Plant Polyphenolic-Protein Conjugates Activate Murine Spleen Cells and Bind to Multiple Cell Surface Components (43800)

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Abstract. **Previous studies have shown that a flavonoid-phenolic-rich glycoprotein (TGP) is a mitogen to murine B cells. To test the hypothesis that this could be duplicated by a protein conjugate of the phenolic moiety, the response to rutin (R)-BSA was studied. It was demonstrated that: (i) R-BSA, like TGP, activates mouse B cells to proliferate; (ii) the kinetics of proliferation induced by R-BSA and TGP are similar; (iii) the mitogenic effects of neither R-BSA nor TGP are inhibitable by free flavonoids or phenolics; (iv) there is no age-dependent decrease in the proliferative response to either R-BSA or TGP; (v) both R-BSA and TGP induce spleen cells to secrete IL-6 by 2 hr of culture. They differ in that the proliferative response of congenitally athymic mice to R-BSA, but not to TGP, is significantly lower than that of euthymic mice, and in that TGP seems to stimulate a small subpopulation of T cells to enter cell cycle.**

The importance of the phenolic moiety in the response to TGP is supported by the observation that R-BSA immunoprecipitates cell surface components that seem to be identical to those precipitated by TGP. The apparent molecular sizes of the bands are ~110, 70, 55, 43, 34, 29, and 25–23 kDa. The 2-D analyses of the TGP and R-BSA precipitates are also striking in their similarity. The isoelectric point (pI) of the 28-kDa band is between pH 6.3 to 6.6. A band at ~23 kDa has a pI of 6.0, one band at ~25 kDa has a pI of ~5.4, and five bands ranging in size from ~26 to ~110 kDa all have a pI in the pH range of 4.6 to 5.4. The presence of multiple binding sites suggests that these compounds might activate cells via multiple distinct pathways.[P.S.E.B.M. 1994, Vol 207]

The plant-derived lectins, PHA and Con A, have played a major role in the studies of T-cell activation. Another class of plant-derived products, which might turn out to be of similar importance in elucidating regulatory mechanisms of the immune response, is the flavonoids and phenols. Free and conjugated phenolics and flavones are ubiquitous in plants (1). They are crucial to plant development and growth as signal molecules (2–10), pesticides (11, 12), and constituents of plant cell wall (13). As plant hormones, flavones and phenolics stimulate cell proliferation, growth, and development, raising the possibility that they might also be activators of animal cells. Ames *et al.* have suggested that "natural pesticides" (i.e., the various phenols which are present at high concentrations in edible fruits and vegetables) might be far more important environmental mitogens and/or carcinogens than many of the man-designed molecules which have been implicated in mitogenesis and carcinogenesis (11, 14, 15).

The effects on various biological systems of a phenol-rich glycoprotein (TGP), which was isolated by Becker *et al.* (16) from tobacco leaves and cigarette smoke condensate, lend support to the idea that naturally occurring phenolics might be important activators of mammalian cells in general, and of cells of the immune network in particular. TGP, which is rich in the phenolic group rutin (R), elicits an immediate, IgE mediated, wheal and flare response in approximately one-third of healthy human volunteers (16), and stimulates a preferential, long-lasting, IgE response in ex-

Received June 8, 1993. [P.S.E.B.M. 1994, Vol 207]
Accepted May 25, 1994.

0037-9727/94/2071-0117$10.50/0
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perimental animals (17–19). A role for the phenolic moiety in the regulation of isotype expression was suggested by our observation (19) that a similar IgE anti-BSA response was elicited in mice immunized with BSA, or with R conjugated to BSA (R-BSA), but the hemagglutinating titer to BSA in R-BSA-immunized mice was significantly lower than in BSA-immunized mice. The presence of the phenolic group on BSA apparently altered the distribution of isotypes in the anti-BSA antibodies produced (19). Most tantalizing was our finding (20) that the mechanism underlying the unique IgE response to R-BSA was the preferential activation by R-BSA of Th2 cells, which produce IL-4, an enhancer of IgE production (21). This suggests that the phenol-containing compounds are distinctly different from PHA and Con A with regard to T-cell activation, in that the latter two mitogens activate both Th1 and Th2 cells.

Other biological effects of TGP in which a role for the phenolic moiety has been implicated are the stimulation of bovine arterial smooth muscle cell to proliferate (22), and the activation of the factor XII-dependent pathways (23). Using R-BSA and TGP as model systems, the present study assesses the effects of the phenolic moiety on the mouse immune system.

Materials and Methods

Mice. LAF_1 and C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Young (6–15 weeks) and old (≥18 months) C57BL/6 (B6), BALB/c, and CD-1 nu/+ and nu/nu mice were obtained from Charles River Breeding Laboratories (Kingston, NY). Congenitally athymic (nu/nu) and euthymic BALB/c mice were from Life Science (St. Petersburg, FL).

Lymphoid Cell Preparation. Single-cell suspensions of spleens and thymuses, $CD3^+$-depleted spleen cells, and peripheral blood leukocytes (PBL) were prepared as previously described (24).

Reconstitution Experiments. Congenitally athymic mice, 6–8 weeks old, were reconstituted by the grafting under the skin of thymic lobes from 4-week-old euthymic, syngeneic donors, at a ratio of one thymus per recipient.

Lymphocyte Proliferation Assays. Unless specified otherwise, cells were cultured as previously described (24). Briefly, cells were cultured in triplicates at 5×10^5 per flat-bottom microtiter well in 200 μl RPMI 1640 containing 2 m*M* L-glutamine, 1× essential amino acids, 0.1 m*M* nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Grand Island, NY), 1 m*M* Na pyruvate (M.A. Bioproducts, Walkerville, MD), 5×10^{-5} *M* 2-ME (Eastman Kodak, Rochester, NY) and 1% syngeneic serum. The cells were incubated with or without the indicated mitogens at 37°C in a humidified atmosphere of 5% CO_2 in air, the last 8 hr in the presence of 1 μCi of [^{3}H]TdR (Amersham Corp., Arlington Heights, IL). Day 0 was defined as the day on which the cultures were initiated. Data are presented as increase in counts per minute (Δcpm) which is the mean cpm of triplicate cultures in the presence of a mitogen less than the mean cpm for cultures in the absence of mitogen. For cells cultured with R-BSA, Δcpm is the mean cpm for cultures in the presence of R-BSA less the mean cpm for cells cultured with BSA.

