cell culture experiments, paired *t* tests (Student's *t* test; two-way) were used to test for differences among means in bromelain-treated cells compared with its paired PBS control. Analysis of variance (ANOVA) was used when comparing the mean of more than one treatment against PBS controls. In animal experiments, independent *t* tests (Student's) were used to test for differences among means in bromelain-treated animals versus saline controls. ANOVA was used when comparing the mean of more than one treatment against saline controls.

RESULTS

Bromelain Increases Splenocyte Proliferation and Inhibits IL-2 Production Following TCR Activation

To assess the effect of bromelain on murine T cell proliferation and IL-2 production, splenocytes were treated with bromelain (0–200 μg/ml), washed, and then stimulated with immobilized anti-CD3ϵ and soluble anti-CD28 mAbs. Control cells were cultured in medium alone, with anti-CD28 mAb alone, or with isotype mAbs. Data in Fig. 1A show that bromelain dose-dependently increases anti-CD3ϵ and anti-CD28-mediated T cell proliferation. Cells cultured in the presence of medium alone (Fig. 1A), anti-CD28, or isotype mAb alone did not proliferate (data not shown). Surprisingly, despite the fact that bromelain increased T cell proliferation, it inhibited anti-CD3ϵ and anti-CD28-mediated IL-2 production (Fig. 1B, $P < 0.005$). Bromelain did not adversely affect cell viability as determined by trypan blue dye exclusion even at the highest dose level (data not shown).

To confirm that the bromelain-induced increase in splenocyte proliferation was not caused by potentially contaminating endotoxin, cells were treated with heat-inactivated bromelain (50 μg/ml) and then stimulated

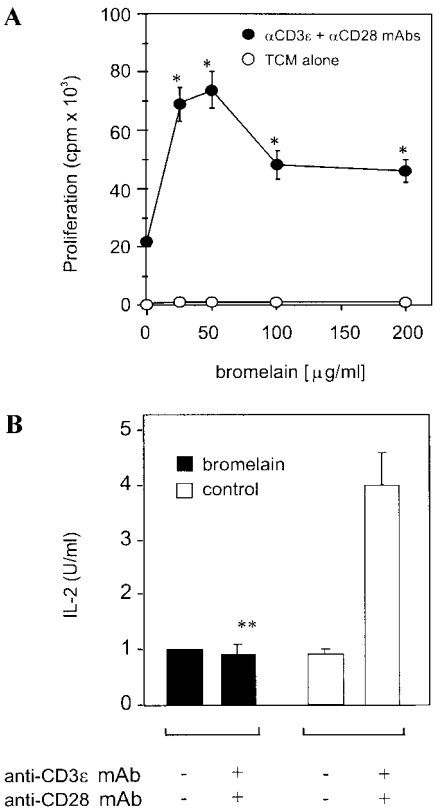

FIG. 1. Bromelain increases T cell proliferation in splenocyte cultures and inhibits IL-2 production. (A) Murine splenocytes (1×10^6) were treated with bromelain (0 to 200 μg/ml) or an equal volume of PBS (diluent for bromelain), washed in RPMI, and then resuspended in TCM. T cells were then stimulated (10^5 per well) with immobilized anti-CD3ϵ and soluble anti-CD28 monoclonal antibodies in 96-well plates and cultured for 30 h. Cells were pulsed with [^{3}H]thymidine 12 h prior to harvesting. (B) Splenocytes were treated with bromelain (50 μg/ml) and stimulated with mAb as above, and culture supernatants were harvested after 24 h. IL-2 levels were determined using the IL-2-dependent CTL-L cell line. Data represent the means ± SE of triplicate cultures and are representative of $n = 4$ experiments performed. *$P < 0.001$, ANOVA, **$P < 0.001$, paired t test.

with anti-CD3ϵ and anti-CD28 mAbs. Heat-inactivation of bromelain completely abrogates the specific activity of bromelain (specific activity, 5 nmol/min/mg), while the mitogenic effect of heat stable endotoxin remains intact. Heat-inactivation of bromelain completely abrogated its immunostimulatory effect (proliferation, cpm $\times 10^3$; control, 59.2 ± 4.1; bromelain, 85.6 ± 2.2; heat-inactivated bromelain, 55.0 ± 4.7), indicating that potentially contaminating endotoxin is not responsible for its ability to increase proliferation. These data also suggest that bromelain's mitogenic activity is dependent on its proteolytic activity. We also tested whether trypsin, a serine protease that recognizes similar cleavage sites to bromelain, would also increase splenocyte proliferation. Trypsin (at the same specific activity as bromelain; 77 nmol/min/mg) increased the proliferation of splenocytes (proliferation, cpm $\times 10^3$; control, 127.8 ± 1.3; bromelain, 197.1 ± 18.0; trypsin, 211.1 ± 3.7), thereby confirming that proliferative activity correlates with proteolytic activity.

