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Immunomodulatory activity of resveratrol: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production

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Abstract

trans-Resveratrol, a phytoalexin found in grapes, wine, and other plant products, has been shown to have anti-inflammatory, antioxidant, and antitumor activities. Many of these beneficial effects of resveratrol require participation of the cells of the immune system; however, the effect of resveratrol on the development of immunological responses remains unknown. We have investigated the effect of resveratrol on mitogen/antigen-induced proliferation of splenic lymphocytes, induction of cytotoxic T lymphocytes (CTLs) and lymphokine activated killer (LAK) cells, and the production of the cytokines interferon (IFN)-γ, interleukin (IL)-2, tumor necrosis factor (TNF)-α, and IL-12. We found that mitogen-, IL-2-, or alloantigen-induced proliferation of splenic lymphocytes and the development of antigen-specific CTLs were suppressed significantly at 25–50 μM resveratrol. The generation of LAK cells at similar concentrations was less sensitive to the suppressive effect of resveratrol. The suppression of cell proliferation and CTL generation by resveratrol was not only reversible, but in some cases the response (mitogen/IL-2-induced proliferation and CTL generation) was actually enhanced following pretreatment of cells with resveratrol. Resveratrol also inhibited the production of IFN-γ and IL-2 by splenic lymphocytes, and the production of TNF-α and IL-12 by peritoneal macrophages. The inhibition of cytokine production by resveratrol was irreversible. Further, resveratrol blocked the activation of the transcription factor NF-κB without affecting basal NF-κB activity. The latter result suggests that resveratrol inhibits cell proliferation, cell-mediated cytotoxicity, and cytokine production, at least in part through the inhibition of NF-κB activation.

Keywords: Resveratrol; Immuno-modulation; Lymphocyte proliferation; Cell-mediated cytotoxicity; Cytokines

1. Introduction

Epidemiological and animal studies have demonstrated that plant-derived dietary constituents of food play an important role in the prevention of disease [1,2]. A number of food components that inhibit the initiation and progression of cancer or otherwise influence the potential for disease outcome have been identified [3–5]. The beneficial effects of these dietary compounds have been attributed partly to the presence in food materials of numerous polyphenolic compounds with antioxidant and free radical scavenging properties [6,7]. This conclusion is best supported by epidemiological studies showing a close association between low incidence of coronary heart disease and breast cancer [8–10] and moderate consumption of red wine containing natural polyphenolic compounds.

Resveratrol is a non-flavonoid polyphenolic compound found in grapes and the red wine prepared from them. Resveratrol has been shown to modulate lipoprotein metabolism [11,12], eicosanoid synthesis [13–15], lipid oxidation [16], and platelet aggregation [13,17]. Resveratrol also suppresses the induction of nitric oxide synthase and disrupts arachidonic acid metabolism by inhibiting cyclooxygenase-2 [18,19]. Recently, resveratrol was shown to inhibit cellular processes associated with tumor initiation, promotion, and progression *in vivo*, and the development of pre-

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Abbreviations: CTLs, cytotoxic T lymphocytes; LAK cells, lymphokine activated killer cells; IL-2, interleukin-2; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; NF-κB, nuclear factor kappa B; Con A, concanavalin A; HBSS, Hanks' balanced salt solution; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; RT–PCR, reverse transcription–polymerase chain reaction; LPS, lipopolysaccharide; and EMSA, electrophoretic mobility shift assay.

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neoplastic lesions in mouse mammary glands *in vitro* [20]. In other studies, resveratrol inhibited the proliferation of breast, oral, liver, prostate, and colon cancer cell lines in a dose- and time-dependent manner [21–24]. Resveratrol has also shown cardioprotective and neuroprotective activity in rats [25,26].

We have reported previously that although resveratrol inhibits the growth of both normal hematopoietic progenitor cells and leukemia cell lines in a dose-related manner, the anti-proliferative effect of resveratrol on normal hematopoietic progenitor cells is less dramatic and reversible compared with that on leukemia cells [27]. Resveratrol induced apoptosis in leukemia cells, but not in normal hematopoietic cells. Additionally, hematopoietic progenitor cells treated with resveratrol fully maintained their capacity to reconstitute lethally irradiated mice hematologically. The antiproliferative and anti-inflammatory activity of resveratrol has been well documented in many studies, but its effect on the development of cellular immune responses and the production of cytokines has not been established. In the present study, we examined the effect of resveratrol on mitogen/antigen-induced proliferation of splenic lymphoid cells, the development of cell-mediated cytotoxicity, and the production of cytokines by lymphocytes and macrophages.

2. Materials and methods

2.1. Agents

Trans-resveratrol (*trans*-3,4′,5-trihydroxy stilbene), mouse interleukin-2 (mIL-2) (2.5×10^8 U/mg), and Con A were purchased from the Sigma Chemical Co. A 100 mM solution of resveratrol was prepared in DMSO, and all test concentrations were prepared by diluting the appropriate amount of stock solution in tissue culture medium.

2.2. Mice

Eight- to ten-week-old male C3H (H-2^k) and C57BL/6 (H-2^b) mice were purchased from the Taconic Laboratories. Mice were maintained in the Bioresource Facility of the Henry Ford Health System. All animals were given mouse chow and acidified water *ad lib.* Mice were housed for at least 1 week before experimental use, and age-matched animals were used within any given experiment.

2.3. Tissue culture medium

All *in vitro* cell cultures were carried out in RPMI-1640 medium (Grand Island Biological Co.), supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin/streptomycin, 25 mM HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol. Hereafter, this medium will be referred to as complete RPMI-1640 medium.