In experiments to determine the effect of free flavones and phenols on the mitogenic response to TGP and R-BSA, the controls included spleen cells cultured in media alone or in media containing free phenols, TGP (50 μg/ml), R-BSA (100–400 μg/ml), LPS (5 μg/ml; LPS B., *E. coli* 0127:B8; Difco Lab. Detroit, Mich.), Con A (2 μg/ml; Burrough-Wellcome, Triangle Park, NC), or PHA (2 μg/ml; Sigma Chemical Co., St. Louis, MO). Experimental cultures contained a mixture of a phenol or flavone and one of the mitogens. Rutin (Sigma) was tested at 5×10^{-8} to 2×10^{-4} *M;* caffeic acid, chlorogenic acid, and naringin (Sigma) at 3×10^{-8} to 1×10^{-4} *M*. Kaempferol (Sigma), which was toxic to the spleen cells at 5×10^{-5} *M*, was assayed at 1×10^{-5} and 1×10^{-6} *M*. [^{3}H]TdR uptake by cells cultured with the mitogens alone was defined as 100% response.

Two Parameter Flow Cytometry (FCM) Analysis of Cell Surface Markers and DNA Content. Spleen cells from B6 mice, at 5×10^6 cells/ml, were cultured with media or with media containing 75 μg/ml TGP, 150 μg/ml BSA, or 150 μg/ml R-BSA. Cells were collected daily, from Day 1 through Day 4 of culture; stained with FITC-anti CD-3 (Becton Dickinson, Mountain View, CA) or FITC-goat $F(ab')_2$ anti-mouse Ig (Cappel Laboratories, West Chester, PA); washed three times with cold PBS; fixed for 10 min in ice cold 70% ethanol; washed twice with PBS; incubated for 30 min at 37°C in 1 ml PBS containing 189.6 units/ml RNAse (Sigma); and counterstained with propidium iodide (Polyscience, Warring, PA), at a final concentration of 25 μg/ml, as previously described (25). The expression of each of the surface markers was correlated with cell cycle phase, using Epics V cell sorter (Coulter Electronic Corp., Hialeah, FL).

TGP and R-BSA. R-BSA was prepared by conjugating CNBr-activated R with BSA (23). TGP was isoelectricly precipitated at pH ≤ 4.5 from a slightly alkaline (pH ~ 8) PBS extract of delipidated cured tobacco leaves, followed by gel filtration and ion exchange chromatography (16, 24, 26, 27). Optimum concentration for mitogenic effect was determined by culturing spleen cells with serial dilution of TGP (200 to 3.1 μg/ml) or R-BSA (400 to 12.5 μg/ml). For TGP it

ranged from 50 to 100 μg/ml, and for R-BSA from 80 to 200 μg/ml. TGP and R-BSA were the generous gift of Dr. Carl G. Becker (Medical College of Wisconsin, Milwaukee, WI; previously at Cornell University Medical College, New York, NY).

Reagents. BSA, R-BSA, TGP, and Con A were conjugated to CNBr-Sepharose 4B beads (Pharmacia Fine Chemicals Uppsala, Sweden) using standard procedures (28). Protein A-Sepharose 4B (SPA) was from Pharmacia, rabbit C (Low Tox) from Accurate Chemical and Scientific Corp. (Westbury, NY), and anti-CD3 was purified from ascites fluid of pristane-treated mice, bearing the 30-H-12 hybridoma, as previously described (24, 29). IL-6 ELISA with a detection limit of 15 pg/ml was from Endogen (Boston, MA).

Immunoprecipitation. Spleen cells were labeled and immunoprecipitated following published procedures (30). Briefly, to 2×10^7 spleen cells in 500 μl PBS were added 100 μl of 1 mg/ml lactoperoxidase (Sigma), 200 μl of 100 m*M* β-D-glucose, 80 μl of 0.5 *M* Na-phosphate buffer pH 7.4, 1 mCi (~20 μl) of ^{125}I as sodium salt (ICN Biomedicals, Inc., Costa Mesa, CA), and 100 μl of glucose oxidase (Sigma) at 0.025 units/μl. Following a 15-min incubation at room temperature and three washes with PBS, the cells were suspended in 1 ml of lysis buffer (150 m*M* NaCl, 50 m*M* Tris pH 8.0, 1% NP-40, 50 μg/ml PMSF), vortexed and kept on ice for 30 min. The lysate was spun in a microfuge at 14,000 RPM for 3 min; the supernatant was collected, respun, and transferred to a clean tube. The lysate was precleared by incubating 1 ml lysate with 100 μl of rabbit anti-mouse IgG (Pel Freeze) 1 hr on ice. One hundred microliters of packed Protein A Sepharose CL-4B beads, prewashed in lysis buffer, were added to the tube, and it was incubated on ice for 30 min with constant mixing. The suspension was spun at 14,000 RPM for 1 min; the supernatant was collected and incubated with a second aliquot of 100 μl prewashed Protein A Sepharose. The precleared supernatant, collected by a 3-min spin at 14,000 RPM, was divided into 100-μl aliquots to which were added 20 μl of either prewashed Sepharose beads, or of Con A-, TGP-, BSA-, or R-BSA-coupled Sepharose beads. The tubes were incubated on ice with constant mixing for 1 hr; the beads were collected, by spinning at 14,000 RPM for 2 min, and washed five times with lysis buffer. After the last wash, the supernatant was completely removed, and 50 μl of elution buffer (10 m*M* Tris, 0.01% Bromphenol Blue, 35% saturated sucrose, 100 m*M* Dithiothreathol, 2.25% SDS) was added to each pellet. The samples were incubated with constant mixing for 10 min at room temperature and were spun at 14,000 RPM for 2 min; the supernatants were transferred to clean tubes, heated at 100°C for 3 min, and subjected to 7.5% or 12% SDS-PAGE analysis on minigels (Bio-Rad). The gels were stained with Coomassie Blue R-250, dried, and autoradiographed.