Bromelain Inhibits IL-2 Production by CD4$^+$ T Cells, but Has No Effect on CD4$^+$ T Cell Proliferation

Since bromelain inhibits IL-2 production in splenocyte cultures, we next examined whether bromelain would block IL-2 production in purified T cells. Highly purified CD4$^+$ and CD8$^+$ T cells isolated from splenocytes were treated with bromelain and stimulated in culture with anti-CD3ϵ and anti-CD28 mAbs. Results show that bromelain (50 μg/ml) has no effect on CD4$^+$ (Fig. 2A) or CD8$^+$ T cell proliferation (data not shown), despite bromelain at this dose having a significant stimulatory effect on T cells in splenocyte cultures (Fig. 1A). Bromelain also significantly ($P < 0.001$) inhibited

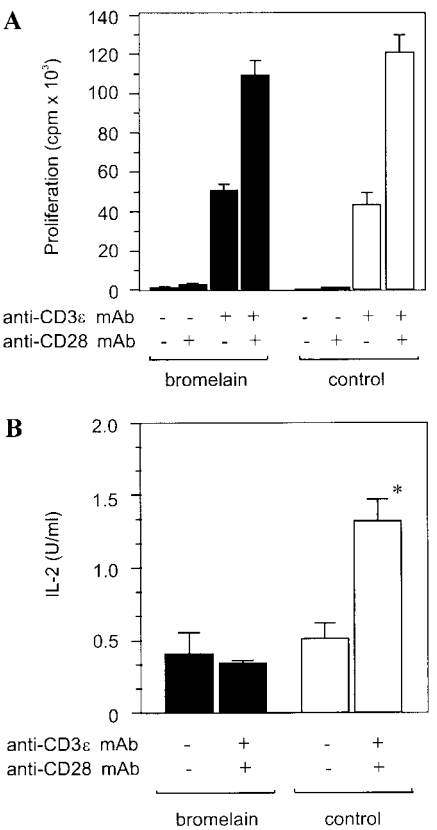

FIG. 2. Bromelain does not affect CD4$^+$ T cell proliferation, but inhibits IL-2 production. (A) Purified CD4$^+$ T cells were treated with bromelain (50 μg/ml) or an equal volume of PBS (diluent for bromelain), washed, and then cultured in RPMI medium alone, anti-CD3ϵ or anti-CD28 mAb alone, or combined immobilized anti-CD3ϵ and soluble anti-CD28 mAbs and cultured for 36 h. Cells were pulsed with [^{3}H]thymidine 12 h prior to harvesting. (B) Purified CD4$^+$ T cells were treated with bromelain and stimulated with mAb as above. Culture supernatants were harvested after 24 h and IL-2 levels were determined using the IL-2-dependent CTL-L cell line. Data represent the means ± SE of triplicate cultures and are representative of $n = 4$ experiments performed. *$P < 0.05$, paired t test.

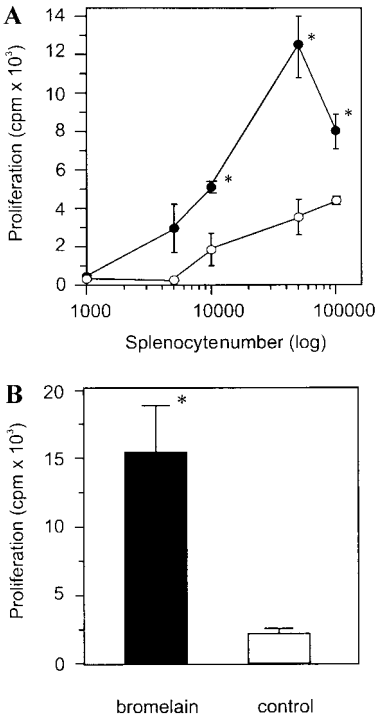

FIG. 3. Bromelain increases the costimulatory activity of irradiated splenocytes and purified B cells. Purified $CD4^+$ T cells (1×10^5 per well) were stimulated with (A) combined anti-CD3ϵ mAb and bromelain-treated (50 μg/ml), irradiated splenocytes or (B) anti-CD3ϵ mAb plus bromelain-treated, irradiated B cells. Cells were mock treated with an equal volume of PBS. Cells were then cultured in 96-well plates for 36 h. Cells were pulsed with [^{3}H]thymidine 12 h prior to harvesting. Data represent the means ± SE of triplicate cultures for each treatment and are representative of $n = 3$ experiments. $*P < 0.05$, paired t test.

IL-2 production by $CD4^+$ T cells (Fig. 2B), similar to its inhibitory effect on IL-2 production in splenocyte cultures (Fig. 1B). Since bromelain did not affect proliferation of purified $CD4^+$ or $CD8^+$ T cells, these data indicate that the bromelain-induced increase in splenocyte proliferation (Fig. 1A) is not a result of a direct effect of bromelain on T cells. The inhibitory effect of bromelain on IL-2 production in splenocyte cultures, however, is caused by a direct effect of bromelain on T cells.