2.4. Preparation of spleen cells

Mice were killed by CO_2 inhalation, and spleens were removed aseptically. Spleens were placed in cold HBSS and teased apart with a pair of forceps and a needle. A single-cell suspension from the teased tissue was obtained by passing it through a 20-gauge needle. Cells were washed two times in cold HBSS and finally resuspended in complete RPMI-1640 medium. Cell viability was determined by trypan blue dye exclusion.

2.5. [^{3}H]thymidine incorporation assay

To determine the effect of resveratrol on the proliferation of lymphocytes, 5×10^6 spleen cells were cultured in 5 mL RPMI-1640 in a 25-cm^2 tissue culture flask in the absence or presence of Con A (1.5 μg/mL) or mIL-2 (150 ng/mL) or allogeneic spleen cells (1:1 ratio) as a stimulator. Resveratrol was added to the cultures in concentrations as described in the individual experiments. After incubation for 4 days at 37^o in 95% humidity and 5% CO_2, cells were washed once with cold PBS and resuspended in RPMI-1640 at 2×10^6 cells/mL. A cell suspension of 0.1 mL was added to each well of a 96-well microtiter tissue culture plate in triplicate; 0.25 μCi of [^{3}H]thymidine (ICN Pharmaceuticals; sp. act. 74 Ci/mmol) in 20 μL of HBSS was added to each well, and the plate was incubated for an additional 8 hr. Cultures were harvested with an automatic cell harvester using distilled water. The amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer.

2.6. In vitro generation of cytotoxic cells

2.6.1. CTLs

For the generation of alloantigen-specific CTLs, 10^7 responder spleen cells (C3H) (H-2^k) and an equal number of irradiated (20 Gy) C57BL/6 (H-2^b) spleen cells (stimulators) were cultured in 10 mL of complete RPMI-1640 tissue culture medium. After incubation for 5 days, cells were harvested, and viability was determined by trypan blue dye exclusion. Cells were tested for cytotoxicity against ^{51}Cr-(ICN Pharmaceuticals) labeled C57BL/6 splenic blast cells in a 4-hr ^{51}Cr-release assay.

2.6.2. LAK cells

For the generation of nonspecific LAK cells, C3H splenic cells at a concentration of 5×10^6/mL were cultured in RPMI-1640 medium containing IL-2 (200 ng/mL). After incubation for 72 hr, cells were harvested and tested for cytotoxicity against 32Dp210 leukemia cells of C3H origin in a 4-hr ^{51}Cr-release assay.

2.6.3. Cytotoxicity assay

Target cells were resuspended in RPMI-1640 medium at 1×10^7 cells/mL, and 150 μCi of sodium ^{51}chromate was added to the cells. Cells were incubated for 90 min at 37^o

with intermittent shaking. Following incubation, cells were washed three times in PBS to remove unbound radioactivity. The effector and labeled target cells (100 μL each) were added to wells of a U-bottomed 96-well microtiter plate in duplicate to obtain effector:target (E:T) ratios of 100:1 to 10:1. For maximum release of radioactivity, target cells were lysed in 200 μL of 1% SDS solution. For spontaneous release of radioactivity, target cells were incubated in 200 μL of medium alone. Plates were centrifuged at 150 *g* for 2 min at 25° and incubated at 37° for 4 hr. One hundred microliters of the supernatant from each well was removed to measure the amount of radioactivity. Percent cytotoxicity was calculated by the formula:

$$\text{Percent cytotoxicity} = \frac{\text{Exp. release (cpm)} - \text{Spon. release (cpm)}}{\text{Max. release (cpm)} - \text{Spon. release (cpm)}} \times 100$$

2.7. Production of cell supernatants for cytokine determination

2.7.1. Spleen cell supernatant

Spleen cells (2.5×10^6/mL of RPMI-1640) were treated with Con A (1 μg/mL) in the absence or presence of 50 μM resveratrol for 20 hr. In separate cultures, spleen cells were first treated with 50 μM resveratrol for 20 hr and then washed three times with PBS. Cells were then treated with Con A (1 μg/mL) for 20 hr, and the supernatant was collected by centrifugation. The concentrations of mIL-2 and mIFN-γ in culture supernatants were determined by using commercially available cytokine-specific ELISA kits (Bioresource International).

2.7.2. Macrophage supernatant

Thioglycolate-induced peritoneal exudate cells (1×10^6) were plated in 16-mm Petri dishes and allowed to adhere to the plastic surface for 1 hr. Cells were treated with LPS (500 ng/mL) for 20 hr in the absence or presence of resveratrol (50 μM). In separate dishes, cells were pretreated with resveratrol (50 μM) for 20 hr before stimulation with LPS (500 ng/mL) for 20 hr in the absence of resveratrol. Culture supernatants were collected by centrifugation (500 *g* for 10 min at 25°) and analyzed for mTNF-α and IL-12 (p40) by ELISA.

2.8. RT–PCR

Total cellular RNA was extracted from cells with the TRIzol Reagent (GIBCO) according to the recommendation of the manufacturer. Then 5 μg of RNA was reverse transcribed by using random primers (Boehringer Mannheim) and reverse transcriptase to generate cDNA. Using gene-specific primers for mIL-2 (upstream, 5′-GTCACATTGACACTTGTCGTCC-3′; downstream, 5′-AGTCAAATCCAGAACATGCCG-3′), 1 μg of cDNA was amplified by PCR for 35 cycles of denaturation (94° for 1 min), annealing (61° for 1 min), and polymerization (72° for 2 min). The PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The primers amplified DNA fragments of 294 bp.