Isoelectric Focusing (IEF) and 2-D Gels Analysis. Isoelectric focusing (IEF) was done in tubes as previously described (31). 2-D gels were done as described by O'Farrel (32).

Data Analysis. Data were analyzed by the Mann-Whitney test or by Student's *t* test. The square root of the Δcpm was used as a normalizing and variance stabilizing transformation for Student's *t* test analysis. Values of $P \leq 0.05$ were considered significant.

Results

Proliferative Response of Murine Lymphoid Cells to R-BSA. Spleen cells from individual LAF_1, B6, and C3H/HeJ mice were cultured with various concentrations of R-BSA, and [^{3}H]TdR uptake was analyzed daily for up to 6 days (Fig. 1). [^{3}H]TdR incorporation was observed by Day 2 of culture. The peak of the response varied from Day 3 (Fig. 1d) to Day 5 of culture (Fig. 1a and f). In some mice a secondary peak of proliferation was observed (Fig. 1b and f). The differences in the kinetics of the response were between individual mice rather than strain related (Fig. 1, LAF1 [a] vs [b]; B6 [c] vs [d]; and C3H/HeJ [e] vs [f]), and were seen in independent preparations of spleen cells from individual mice of the same strain cultured on the same day. This pattern of response differed markedly from the responses to PHA or LPS, the classical T- and B-cell mitogens, respectively (Fig. 1), but was reminiscent of the kinetics observed for TGP (24).

The kinetics of the proliferative response of PBL to R-BSA also differed from that of PHA and LPS (Fig. 2), but was similar to TGP (24). Similarly, the lack of [^{3}H]TdR uptake by thymocytes cultured with R-BSA (data not shown) was also reminiscent of the response to TGP (24). The similarity in the mitogenic responses to R-BSA and TGP suggested that the proliferative response to TGP might be associated with the phenolic moiety. This raised the possibility that free phenols and flavones might inhibit the mitogenic response of spleen cells to R-BSA and to TGP.

Free phenolics and Flavones Do Not Inhibit the Mitogenic Effects of Either TGP or R-BSA. Initially, B6 spleen cells were cultured with TGP (50 μg/ml), R-BSA (200 μg/ml), PHA (2 μg/ml) or LPS (5 μg/ml), with or without rutin at concentrations varying from 5×10^{-8} to 2×10^{-4} *M*. [^{3}H]TdR uptake was not increased in cells cultured with free rutin at any of the doses analyzed, neither did the rutin alter the response to any one of the mitogens tested. For example, in four independent experiments the proliferative responses of spleen cells cultured with TGP in the presence of 1×10^{-4} *M* rutin were 92%, 88%, 116%, and 135% of