Bromelain Increases Costimulatory Activity of B Cells

Since bromelain did not affect $CD4^+$ or $CD8^+$ T cell proliferation, we next examined whether the bromelain-mediated increase in T cell proliferation in splenocyte cultures was due to effects on a non-T-cell population. Highly purified $CD4^+$ T cells were cultured in the presence of anti-CD3ϵ mAb and costimulated with irradiated splenocytes that had been treated with bromelain or were mock-treated. Data in Fig. 3A show that bromelain-treated, irradiated splenocytes significantly increased $CD4^+$ T cell proliferation, compared with mock-treated, irradiated cells. When highly purified, irradiated $B220^+$ B cells were used, instead of irradiated splenocytes, again bromelain significantly increased $CD4^+$ T cell proliferation (Fig. 3B). Together, these data indicate that bromelain acts on accessory cell populations to enhance their ability to provide costimulatory signals to T cells.

Bromelain Selectively Cleaves Cell Surface Molecules

In a previous study, bromelain treatment of human PBMCs at 1000 μg/ml for 60 min removed several cell surface molecules (5). It removed CD44 (by 97%), CD45RA, and CD8, among other cell surface molecules. At this high dose, bromelain also reduces CD3, CD28, and MHC class II by 25% and CD4 by 50%. In other studies, bromelain treatment of T cell hybridomas or B cell tumor cell lines (50 μg/ml for 2 h) reduced surface expression of CD4, CD44, B7-1, B7-2, and ICAM-1 while not affecting expression of CD3, MHC class I, MHC class II, and LFA-1 (9). In the present study, we show that bromelain modulates T cell responses *in vitro* at much lower doses (50 μg/ml, or 20 times less) and incubation times (30 min, or 2 to 4 times less) than that previously reported (5, 9). Therefore, we reexamined the effect of bromelain on the expression of various cell surface molecules using the less stringent treatment regimen where we observed increased splenocyte proliferation and a concomitant decrease in IL-2 production. Purified murine splenic T cells or purified B cells were treated with bromelain (50 μg/ml for 30 min) and washed three times in RPMI, and then the level of cell surface markers was assessed by FACS analysis. In contrast to results obtained earlier (5, 9), bromelain did not reduce CD4, CD45 (isoform RB), CD80, or CD86 cell surface expression on splenic T cells or B cells. Bromelain also did not affect MHC class II or CD25 expression (data not shown). It also did not completely remove CD44 (reduced expression by 50%) and only marginally decreased CD8 expression (Fig. 4A). Interestingly, bromelain increased surface expression of CD3 and CD28 molecules and marginally increased expression of CD4 (Fig. 4A). Trypsin, shown earlier to increase splenocyte proliferation, marginally increased the surface expression of CD3 and CD28 (Fig. 4B). These data show that bromelain selectively modulates surface expression of certain cell surface molecules.

Since bromelain does not remove CD3ϵ and CD28 molecules, these data indicate that bromelain does not reduce IL-2 production by simply removing CD3ϵ and CD28 molecules from the T cell surface and thus preventing optimal stimulation of T cells. Although bromelain increased the surface expression of CD4, CD3, and CD28 on $CD4^+$ T cells, surprisingly it did not affect their proliferation. Also, since bromelain did not alter expression of MHC class II, CD80, or CD86, the data