2.9. EMSA

Nuclear extracts were prepared by the modified procedure of Dignam *et al.* [28]. Following treatment, cells were washed three times with PBS, resuspended, and incubated on ice for 15 min in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% NP-40). Cells were vortexed gently for lysis, and nuclei were separated from cytosolic components by centrifugation at 12,000 *g* for 1 min at 25°. Nuclei were resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and shaken for 30 min at 4°. Nuclear extracts were obtained by centrifugation at 12,000 *g* for 10 min at 25°. Protein concentration was measured by the Bradford assay (Bio-Rad).

For binding reactions, nuclear extracts (10 μg of protein) were incubated in a 25-μL total reaction volume containing 20 μM HEPES, pH 7.9, 50–80 mM NaCl, 0.1 mM EDTA, 1 mM DDT, 8% glycerol, and 2.55 μg/mL of poly(dl-dC) (Pharmacia). Double-stranded radiolabeled NF-κB oligonucleotide probe (5′-CGCTTGATGAGTCAGCCGGAA-3′) was added to the mixture after preincubation for 15 min at 4°, and the reaction mixture was then incubated for 20 min at room temperature. Samples were loaded on 6% polyacrylamide gels in low-ionic-strength 0.25× TBE buffer (22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA) and run at 150 V/cm with cooling. The gels were dried and analyzed by autoradiography.

3. Results

3.1. Antiproliferative effect of resveratrol

The effect of resveratrol on induced proliferation of splenic lymphocytes was examined by [^{3}H]thymidine incorporation. The desired concentration of resveratrol was incorporated in culture medium at the initiation of the cultures. The results presented in panels A–C of Fig. 1 demonstrate the effect of different concentrations of res-

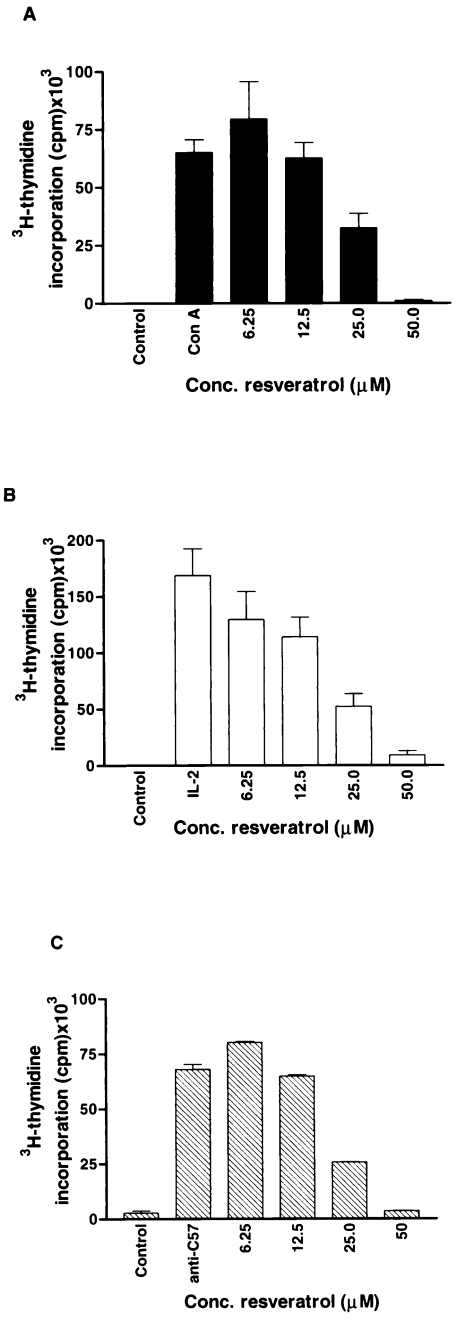

Fig. 1. Effect of resveratrol on the proliferation of spleen cells. C3H spleen cells (1×10^6/mL) were stimulated with Con A (1.5 μg/mL) (A) or IL-2 (150 ng/mL) (B) or irradiated C57BL/6 spleen cells (1:1) (C) for 4 days in the absence or presence of resveratrol (6.25 to 50 μM). Cells (2×10^5) from each culture were transferred to the wells of a 96-well microtiter tissue culture plate in triplicate. Cultures were pulsed with [^{3}H]thymidine (0.25 μCi/well) for 8 hr. [^{3}H]Thymidine incorporation was determined by liquid scintillation spectrometry. Data are presented as means ± SEM of three to four experiments.

veratrol (range, 6.25 to 50 μM) on the proliferation of splenic lymphocytes induced by Con A (A), IL-2 (B), and alloantigen (C). There was an insignificant increase (<15%) in the Con A- (Fig. 1A) and alloantigen- (Fig. 1C) induced proliferation of splenic cells following exposure to 6.25 μM resveratrol. Proliferation of spleen cells with each of the three stimuli was reduced markedly at 25 μM and inhibited almost completely at 50 μM resveratrol. The inhibition of Con A-, IL-2-, and alloantigen-induced proliferation of cells was concentration-dependent, since an increasing level of suppression was observed at increasing concentrations of resveratrol. Inhibition of IL-2 induced cell proliferation was observed at all resveratrol concentrations used, whereas alloantigen- and Con A-induced proliferation was suppressed only at the higher resveratrol concentrations, i.e. 25 and 50 μM (from 52 to 98%). These data demonstrate that resveratrol markedly inhibits mitogen-, cytokine-, and alloantigen-induced proliferation of splenic lymphocytes at concentrations of 25–50 μM.