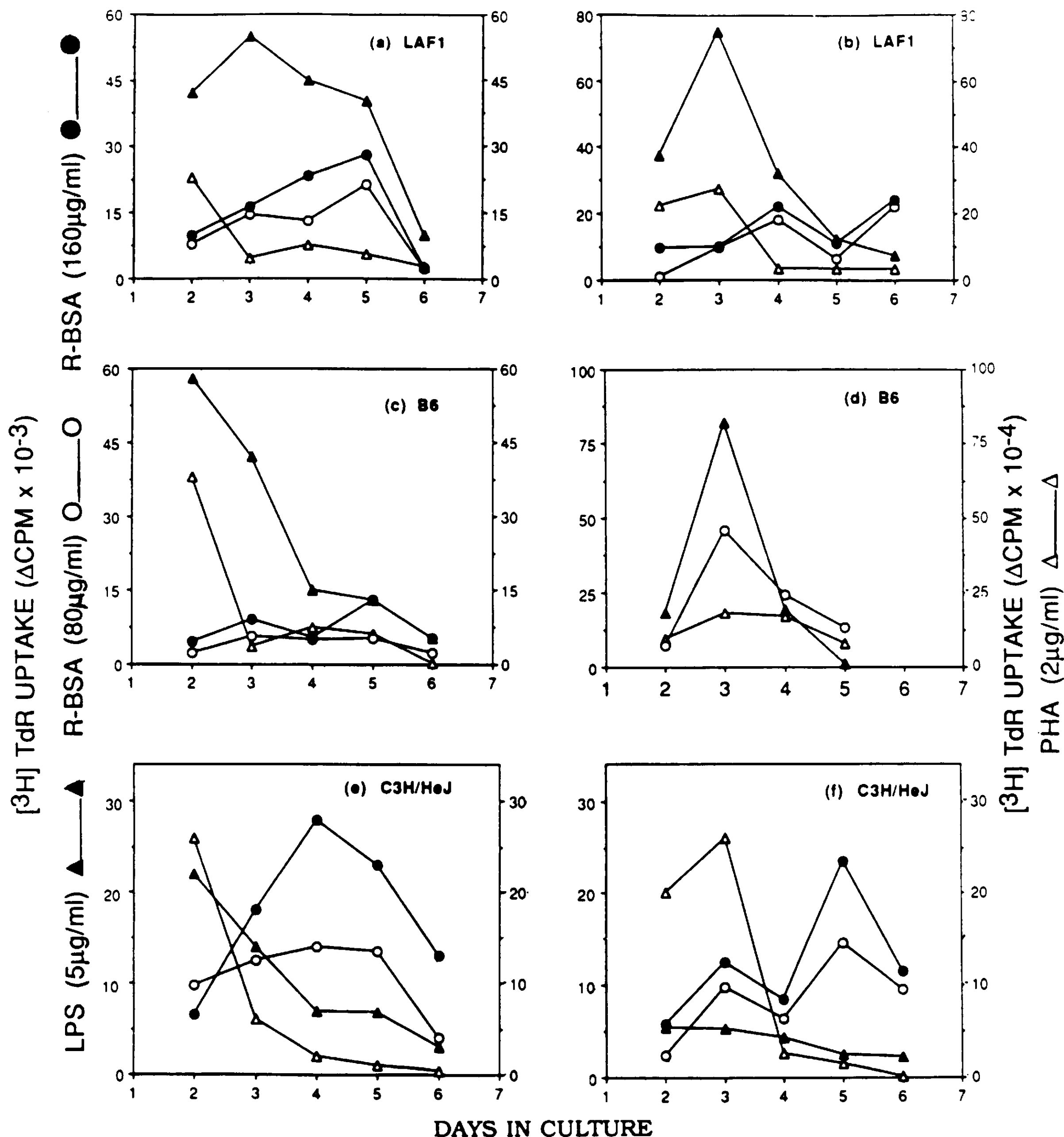

Figure 1. R-BSA stimulation of murine spleen cells. Spleen cells were cultured for the time indicated on the abscissa at 5×10^5 cells per well with media alone or with media containing the indicated mitogen, the last 8 hr in the presence of 1 µCi of [^{3}H]TdR, as described in Materials and Methods. The ordinate represent Δcpm. Δcpm is the mean cpm of triplicate cultures in the presence of a mitogen less the mean cpm for cultures in the absence of mitogen. For cells cultured with R-BSA, Δcpm is the mean cpm for cultures in the presence of R-BSA less the mean cpm for cells cultured with BSA.

the response to TGP alone. Similarly, the response of cells cultured with R-BSA and rutin was 93%, 106%, 102%, and 101% of that to R-BSA by itself. Comparable results were obtained for LPS and PHA, as well as when spleen cells were incubated for 1 hr with free rutin, prior to the addition of the mitogens.

Though rutin is an important constituent of tobacco leaves, other phenolics and flavones are present. Prominent among them are quercetin, the aglucan backbone of rutin, caffeic acid, chlorogenic acid, kaempferol, and naringin. It was conceivable that any of these compounds might be inhibitory to the mitogenic effect of TGP. None of these compounds, at any of the concentration analyzed (3×10^{-8} to 1×10^{-4} *M;* and Kaempferol at 1×10^{-5} and 1×10^{-6} *M*), showed any effect on the mitogenic response to either TGP, or to the other mitogens. For example, the response of spleen cells to TGP in the presence of 1×10^{-5} *M* phenolic was: 108%, 114%, 113%, 103%, and 109% of control for caffeic acid, chlorogenic acid,

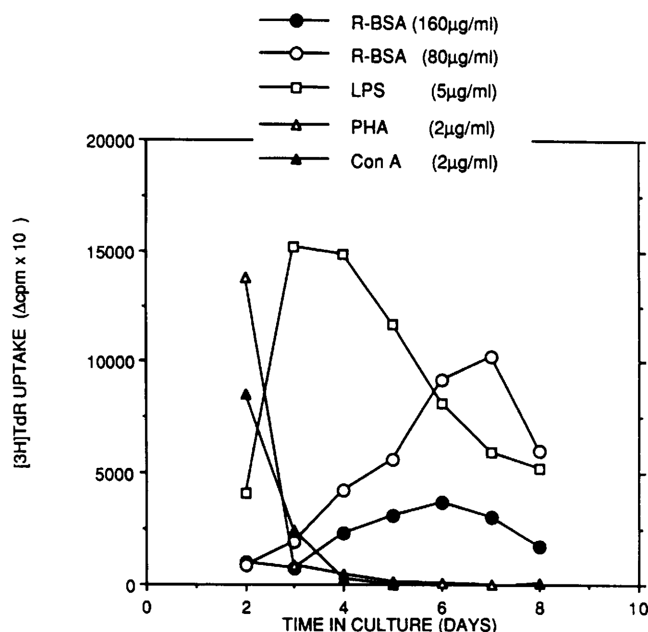

Figure 2. Stimulation of B6 PBL by R-BSA. Experimental details as described for Figure 1.

Kaempferol, quercetin, and naringin, respectively. Noteworthy was the observation that spleen cells cultured for 4 days with 1×10^{-4} *M* caffeic acid, chlorogenic acid, naringin, or quercetin showed a 2- to 3-fold increase in [^{3}H]TdR uptake.

Proliferative Response to TGP and R-BSA Is Not Decreased with Age. Although immune responses to many mitogens usually decrease with age, we observed no age-related decline in the response of human PBL to TGP, and a marginal decline in the response to R-BSA (33). To further probe the correlation between the effects of TGP and R-BSA in the mouse system, the proliferative responses of old mice to both antigens were investigated. The results in Table I show that [^{3}H]TdR uptake by TGP-stimulated spleen cells from individual, 18 to 24 months old, B6 or BALB/c mice did not differ significantly from that of spleen cells from 3-month-old syngeneic mice. Similarly, the response to R-BSA of spleen cells from young and old BALB/c or B6 mice did not differ (Table I). This observation suggested that the R-BSA and TGP might be activating the same subpopulation of cells. Having demonstrated that TGP was a T-independent B-cell mitogen (24), the possibility that the same holds true for R-BSA was considered.

Proliferative Response of T Cell-Depleted Spleen Cells to R-BSA. Spleen cells treated once or twice with anti-CD3 and C, were resuspended at 5×10^6 viable cells/ml and were cultured with R-BSA, PHA, or LPS. As shown in Table II, the response to PHA, a T-cell mitogen, was essentially obliterated. The responses to R-BSA and to the B-cell mitogen LPS, were not reduced following T-cell lysis, but rather seemed to increase, probably due to the increase in the relative number of B cells present, as the same number of viable cells was cultured. Taken together, the data suggested that R-BSA, like TGP (24), might be a T-independent B-cell mitogen.