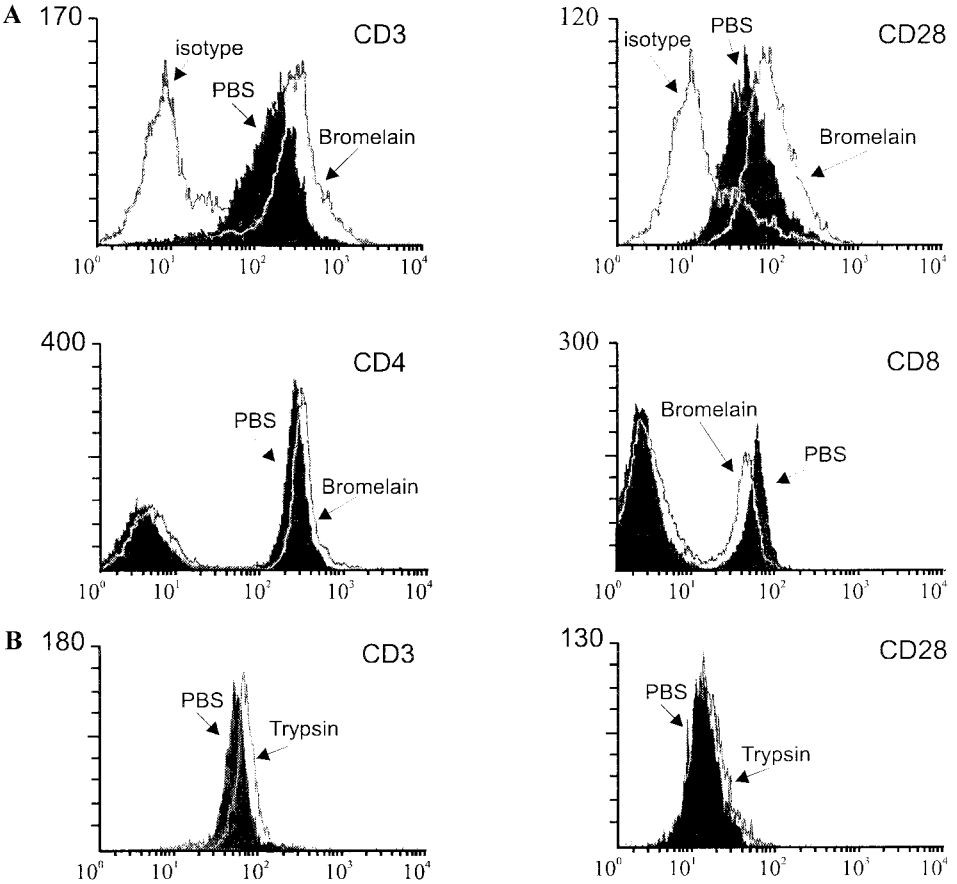

FIG. 4. Bromelain and trypsin increase surface expression of CD3 and CD28 T cell surface molecules. Splenic T cells were treated with (A) bromelain (50 μg/ml, 77 nmol/min/mg), (B) trypsin (12 μg/ml, 77 nmol/min/mg), or PBS for 30 min at 37°C. Cells were then washed twice in RPMI, suspended in FACS buffer, and then stained with anti-CD3ϵ-chain mAb, CD28 mAb, anti-CD4 mAb, or anti-CD8 mAb where indicated or an appropriate isotype control mAb, followed by FITC-conjugated mAb. Data shown are the fluorescence intensity of cells from a single experiment, representative of three performed.

indicate that bromelain acts on accessory cell populations to enhance their ability to provide costimulatory signals to T cells but not via the major TCR signaling molecule (MHC class II) or the important costimulatory molecules, CD80 and CD86.

Bromelain Increases T-Cell–Dependent Antibody Responses in Vivo

The above experiments show that bromelain can directly block T-cell-dependent responses and also enhance B cell activity. Therefore, bromelain has the potential to both stimulate and inhibit immune function *in vitro.* Since immune responses *in vivo* are dependent on many factors, including specific cell–cell interactions, tissue site, and the cytokine microenvironment within these sites, we wished to test the *in vivo* activity of bromelain in a model T-cell-dependent system. Therefore, we examined the effects of bromelain on immune function by assessing its ability to modulate T-cell-dependent antibody responses *in vivo.* Mice were administered a single injection (iv) of bromelain (200 μg, sp. act., 308 nmol/min/200 μl) and then immunized with a single injection (ip) of SRBC, a T-cell-dependent antigen. The dose rate of 200 μg/20 g mouse is equivalent to approximately one-third of the LD_{50} (30–35,000 μg/kg) for bromelain (Sigma Aldrich Ltd.; Material Safety Data Sheet). Control mice were untreated (naive) or immunized with saline alone. Specific antibody responses were assessed by counting the number of plaque-forming cells (B cells secreting Ab specific for SRBC) at 7 days postimmunization. In some control experiments, mouse red blood cells (MRBCs) were substituted for SRBCs as the target antigen in the *in vitro* plaque assay since bromelain has been shown to induce autoantibody responses. This was to ensure that PFC formation was SRBC-antigen-specific and not a result of bromelain inducing autoantibody responses against newly exposed MRBC antigenic sites, which might cross-react with SRBC.

Data in Fig. 5 show that bromelain dose-dependently increases the frequency of antigen-specific B cells in comparison with mice treated with saline alone ($P <$