3.2. Effect of resvertrol on the development of cell-mediated cytotoxicity

The inhibition of proliferation of splenic lymphocytes by resveratrol suggests that this compound may also affect the generation of cell-mediated cytotoxic responses. To test this, we examined the effect of resveratrol on the production of antigen-specific cytotoxic T lymphocytes (CTLs) and IL-2-induced non-specific LAK cells. For alloantigen-induced CTLs, spleen cells from the C3H strain of mice (H-2^k) were stimulated with the spleen cells (irradiated) of C57BL/6 (H-2^b) mice for 5 days in the absence and presence of resveratrol. The cytolytic activity of effector cells against Con A-induced C57BL/6 lymphoblast target cells was determined in a 4-hr ^{51}Cr-release assay. In a concentration–response study, we found no effect of resveratrol on the generation of CTLs at 6.25 or 12.5 μM (Fig. 2A). The response was ~50% suppressed at 25 μM resveratrol, and it was almost completely abrogated at a concentration of 50 μM (Fig. 2A). To investigate the effect of resveratrol on the development of non-specific cytotoxic cells, LAK cells were generated by culturing spleen cells with IL-2 in the absence and presence of various concentrations of resveratrol. The cytotoxic activity of LAK cells was determined in a 4-hr ^{51}Cr-release assay using 32Dp210 mouse leukemia target cells. The effect of resveratrol on the development of LAK cell-mediated cytotoxicity was insignificant at concentrations ranging from 6.25 to 25 μM (Fig. 2B). At 50 μM resveratrol, LAK cell production was 40–50% inhibited. These data indicate that the generation of LAK cells is less sensitive than the generation of CTLs to suppression by resveratrol.

3.3. Reversibility of the effect of resveratrol

To investigate whether the inhibitory effect of resveratrol on the proliferation of lymphocytes and the production of CTLs is reversible, spleen cells were either pretreated with or continuously exposed to resveratrol before induction of proliferation. For the pretreated, spleen cells were exposed to 50 μM resveratrol for 20 hr and then washed three times

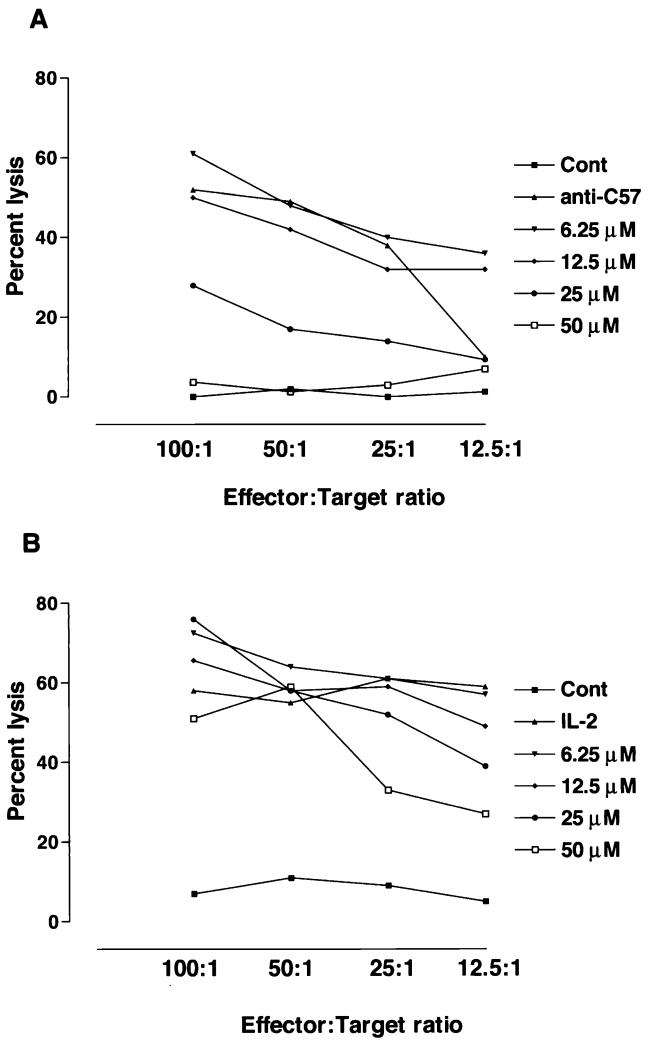

Fig. 2. Effect of resveratrol on the development of cell-mediated cytotoxicity. To determine the effect of resveratrol on the generation of CTLs, 10^7 C3H spleen cells were co-cultured with an equal number of irradiated C57BL/6 spleen cells for 5 days in the absence or presence of resveratrol (6.25 to 50 μM). Cytotoxicity of the effector against C57BL/6 splenic blast cells was determined in a 4-hr ^{51}Cr-release assay (A). The effect of resveratrol on LAK cell generation was examined by incubating C3H spleen cells (5×10^5/mL) with IL-2 (200 ng/mL) for 3 days in the absence or presence of resveratrol (6.25 to 50 μM). Cytotoxicity of effector cells against 32Dp210 mouse leukemia cells was measured in a 4-hr ^{51}Cr-release assay (B). In each panel, the results from a representative experiment are presented as percent lysis at different E: T ratios. Similar results were obtained in three separate experiments.