The Proliferative Response to R-BSA by Spleen Cells from Congenitally Athymic Mice is Significantly Reduced. The second approach used to determine a role for T cells in the R-BSA response was to examine the response of congenitally athymic mice. As expected, these mice lacked responses to PHA, confirming that they did not have functional T cells (Table III). It was also evident from the results (Table III), that the proliferative response to R-BSA-stimulated spleen cells from congenitally athymic mice was significantly lower than the response of cells from euthymic mice. [^{3}H]TdR incorporation by the nu/nu cells (n = 9) cultured with 80 μg/ml R-BSA ranged from 900 to 33,500 (mean ± SE, 8,400 ± 3,300) Δcpm. Under the same condition the uptake by cells from +/nu mice (n = 10) ranged from 4,300 to 48,400 (mean ± SE, 25,200 ± 4,900) Δcpm; P = 0.006. The response

Table I. Proliferative Response of Spleen Cells from Young and Old Mice To TGP and R-BSA[a]

Parameter	BALB/c				C57BL/6			
	TGP		R-BSA		TGP		R-BSA	
	Young	Old	Young	Old	Young	Old	Young	Old
	[^{3}H]TdR uptake (Δcpm × 10^3)[b]							
Range	0.3 to 96	2 to 69	14 to 75	4 to 39	2 to 109	1 to 84	2 to 42	1 to 57
Mean	31	24	41	22	41	29	18	21
SE	9	7	9	7	8	6	4	7
(n)	13	12	6	4	21	17	12	10
P^c	NS		NS		NS		NS	

[a] Spleen cells from young (3 months) and old (18 to 24 months) mice were cultured in triplicates with or without the indicated mitogen, as described in Materials and Methods.
[b] Δcpm: for TGP cultures = mean cpm for TGP stimulated cells minus mean cpm for cells cultured in media alone; for R-BSA cultures = mean cpm for R-BSA stimulates cells minus mean cpm for BSA-cultured cells.
[c] Student's t test using square root of Δcpm and Mann-Whitney U gave similar results.

Table II. Proliferative Response of B6 Spleen Cells to R-BSA[a]

Day	Mitogen (μg/ml)	Treatment		
		Anti-CD3 + C		
		None	Once	Twice
		[^{3}H]TdR uptake (Δcpm)[b]		
2	PHA (2)	180,144	1,806	−1,097
	LPS (5)	37,962	40,870	85,226
	R-BSA (160)	4,342	5,165	14,161
3	PHA (2)	54,459	2,354	−192
	LPS (5)	18,463	28,821	61,581
	R-BSA (16)	5,708	3,940	9,274

[a] Cells were treated with anti-CD3 and C, and cultured with or without the indicated mitogens as described in Materials and Methods.
[b] [^{3}H]TdR uptake by cells cultured in media alone was 688, 1172, and 2980 on Day 2, and 1292, 712, and 1536 on Day 3 for untreated, treated once, or treated twice with CD3 + C, respectively.

of nu/nu cells to 160 μg/ml R-BSA was also significantly lower ($P = 0.022$) (Table III). Thus, congenitally athymic mice responded differently to TGP (24) and R-BSA. Addition of 2×10^5 thymocytes to 4×10^5 nu/nu spleen cells did not increase proliferative response to 80 μg/ml R-BSA. Spleen cells incorporated 20,648 and 21,254 Δcpm, when cultured with or without syngeneic thymocytes, respectively. Spleen cells from normal littermates cultured with or without thymocytes incorporated 31,097 and 32,342 Δcpm, respectively.

Congenitally athymic mice implanted with thymic lobes from 4-week-old syngeneic donors, did not differ from euthymic mice in their spleen cells response to R-BSA (Table IV). Taken together the data suggested that, unlike TGP (24), the presence of a thymus seemed essential for a full response to R-BSA. To determine whether R-BSA-stimulated T-cell proliferation, bivariate analysis using FCM was carried out.

Bivariate FCM Analysis to Identify the Cycling Cells. Spleen cells from B6 mice cultured for up to 4 days in media with or without BSA (150 μg/ml), R-BSA (150 μg/ml), or TGP (75 μg/ml) were harvested daily, were stained with FITC anti-CD3 or FITC anti-Ig, and counter stained with propidium iodide. A representative experiment is shown in Figure 3. The incidence of cycling B cells in TGP stimulated cultures was 15.36% on Day 1 and increased to 58.53% by Day 3 of culture, as compared with 11.74% and 22.03% on Day 1 and 3, respectively, for cells in media alone. In R-BSA-stimulated cultures, 81.23% of the B cells were cycling on Day 2 of culture, as compared with 12.45% and 15.94% for cells cultured in media or BSA, respectively. These data confirmed our observations that TGP (24) and R-BSA activate B cells to enter cell cycle. Unexpected was the finding that on Day 1 of culture 12.88% of the T cells in TGP-stimulated cultures were cycling, while the incidence was 8.84%, 8.12%, and 8.52%, for cells cultured in media, BSA, and R-BSA, respectively. That a small subpopulation of T cells is stimulated by TGP, but not by R-BSA, was confirmed by a second independent experiment (data not shown).

IL-6 Production by TGP and R-BSA-Stimulated Spleen Cells. Although T cells were not stimulated by R-BSA to enter cell cycle, T cells could participate by producing a factor(s) essential for the B-cell response. Given our earlier observation that R-BSA induces spleen cells to secrete IL-4, but not IL-2, suggesting a preferential activation of Th2 cells (20), the B-cell stimulatory factor IL-6, which is another Th2 cell product (21), was examined. Supernatants obtained from B6 spleen cells, cultured at 5×10^6 cells/ml with media, or media containing TGP (75 μg/ml), R-BSA (150 μg/ml), or BSA (150 μg/ml), were assayed for IL-6. The results demonstrated that IL-6 was detectable in the supernatants of TGP and R-BSA stimulated cells by 2 hr. of culture (Fig. 4A), its concentration increased until 24 hr. of culture when it plateaued (Fig. 4A and B). IL-6 concentration in supernatants collected at 24 hr of culture ($n = 4$) ranged from 515 to 2875 (mean ± SE, 1498 ± 495) pg/ml and 565 to 830 (mean ± SE, 729 ± 62) pg/ml for TGP and R-BSA stimulated cells, respectively. These values were significantly ($P \leq 0.001$) higher than the <15 to 69 (mean ± SE, 35.5 ± 11.7) pg/ml and 22 to 250 (mean ± SE, 92 ± 54) pg/ml, for media and BSA controls, respectively.