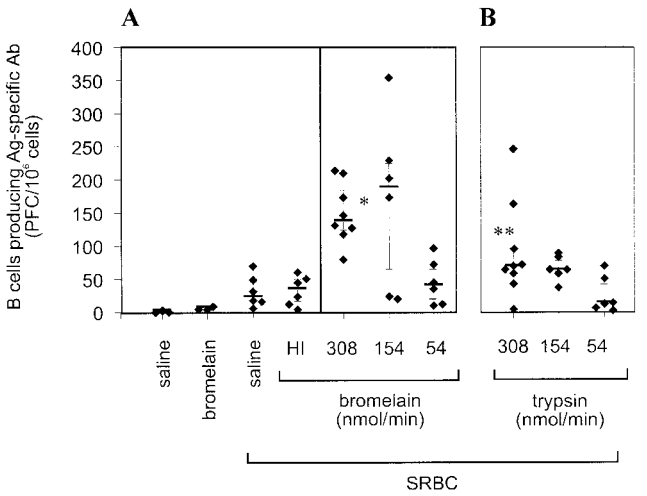

FIG. 5. Bromelain dose-dependently increases the frequency of antigen-specific B cells *in vivo.* Mice ($n = 6$ or 9) were administered a single injection (iv) of (A) bromelain (35, 100, 200 μg/200 μl; sp. act. 54, 154, 308 nmol/min/mg, respectively), heat-inactivated bromelain (200 μg/200 μl; sp. act., <5 nmol/min/mg), or saline and then immunized (ip) with SRBC (1×10^7 cells), as indicated. (B) Mice were administered trypsin at the same sp. act. (54, 154, or 308 nmol/min/mg) as bromelain. Data represent the median plus the interquartile range of the number of plaque-forming cells (PFCs) in assays performed in triplicate for each mouse. The frequencies of antigen-specific B cells formed in trypsin and bromelain-treated animals are significantly different from controls (*$P < 0.0005$, **$P < 0.003$; ANOVA).

0.0005, ANOVA). No PFCs were observed in samples obtained from naive mice or those immunized with saline alone (SRBC diluent). Also, bromelain alone did not induce plaque formation. No PFCs were observed in plaque assays when MRBCs were used instead of SRBCs, confirming that the increase in PFCs is an antigen-specific response.

To confirm that the bromelain-induced increase in the frequency of antigen-specific B cells was not caused by potentially contaminating endotoxin, mice were treated (200 μg) with heat-inactivated bromelain (200 μg/200 μl). Data in Fig. 5 show that heat inactivation of bromelain completely abrogates its immunostimulatory effect, indicating that potentially contaminating endotoxin is not responsible for its immunostimulatory effect. These data also suggest that bromelain's *in vivo* immunostimulatory activity is dependent on its proteolytic activity.

Earlier we showed that trypsin, a serine protease that shares common cleavage sites to bromelain, could stimulate splenocyte proliferation *in vitro.* Therefore, we next investigated whether the stimulatory effect of bromelain on Ag-specific antibody production *in vivo* was bromelain specific or caused by nonspecific proteolysis. Mice were treated with proteolytically equivalent amounts of trypsin (sp. act. of 308, 154, and 84 nmol/min/200 μl) and then immunized with SRBC as described above. Data in Fig. 5 show that trypsin does increase the frequency of antigen-specific B cells ($P < 0.003$, ANOVA) when compared to controls, but its immunostimulatory activity is much less than that of bromelain. These data indicate that SRBC-specific Ab production does not simply correlate with proteolytic activity in enzyme preparations.

In the above studies, bromelain was administered to mice at the same time as they were immunized with antigen (i.e., at day 0). Given that bromelain has a half-life in plasma of 6 to 9 h (19), we wished to determine whether bromelain could either precondition the immune system or modify an ongoing response. Bromelain (200 μg/200 μl; sp. act., 308 nmol/min) was administered 3 days before SRBC immunization (d −3) or 3 days postimmunization (d +3). In addition, multiple doses of bromelain were also administered (d −3, d0; d −3, d +3; or d −3, d0, d +3) to determine whether we could increase the frequency of antigen-specific B cells. Data in Fig. 6 show that bromelain administered 3 days before or after immunization still increases the frequency of antigen-specific B cells when compared to control treated mice. Multiple doses of bromelain did not improve Ab responses compared to a single dose. Together, these data suggest that the immune system can be preconditioned by bromelain to generate enhanced T-cell-dependent Ab responses and can also modulate ongoing immune responses.

Bromelain Decreases IL-2 Production in Vivo

In experiments described above, we showed that bromelain could enhance B cell responses (Fig. 3) and, simultaneously, inhibit IL-2 production by T cells (Figs. 1B and 2B) *in vitro.* Also, we showed that bro-

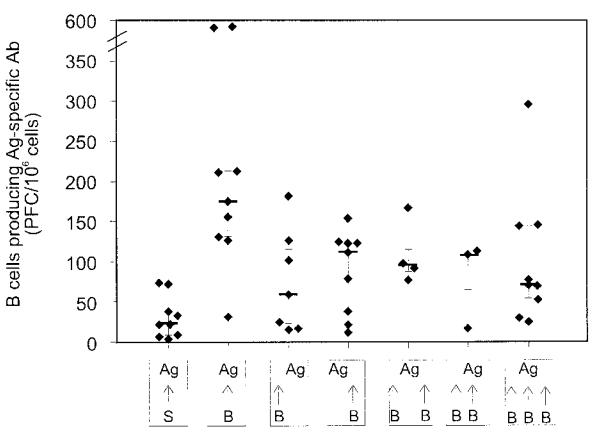

FIG. 6. Comparison of different bromelain treatment regimes on the number of antigen-specific B cells *in vivo.* Mice were administered (iv) bromelain (B) (200 μg) or saline (S) 3 days prior to immunization with SRBC (Ag), the day of SRBC immunization, or 3 days after SRBC. Data represent the median plus the interquartile range of the number of plaque-forming cells (PFCs; number of B cells producing Ab against SRBC) in assays performed in triplicate for each mouse. The number of plaques formed is significantly different from controls ($P < 0.05$, independent t test).