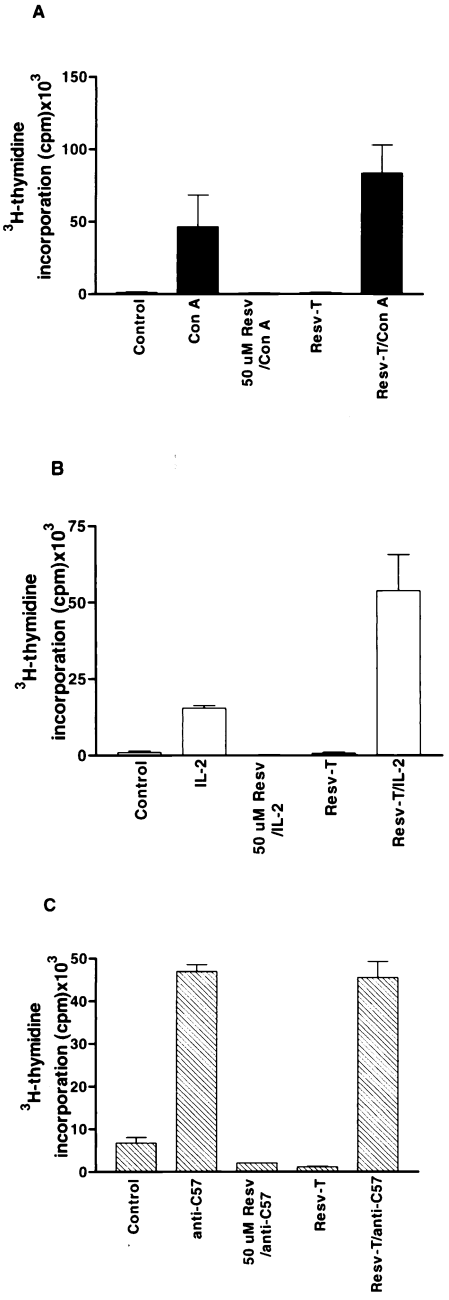

Fig. 3. Reversibility of the effect of resveratrol on splenic cell proliferation. Spleen cells (1×10^7/mL) were treated with resveratrol at 50 μM for 20 hr, washed three times in PBS (Resv-T), and then stimulated with Con A (A) or IL-2 (B) or irradiated C57BL/6 spleen cells (C) for 4 days as described in Fig. 1. In parallel, untreated cells were stimulated with the inducers in the absence or the continuous presence of 50 μM resveratrol. Proliferation of cells was determined by [^{3}H]thymidine incorporation as described. Data represent means ± SEM of three experiments.

to remove the resveratrol. Cultures were initiated for Con A-, IL-2-, or alloantigen-induced proliferation or production of CTLs against C57BL/6 cells as described earlier. Figure 3 shows that Con A- (A), IL-2- (B), and alloantigen- (C) induced spleen cell proliferation was completely suppressed by continuous exposure to 50 μM resveratrol. In contrast, when resveratrol was removed from the cultures after 20 hr of treatment, there was a complete reversal of the suppression (Fig. 3, A–C). In fact, compared to the proliferation of untreated control cells, the proliferation of spleen cells that had been pretreated with resveratrol for 20 hr was increased markedly in response to Con A and IL-2 (Fig. 3, A and B). The alloantigen-induced proliferative response in pretreated spleen cells was not enhanced compared with that of untreated cells (Fig. 3C).

Since production of LAK cells was inhibited only partially at 50 μM resveratrol, we only studied the reversibility

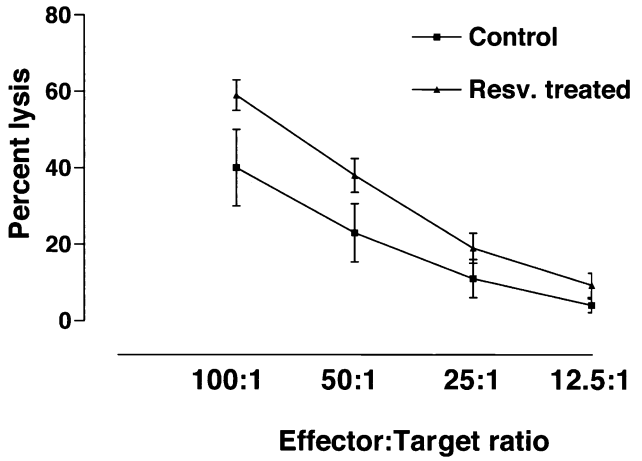

Fig. 4. Reversibility of the effect of resveratrol on CTL development. C3H spleen cells (1×10^7/mL) were treated with resveratrol at 50 μM for 20 hr. Cells were washed with PBS three times prior to stimulation with irradiated C57BL/6 spleen cells for 5 days as described in Fig. 2. The cytotoxicity of effector cells against ^{51}Cr-labeled C57BL/6 splenic blast cells was determined in a 4-hr ^{51}Cr-release assay. Data represent means ± SEM of three experiments.

of the suppression of CTL development. Consistent with the observation that the antiproliferative effect of resveratrol is reversible, data in Fig. 4 demonstrate that inhibition of the development of CTLs by resveratrol was also reversible. The cytolytic activity of effector cells generated from spleen cells pretreated with resveratrol was 10–20% higher at all E:T ratios compared with that of the untreated cells (Fig. 4). Thus, suppression of proliferative and cell-mediated cytotoxic immune responses by resveratrol is reversible.

3.4. Effect of resveratrol on the production of cytokines

To determine whether resveratrol affects cytokine production, we examined the production of IFN-γ and IL-2 by spleen cells or TNF-α and IL-12 by macrophages in the continuous presence of resveratrol or by cells that were pretreated with resveratrol for 20 hr and then washed free of it before inducing the production of cytokines. Data in Fig. 5A demonstrate that production of IFN-γ and IL-2 by spleen cells, as measured by cytokine-specific ELISA, was abolished in the presence of resveratrol. While suppression of IL-2 production was slightly reversed (~20%) following removal of resveratrol, production of IFN-γ remained completely suppressed. Production of TNF-α and IL-12 by macrophages was only partially blocked by resveratol (36 and 47%, respectively) even when present continuously in the cultures (Fig. 5B). Suppression of both of these cytokines was not reversed following the removal of resveratrol from the cultures (Fig. 5B).