TGP and R-BSA Immunoprecipitate Similar Membrane Components from Spleen Cells. Similarity in the cell activation pathway is implied when different compounds bind to the same cell surface site(s). This is usually evaluated by competitive binding assays. Binding studies with [^{125}I]-TGP or [^{125}I]-R-BSA yielded unexpected results. The binding of either compound to spleen cells was essentially unsaturable and did not follow the kinetics expected for one or two binding sites (data not shown). Three possible explanations come to mind. First, that the binding is "nonspecific"; second, that although the initial binding is specific, there is additional "nonspecific" stacking of molecules, due to the hydrophobicity of the aromatic rings; and, third, as is the case of LPS (34–36), TGP and R-BSA bind at multiple sites. To distinguish between these possibilities, [^{125}I]-labeled BALB/c spleen cells were lysed; the supernatant was immunoprecipitated with Protein A-Sepharose CL-4B beads or with beads coupled to TGP, R-BSA, BSA, or Con A; and the precipitates were examined by SDS-PAGE. As can be seen in Figure 5, the most striking result was the

Table III. Proliferative Response of Congenitally Athymic Mice to R-BSA[a]

Parameter	R-BSA (80 μg/ml)		R-BSA (160 μg/ml)		PHA (2 μg/ml)	
	+/nu	nu/nu	+/nu	nu/nu	+/nu	nu/nu
			[^{3}H]TdR uptake (Δcpm × 10^3)[b]			
Range	4.3 to 48.4	0.9 to 33.5	6.7 to 81.9	1.4 to 29.5	37 to 224	−9.5 to 7.9
Mean	25.1	8.4	23.7	8.2	101	−0.4
SE	4.9	3.3	7.2	2.8	20	1.6
P^c	0.006		0.022		<0.00001	

[a] Results are from seven independent experiments; 10 +/nu and 9 nu/nu mice. CD-1 mice were used in two experiments, and BALB/c in five. The data were pooled since both strains gave similar results.
[b] Δcpm R-BSA = mean cpm for R-BSA stimulates cells minus mean cpm for BSA-cultured cells. Δcpm PHA = mean cpm for PHA stimulates cells minus mean cpm for media control.
[c] Student's *t* test using square root of Δcpm.

Table IV. Proliferative Response to R-BSA of Congenitally Athymic Mice Reconstituted with Thymic Lobes[a]

Experiment	Mice	PHA 2 μg/ml	R-BSA 25 μg/ml	R-BSA 50 μg/ml	R-BSA 100 μg/ml
		[^{3}H]TdR uptake (Δcpm × 10^3)			
1	nu/+	104.9	8.4	11.5	16.3
	nu/+	105.8	2.3	3.0	6.8
	nu/nu + thymus	60.9	6.7[b]	11.6[b]	11.7[b]
	nu/nu + thymus	91.0	12.7	7.1	9.2
2	nu/+	130.8	8.8	14.5	22.0
	nu/+	139.2	9.2	13.1	13.3
	nu/+	127.7	4.2	5.0	8.2
	nu/nu + thymus	96.2	2.1	3.7	5.6
	nu/nu + thymus	−0.5	3.9	1.5	5.4

[a] Thymus lobes from 4-week-old euthymic B6 donors were implanted under the skin of syngeneic nu/nu recipients. Their spleen cells were assayed 6 months later for their proliferative response, as indicated.
[b] The responses of the reconstituted nu/nu did not differ significantly from that of nu/+ (P = 0.931, 0.322, and 0.161 for the response to 25, 50, and 100 μg/ml R-BSA, respectively.

essential identity in the pattern of bands from the TGP and R-BSA precipitates. Specificity for the binding was suggested by the fact that a different pattern was seen in the Con A precipitate, and the fact that no radiolabeled material was precipitated by either Protein A- or by BSA-Sepharose CL-4B beads. The approximate molecular masses of the various bands are 110, 70, 55, 43, 34, 29, and 25–23 kDa. The 2-D analysis of the TGP and R-BSA precipitates, shown in Figure 6, are also striking in their similarity. The pI of the 28 kDa band is between pH 6.3 to 6.6. A band at ~23 kDa has a pI of 6.0, one band at ~25 kDa has a pI ~5.4, and five bands ranging in size from ~26 to ~110 kDa all have a pI in the pH range of 4.6–5.4.

Discussion

This study was designed to test the hypothesis that the effects of TGP, a flavone-phenolics-rich glycoprotein from tobacco, on murine spleen cells could be duplicated by a conjugate of rutin to a carrier protein. A compound similar to TGP was previously isolated by Wright, who identified rutin as a major component (37). For that reason rutin conjugates to BSA were used as a model to study the role of phenolics in the effects of TGP (23). R-BSA was shown to share many of the biological effects of TGP (19, 22, 23, 27). Here I show that R-BSA, like TGP, activates mouse B cells to proliferate with similar kinetics; their mitogenic effects are not inhibitable by free flavonoids or phenolics; there is no age-dependent decrease in the proliferative response to either compound; they both induce spleen cells to secrete IL-6 by 2 hr of culture; and they immunoprecipitate similar cell surface components. They differ in that the proliferative response of spleen cells from congenitally athymic mice to R-BSA, but not to TGP, is significantly lower than that of euthymic mice. In addition, TGP seems to stimulate a small subpopulation of T cells to enter cell cycle. The reason for these differences are not clear. One possibility is that TGP contains phenolics other than rutin, and those might contribute to its effect. In this context, it should be noted that free caffeic acid, chlorogenic acid, nar-