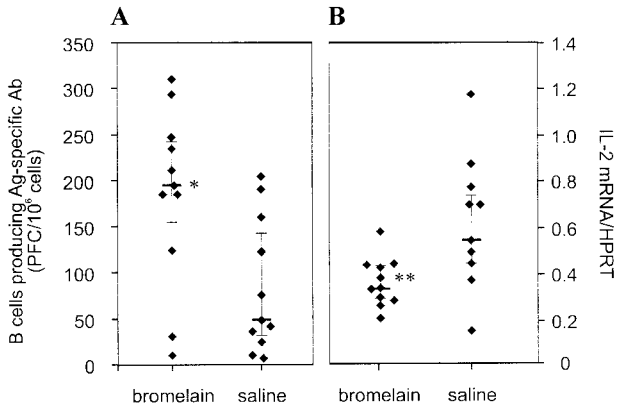

FIG. 7. Bromelain inhibits IL-2 production *in vivo.* Mice (n = 11) were administered a single injection (iv) of bromelain (200 μg/200 μl; sp. act. 308 nmol/min) or saline and then immunized (ip) with SRBC (1×10^7 cells). (A) Data represent the median plus the interquartile range of the number of PFCs in assays performed in triplicate for each mouse. The frequency of antigen-specific B cells formed in bromelain-treated animals is significantly different from controls (*$P < 0.009$, independent t test). (B) IL-2 mRNA accumulation in splenocytes was measured using a semi-quantitative RT-PCR assay and standardized with the hypoxantine-quaninephosphoribosyl transferase (HPRT) housekeeping gene. The intensity of signals was quantitated by densitometry of bands revealed by chemiluminescence. Data represent the median plus the interquartile range of mRNA/HPRT accumulation in assays performed in duplicate for each mouse. **$P < 0.007$, independent t test.

melain can enhance B cell responses *in vivo* (Figs. 5 and 6). Therefore, to examine whether bromelain could, in addition, inhibit IL-2 production *in vivo,* we measured cytokine mRNA levels in mice immunized with SRBC. Results in Fig. 7A show that bromelain, as shown earlier, significantly ($n = 11$, $P < 0.007$, independent t test) increases the frequency of antigen-specific B cells. In the same animals, however, bromelain significantly reduces IL-2 mRNA accumulation in mice immunized with SRBC ($n = 11$, $P < 0.009$, independent t test, Fig. 7B). These data show that bromelain can increase the frequency of antigen-specific B cells and, simultaneously, inhibit IL-2 production *in vivo.* These data also show that bromelain can inhibit antigen-induced IL-2 production *in vivo* as well as anti-CD3ϵ (TCR)-induced IL-2 production *in vitro.*

DISCUSSION

The modulation of immune responses has become a principal aim of many therapeutic and vaccine strategies against disease. Recently, attention has focused on extracellular proteases with immunomodulatory properties. In particular, both cysteine and serine proteases have been identified that stimulate proliferation, apoptosis, or cytokine production by immune effector cells (reviewed in 20). In this study, we show that bromelain, a mixture of cysteine proteases from pineapple stems, can simultaneously inhibit and stimulate immune responses in different cell populations.

Bromelain increased T cell proliferation in splenocytes but did not affect proliferation of highly purified $CD4^+$ or $CD8^+$ T cells. In fact, bromelain reduced IL-2 production by both splenocytes and $CD4^+$ T cells. This finding is consistent with our previous data that showed that bromelain inhibits ERK-2 activation and IL-2 production in a T cell hybridoma following stimulation with phorbol ester and calcium ionophore (8). When the T cell hybridoma was stimulated via the TCR with anti-CD3ϵ mAbs, however, bromelain reduced ERK-2 activation, but did not affect IL-2 production. Given that bromelain blocks anti-CD3ϵ-mediated IL-2 production in murine splenic $CD4^+$ T cells, but not ERK-2 activation (Mynott, manuscript submitted), these data suggest that IL-2 production can occur in an ERK-2-independent manner in the T cell hybridoma and in primary T cells. We found no evidence that the bromelain-induced reduction in IL-2 production was a result of removal of any specific cell surface receptor. Expression of CD4, CD3ϵ, or CD28 molecules on splenocytes or $CD4^+$ T cells was not reduced following bromelain treatment; in fact, expression of these molecules was increased following bromelain treatment. Despite bromelain marginally removing CD8 molecules, it did not affect the proliferation of highly purified $CD8^+$ T cells.