3.5. Resveratrol inhibition of IL-2 mRNA expression

We examined the effect of resveratrol on Con A-induced IL-2 gene expression in spleen cells by RT–PCR. For this

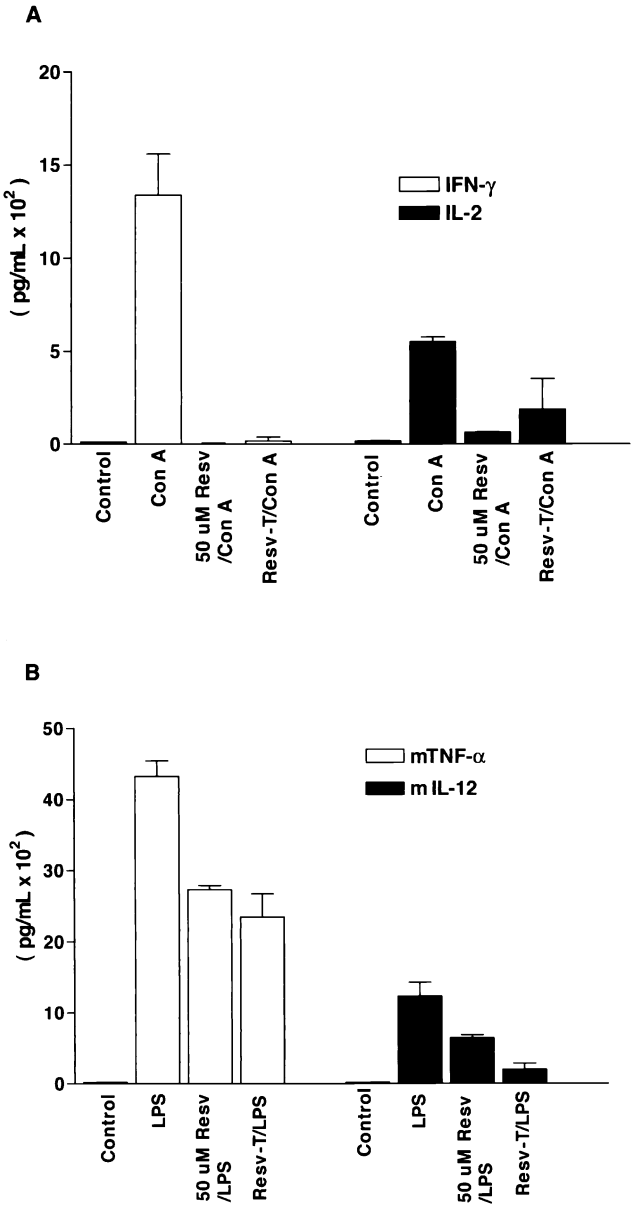

Fig. 5. Effect of resveratrol on cytokine production. C3H spleen cells (2.5×10^6/mL) or peritoneal macrophages (1×10^6 cells) were stimulated with Con A (1 μg/mL) (A) and LPS (500 ng/mL) (B), respectively, for 20 hr in the absence or presence of 50 μM resveratrol. Alternatively, cells were pretreated with resveratrol (50 μM) for 20 hr, washed with PBS three times (Resv-T), and then stimulated with mitogens for 20 hr as described. The concentrations of IFN-γ and IL-2 in spleen cell supernatants (A) or of TNF-α and IL-12 (p40) in macrophage supernatants (B) were determined using commercially available cytokine-specific ELISA kits. Data are means ± SEM of three experiments.

purpose, spleen cells were treated with resveratrol for 4 hr before stimulating them with Con A for 2 hr. Total cellular RNA isolated from these cells was reverse transcribed, amplified, and fractionated on 1% agarose DNA gels. A low level of IL-2 gene expression was detected in normal spleen cells (Fig. 6, lane 2), which increased following stimulation of cells with Con A (lane 3). IL-2 gene expression was suppressed almost completely in cells that were treated with

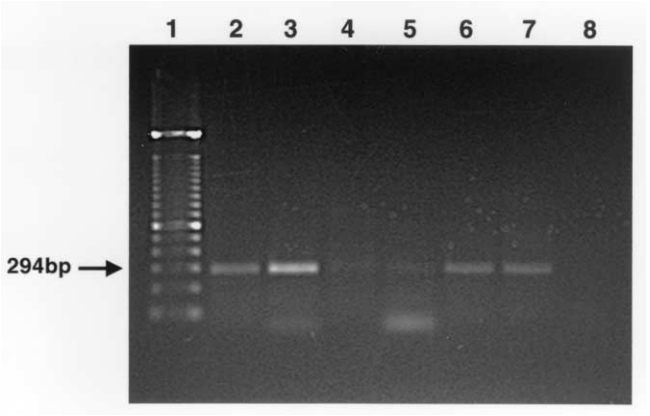

Fig. 6. Effect of resveratrol on IL-2 mRNA expression. Treatment of spleen cells with resveratrol (6.25 to 50 μM) was started 4 hr prior to stimulating the cells with Con A (1 μg/mL) for 2 hr. Total cellular RNA was isolated and reverse transcribed using random primers to generate cDNA. cDNA (1 μg) was amplified by PCR using gene-specific primers. PCR products were separated by 1% agarose gel electophoresis and visualized by ethidium bromide staining. Lane 1, DNA ladder; lane 2, normal spleen cells; lane 3, spleen cells stimulated with Con A; lanes 4–7, spleen cells stimulated with Con A (1 μg/mL) in the presence of resveratrol at 50, 25, 12.5, and 6.25 μM, respectively; lane 8, water control (no cDNA).