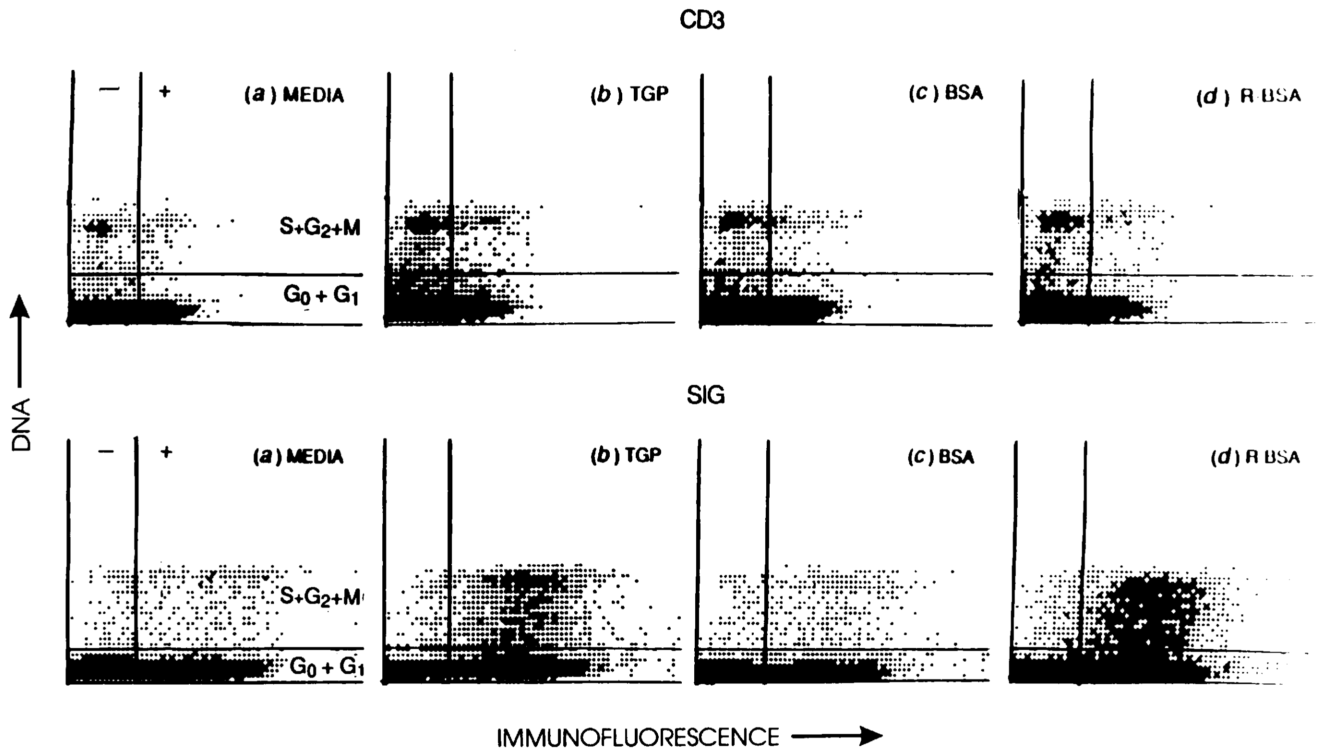

Figure 3. Bivariate FCM analysis to identify the cycling cells. B6 spleen cells were cultured with media alone or media containing TGP (75 μg/ml), BSA (150 μg/ml), or R-BSA (150 μg/ml). The cells were harvested on Day 1 through 4 of culture and stained with FITC-anti-CD3 or FITH-anti-mouse Ig (SIG), followed by propidium iodide, as described in Materials and Methods. The data shown for CD-3 is of Day 1, and for SIG of Day 3. The abscissa shows the intensity of FITC, and the ordinate of propidium iodide.

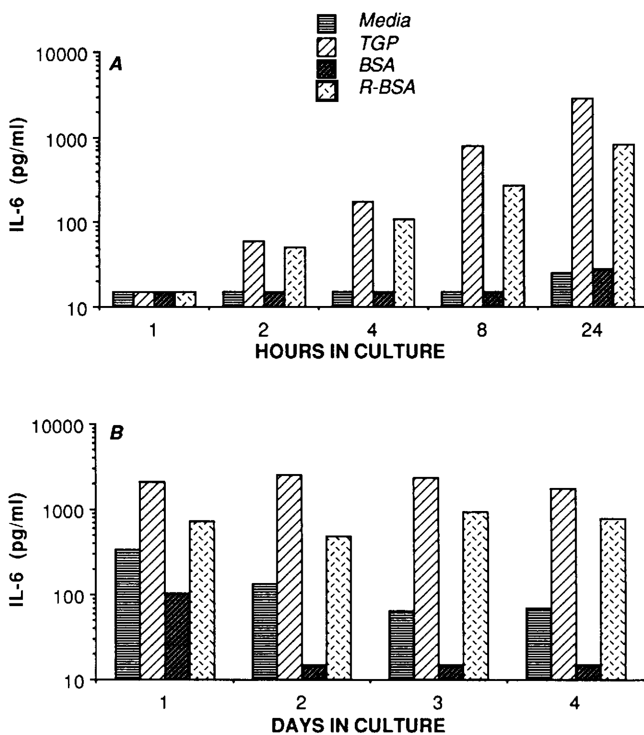

Figure 4. IL-6 production by B6 spleen cells cultured with media alone or media with the indicated mitogen. IL-6 ELISA with a detection limit of 15 pg/ml was used.