Bromelain increased the T cell costimulatory activity of splenocytes and purified B cells. This appeared to compensate for the reduction in IL-2 production in bulk splenocyte cultures treated with bromelain, resulting in enhanced T cell proliferation. Bromelain did not change levels of the major costimulatory molecules CD80 or CD86. In addition, MHC class II expression was unaffected by bromelain treatment, suggesting that bromelain did not enhance signaling via these molecules. There are several possible explanations for the enhanced T cell costimulatory activity of bromelain-treated cells. First, bromelain could be cleaving an as yet unidentified negative regulatory molecule. A number of molecules with these properties, such as CTLA4 (21) and CD95 (fas) (22), have been identified. However, as yet we have no evidence that bromelain affects their expression on the surface of T cells. Second, bromelain may display proliferative activity via an interaction with a proteinase activated receptor (PAR). Trypsin and thrombin induce cell proliferation via proteolytic cleavage and then activation of PAR-2, and PAR-1 and PAR-3, respectively (20, 23). Studies to determine whether bromelain has any such activity are underway. Third, bromelain may alter cell signals to increase costimulatory activity. Our previous finding that the ERK-2 pathway in T cells is blocked following bromelain treatment indicates that this compound is capable of these activities (8). Finally, bromelain might increase T cell proliferation by removing large cell sur-

face molecules, such as CD44 and CD43, and thus removing a potential physical constraint on the interaction of TCR with MHC molecules. The TCR and peptide–MHC are very small (approx. 7 nm) when compared to other cell surface molecules such as CD43 (approx. 43 nm), CD45 (approx. 28–50 nm), and integrins (approx. 25 nm) (24). Therefore, proteolytic cleavage of CD44, CD62-L (7), and CD43 (25) and other as yet unidentified molecules by bromelain, plus the increased surface expression of CD3 and CD28, might contribute to increased TCR–MHC and CD28/CD80/CD86 interactions and thus increased immune responses. Interestingly, trypsin also induced similar proliferative responses *in vitro* and increased cell surface expression of CD3 and CD28 T cell surface markers. However, trypsin did not enhance similar antibody responses *in vivo* as bromelain. These data indicate that the mechanisms responsible for inducing immunostimulatory effects *in vitro* may be different from those operating *in vivo.* All of the above possibilities are not mutually exclusive and all may contribute to immunomodulation mediated by bromelain.

The enhanced antibody response generated in mice treated with bromelain and immunized with SRBC indicates that the negative effect on T cell IL-2 production can be overcome by positive effects on costimulatory activity *in vivo.* However, we cannot rule out the possibility that bromelain may have been acting directly on B cells to improve SRBC-specific Ab production. Nevertheless, bromelain would seem capable of both enhancing and inhibiting T-cell-dependent immune responses *in vivo.* Indeed, a recent study in a murine model of EAE found that treatment with a compound containing bromelain prevented the onset of this T-cell-mediated autoimmune disease (9). Similarly, we have recently found that bromelain inhibited T-cell-dependent IgG production in mice immunized with ovalbumin and KLH administered in Freund's complete adjuvant (Mynott, unpublished data). Therefore, the outcome of bromelain treatment *in vivo* may depend on the particular immune environment into which it is administered.

In summary, we have shown that bromelain can simultaneously stimulate and inhibit the immune system. Bromelain comprises several different proteases (26) and preliminary testing of these proteases indicate that the specific immunostimulatory and inhibitory effects of bromelain are attributed to distinct proteases (Mynott *et al.*, manuscript in preparation). Characterization of these proteases responsible for the particular immunomodulatory activity will allow testing in specific disease situations. Elucidation of the physiological function of exogenous proteases *in vivo* may lead to the development of new adjuvants, immunosuppressive agents, or therapeutic tools.