50 μM (lane 4) or 25 μM (lane 5) resveratrol. At 12.5 μM (lane 6) and 6.25 μM (lane 7), resveratrol suppressed only induced mRNA expression but not the constitutively expressed IL-2 mRNA. This result demonstrates that inhibition of cytokine production by resveratrol involves inhibition of cytokine gene expression.

3.6. Inhibition of NF-κB activation by resveratrol

Activation and nuclear translocation of transcription factor NF-κB are important for expression of genes involved in the development of immune and inflammatory responses. To investigate whether inhibition of various responses by resveratrol results from its interference of NF-κB activation, we measured the activation of NF-κB DNA binding activity in spleen cells treated with resveratrol for 4 hr and stimulated with Con A for 1 hr. Figure 7 demonstrates measurable NF-κB DNA binding activity in untreated cells (lane 1), which increased following stimulation of cells with Con A for 1 hr (lane 2). Treatment of cells with 50 μM resveratrol suppressed the activation of NF-κB induced by Con A (lane 3). Since pretreatment with resveratrol blocked only induced NF-κB activity, resveratrol does not appear to inhibit basal binding activity of NF-κB. This result indicates that suppression of various immune responses by resveratrol may result, at least in part, from suppression of the antigen-induced activation of NF-κB activity.

4. Discussion

Recent studies have indicated that dietary constituents provide chemoprevention against many diseases and reduce

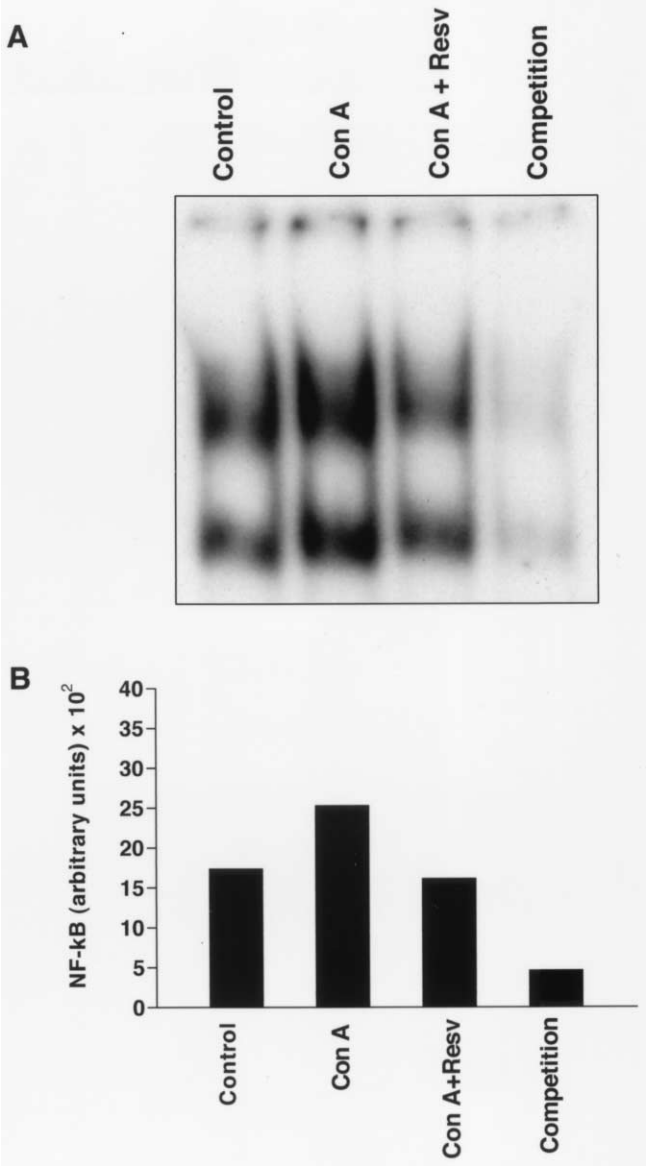

Fig. 7. Effect of resveratrol on the activation of NF-κB. Spleen cells were treated with resveratrol (50 μM) for 4 hr and then stimulated with Con A (1 μg/mL) for 1 hr. Nuclear extract was prepared, and levels of NF-κB were analyzed by EMSA using sequence-specific radiolabeled oligonucleotide (lanes 1–3) (A). The specificity of NF-κB binding was confirmed by competitive inhibition with unlabeled nucleotide (lane 4). (B) Quantitation of NF-κB activity by PhosphorImager.

the severity of others. For instance, epidemiological studies have indicated that moderate consumption of red wine can reduce mortality from coronary heart disease and breast cancer [10,29]. The beneficial effects of red wine against these conditions have been attributed to resveratrol, a polyphenolic compound present in wine [29]. In the plant kingdom, especially in grapes, resveratrol is believed to provide protection against environmental stress and plant pathogens [30]. Although numerous effects of resveratol have been described, e.g. anti-inflammatory activity and chemoprevention, whether the mechanism(s) by which resveratrol provides protection involves modulation of the immune

system is not clear. In the present study, we investigated the effect of resveratrol on the development of several lymphocytic responses *in vitro*, including mitogen/antigen-induced T cell proliferation, cell-mediated cytotoxicity, and production of cytokines. Our studies demonstrated that resveratrol generally inhibits the proliferation of spleen cells induced by Con A, IL-2, or alloantigens. The proliferation of spleen cells was affected minimally at 6.25 or 12.5 μM resveratrol; however, the proliferative response to each of the three inducers was suppressed by more than 50–95% at 25–50 μM resveratrol. Although the proliferative response induced with Con A and allogeneic cells was somewhat increased at 6.25 μM resveratrol, the increase was insignificant and could not be observed at 3.25 μM resveratrol (data not shown). The inhibition of induced proliferation of spleen cells by resveratrol corroborates its previously reported antiproliferative effects against several tumor cell lines [21–24]. The inhibition of ribonucleotide reductase and DNA polymerase activation by resveratrol, two key enzymes involved in DNA synthesis [31,32], as well as its suppression of the cell cycle [33] may account for the inhibition of mitogen/antigen-induced proliferation of spleen cells. In addition, the antiproliferative effect of resveratrol on lymphocytes could also be attributed to the antioxidant properties of resveratrol, since reactive oxygen species have been shown to have a role in the cellular response to cytokines and growth factors [34,35].

Resveratrol also inhibited the induction of cell-mediated cytotoxic responses. The production of alloantigen specific CTLs was reduced only slightly at 6.25 to 12.5 μM resveratrol, whereas at 25 and 50 μM, CTL production was reduced markedly ($>$50 and 90%, respectively). The induction of non-specific cytotoxic LAK cells by IL-2 was less sensitive to suppression by resveratrol than the suppression of CTL production. LAK cell production was reduced $\sim$50% at the highest concentration (50 μM) compared to more than 90% inhibition of CTL production at an equivalent concentration of resveratrol. The disparate effect of resveratrol on the proliferation and induction of LAK cell generation from spleen cells by IL-2 indicates that the two responses are mediated by different subpopulations of lymphoid cells.

To study whether suppression by resveratrol is reversible, spleen cells were treated with resveratrol for 20 hr, and then the drug was removed by washing the cells extensively. The results of these experiments demonstrated that suppression of lymphocyte proliferation and generation of cell-mediated cytotoxicity by resveratrol is rapidly reversible, suggesting that the molecule does not irreversibly interfere with the molecular processes required for the activation/induction of lymphocytes to generate these immune functions. In fact, while alloantigen-induced cell proliferation was not enhanced, proliferative response induced with Con A or IL-2 was enhanced, as was the production of CTLs following pretreatment of spleen cells with resveratrol. Although the mechanism by which pretreatment of cells with resveratrol enhances these responses remains to be determined, resveratrol may promote receptor–ligand interactions by increasing receptor avidity or by increasing receptor density on the cell surface of lymphocytes. Pretreatment with resveratol may also cause pharmacological preconditioning of cells for efficient signal transduction by the ligands.

Since cytokines play a prominent role in the development of immune responses, we also investigated the effect of resveratrol on the production of IFN-γ, IL-2, TNF-α, and IL-12 (p40). Resveratrol was more effective in inhibiting the production of T cell secreted cytokines IL-2 and IFN-γ, compared with the production of TNF-α or IL-12 by macrophages. Furthermore, unlike the reversible effect of resveratrol on mitogen/antigen-induced lymphocyte proliferation or the production of CTLs, inhibition of cytokine production by T cells (IL-2 and IFN-γ) or macrophages (IL-12 and TNF-α) was essentially irreversible. Although this result would appear to contradict the finding that cell proliferation and CTL generation are enhanced following pretreatment of spleen cells with resveratrol, there appears to be enough IL-2, IL-12, and TNF-α produced by pretreated cells to allow activation of these responses. Furthermore, the enhancement of responses may result from priming by resveratrol of T cell subsets that mediate cell proliferation and CTL development, but not of cells that produce cytokines.

Transcription factor NF-κB and other members of the Rel homology family of transcription factors have been shown to play a pivotal role in the transcription of genes involved in immune and inflammatory responses [36], and in cell proliferation and transformation [37,38]. We considered the possibility that suppression of lymphocyte proliferation, CTL development, and cytokine production by resveratrol may result from suppression of NF-κB activation. In resting cells, NF-κB remains sequestered in the cytoplasm in a functionally inactive form noncovalently bound to an inhibitory protein, IκB. Upon stimulation of cells with mitogens, antigens, or cytokines, IκB dissociates from the NF-κB complex, allowing NF-κB to translocate to the nucleus where it binds to κB motifs in the promoter region of the response genes. We have shown that spleen cells constitutively express low levels of activated NF-κB, which is increased further following stimulation with Con A. In the presence of resveratrol, the NF-κB activation that occurs upon stimulation with the mitogen is blocked completely; however, constitutively expressed NF-κB remains unaffected by resveratrol. Therefore, while mitogen-stimulated activation of NF-κB is suppressed by resveratrol, basal NF-κB activity in cells appears to be resistant to resveratrol. These results are consistent with those of other investigators, who have previously reported suppression of NF-κB activation by resveratrol [39,40].

In conclusion, we have demonstrated that resveratrol inhibits mitogen/antigen-induced lymphocyte proliferation, development of cell-mediated cytotoxicity, and production

of cytokines. Suppression of the activation of nuclear factor NF-κB appears to be part of the mechanism by which resveratrol inhibits the *in vitro* development of immunological responses. Animal studies are in progress to study the effect of resveratrol on the development of immunological responses *in vivo*. Based on the results of the present study, it can be suggested that perhaps the reported antitumor activity of resveratrol is due to direct cytotoxic/antiproliferative activity against tumor cells, and not to the augmentation of immune responses against tumors. However, the molecule is potentially an attractive candidate for development as a drug for immunosuppressive therapies.

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