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REFERENCES

1. Chambers, C. A., and Allison, J. P., Co-stimulation in T cell responses. *Curr. Opin. Immunol.* **9,** 396–404, 1997.
2. Lenschow, D. J., Walunas, T. L., and Bluestone, J. A., CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* **14,** 233–258, 1996.
3. Hutloff, A., Dittrich, A. M., Beier, K. C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczek, R. A., ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* **397,** 263–266, 1999.
4. Yoshinaga, S. K., Whoriskey, J. S., Khare, S. D., Sarmiento, U., Guo, J., Horan, T., Shih, G., Zhang, M., Coccia, M. A., Kohno, T., Tafuri-Bladt, A., Brankow, D., Campbell, P., Chang, D., Chiu, L., Dai, T., Duncan, G., Elliott, G. S., Hui, A., McCabe, S. M., Scully, S., Shahinian, A., Shaklee, C. L., Van, G., Mak, T. W., *et al.*, T-cell co-stimulation through B7RP-1 and ICOS. *Nature* **402,** 827–832, 1999.
5. Hale, L. P., and Haynes, B. F., Bromelain treatment of human T cells removes CD44, CD45RA, E2/MIC2, CD6, CD7, CD8, and Leu 8/LAM1 surface molecules and markedly enhances CD2-mediated T cell activation. *J. Immunol.* **149,** 3809–3816, 1992.
6. Desser, L., Rehberger, A., Kokron, E., and Paukovits, W., Cytokine synthesis in human peripheral blood mononuclear cells after oral administration of polyenzyme preparations. *Oncology* **50,** 403–407, 1993.
7. Kleef, R., Delohery, T. M., and Bovbjerg, D. H., Selective modulation of cell adhesion molecules on lymphocytes by bromelain protease 5. *Pathobiology* **64,** 339–346, 1996.
8. Mynott, T. L., Ladhams, A., Scarmato, P., and Engwerda, C. R., Bromelain, from pineapple stems, proteolytically blocks activation of extracellular regulated kinase-2 in T cells. *J. Immunol.* **163,** 2568–2575, 1999.
9. Targoni, O. S., Tary-Lehmann, M., and Lehmann, P. V., Prevention of murine EAE by oral hydrolytic enzyme treatment. *J. Autoimmun.* **12,** 191–198, 1999.
10. Desser, L., Rehberger, A., and Paukovits, W., Proteolytic enzymes and amylase induce cytokine production in human peripheral blood mononuclear cells in vitro. *Cancer Biother.* **9,** 253–263, 1994.
11. Abe, R., Vandenberghe, P., Craighead, N., Smoot, D. S., Lee, K. P., and June, C. H., Distinct signal transduction in mouse CD4+ and CD8+ splenic T cells after CD28 receptor ligation. *J. Immunol.* **154,** 985–997, 1995.
12. Leo, O., Foo, M., Sachs, D. H., Samelson, L. E., and Bluestone, J. A., Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA* **84,** 1374–1378, 1987.
13. Razi-Wolf, Z., Freeman, G. J., Galvin, F., Benacerraf, B., Nadler, L., and Reiser, H., Expression and function of the murine B7 antigen, the major costimulatory molecule expressed by peritoneal exudate cells. *Proc. Natl. Acad. Sci. USA* **89,** 4210–4214, 1992.
14. Napper, A. D., Bennett, S. P., Borowski, M., Holdridge, M. B., Leonard, M. J., Rogers, E. E., Duan, Y., Laursen, R. A., Reinhold, B., and Shames, S. L., Purification and characterization of multiple forms of the pineapple-stem-derived cysteine proteinases ananain and comosain. *Biochem. J.* **301,** 727–735, 1994.

15. Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A., T cell growth factor: Parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027–2032, 1978.
16. Engwerda, C. R., Smelt, S. C., and Kaye, P. M., An in vivo analysis of cytokine production during Leishmania donovani infection in scid mice. *Exp. Parasitol.* **84,** 195–202, 1996.
17. Tomasulo, P. A., Levin, J., Murphy, P. A., and Winkelstein, J. A., Biological activities of tritiated endotoxins: Correlation of the Limulus lysate assay with rabbit pyrogen and complement-activation assays for endotoxin. *J. Lab. Clin. Med.* **89,** 308–315, 1977.
18. Hudson, L., and Hay, F. C., "Enumeration of Antigen-Specific Plasma Cells," 3rd ed., pp. 140–141, Blackwell Sci., Oxford, 1989.
19. Castell, J. V., Friedrich, G., Kuhn, C. S., and Poppe, G. E., Intestinal absorption of undegraded proteins in men: Presence of bromelain in plasma after oral intake. *Am. J. Physiol.* **273,** 139–146, 1997.
20. Altieri, D. C., Proteases and protease receptors in modulation of leukocyte effector functions. *J. Leukocyte Biol.* **58,** 120–127, 1995.
21. Leach, D. R., Krummel, M. F., and Allison, J. P., Enhancement of antitumor immunity by CTLA-4 blockade. *Science* **271,** 1734–1736, 1996.
22. Trauth, B. C., Klas, C., Peters, A. M., Matzku, S., Moller, P., Falk, W., Debatin, K. M., and Krammer, P. H., Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* **245,** 301–305, 1989.
23. Dery, O., Corvera, C. U., Steinhoff, M., and Bunnett, N. W., Proteinase-activated receptors: Novel mechanisms of signaling by serine proteases. *Am. J. Physiol.* **274,** C1429–C1452, 1998.
24. Van der Merwe, P. A., Davis, S. J., Shaw, A. S., and Dustin, M. L., Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition. *Semin. Immunol.* **12,** 5–21, 2000.
25. Weber, S., Babina, M., Hermann, B., and Henz, B. M., Leukosialin (CD43) is proteolytically cleaved from stimulated HMC-1 cells. *Immunobiology* **197,** 82–96, 1997.
26. Rowan, A. D., Buttle, D. J., and Barrett, A. J., The cysteine proteinases of the pineapple plant. *Biochem. J.* **266,** 869–875, 1990.