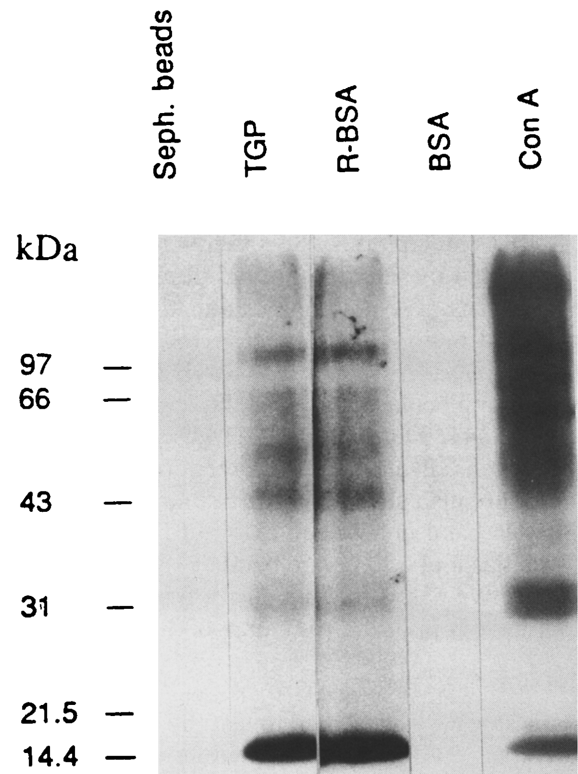

Figure 5. SDS-PAGE (12%) analysis of [^{125}I]-labeled BALB/c spleen cells were lysate immunoprecipitated with Protein A-, TGP-, R-BSA-, BSA-, or Con A-Sepharose CL-4B beads. Yhe rainbow molecular weight markers were used.

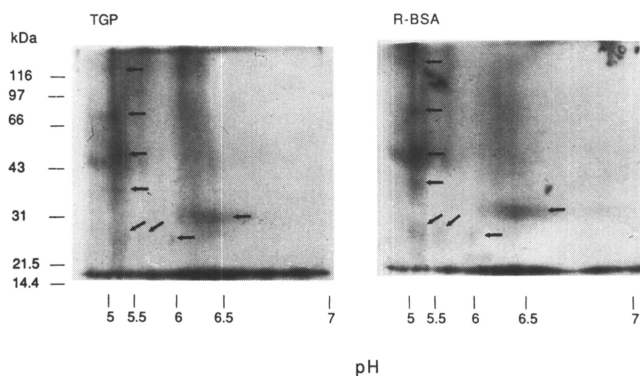

Figure 6. 2-D gel analysis of [^{125}I]-labeled BALB/c spleen cells were lysate immunoprecipitated with Protein A-, TGP-, R-BSA-, or Con A-Sepharose CL-4B beads.

ingin, and quercetin, but not rutin, stimulated a 2- to 3-fold increase in [^{3}H]TdR uptakes by spleen cells. Although the stimulatory effect of the free phenolic was at a high concentration, it seems reasonable to expect that upon conjugation to a carrier they would be effective at a lower concentration due to the possibility of multiple site binding.

The observation that TGP stimulates a small subpopulation of mouse splenic T cells to proliferate seemed surprising in view of our earlier observation that TGP stimulated proliferation of murine B cells in a T cell-independent manner (24). The stimulatory effect on T cells was not detected originally, most likely because of the small percentage of T cells that respond and the method used. Their contribution to the [^{3}H]TdR incorporation by spleen cells is insignificant considering that T cells represent <30% of the spleen cells, while B cells, ~60% of which respond, constitute ≥50% of the spleen cells. Thus, there was no noticeable decrease in [^{3}H]TdR uptake following anti-CD3 plus C treatment of spleen cells (24). The percentage of proliferating T cells (~4%) is similar to that observed in human peripheral blood lymphocytes (25). This raises the possibility that TGP acts on T cell as an antigen, via a specific T-cell receptor, while activating B cells as a mitogen (polyclonal activator). The observation that both TGP and R-BSA bind to multiple cell surface components on murine spleen cells suggest that these compounds might, indeed, activate cells through multiple pathways.

Some of the effects of TGP and R-BSA are similar to those of LPS. Both stimulate mouse B cells (24), activate factor XII-dependent pathways (23), and stimulate human peripheral leukocytes to produce interleukin-1 (IL-1) (31 and T. Francus, unpublished observations). In addition, TGP induces the expression of IL-1, IL-6, and platelet-derived growth factor in human alveolar cells (27). We, therefore, repeatedly tested TGP preparations for LPS contamination (24, 25, 27), compared the kinetics of the responses to LPS and TGP in the same donors (25), and documented that the LPS hyporesponsive mouse (C3H/HeJ) (38) responds to TGP (24) and to R-BSA (this paper) but not to LPS. Thus, it was extremely interesting to discover that TGP and R-BSA, like LPS (36), bind to multiple cell surface sites. However, the cell surface components to which they bind appear to be entirely different. The major LPS-binding protein on mouse spleen cells has a pI of 6.5 and an apparent molecular mass of 70 or 73 kDa (35,36). The pI's of the other 15 or so bands that bind LPS are in the pH range of 6 to 7, with the exception of one component which has a pI of ~5.5 (36). One of the TGP and R-BSA binding components has a pI at the pH range of 6.3 to 6.6, and a second pI of ~6.0. The remainder of the bands have pI's in the range of 4.6 to 5.4. These observations clearly indicate that the effects of TGP and R-BSA are not due to LPS contamination.

The multiple binding sites for TGP and R-BSA most likely underlie our observation that their binding to spleen cells could not be saturated. The possibility of additional "nonspecific" stacking of molecules due to the hydrophobic aromatic rings is not ruled out.

Two of the observations reported here might have far reaching biological ramifications. First, it was shown here that R-BSA is a mitogen. Rutin, and other phenolics, are biochemically active compounds and can oxidize and conjugate to various proteins (11), making them potential activators of cell proliferation. Increased cell division increases the possibility for initiating one or more of the steps leading to neoplasia, like proto-oncogenes activation, and loss of mutation of a tumor suppressor gene (11, 14, 15, 39–42). Thus, phenolics in edible fruits and vegetables might play a role in the development of gastrointestinal tumorigenesis. The second intriguing observation is that TGP and R-BSA induce the synthesis of IL-6. IL-6 is a B cell-stimulating factor (43–46). It is a polyclonal B-cell activator, and was shown to stimulate the secretion of "nonspecific" antibodies, some of which could be autoantibodies (45, 46). This raises the possibility that naturally ingested phenolics might underlie some autoimmune diseases.

This work was supported in part by grants from the National Institutes of Health, United States Public Health Service: AI21357, HL37407, and BRSG SO7 RR05396.

I thank Dr. Carl G. Becker and Mary W. Wagner for the generous gift of TGP and R-BSA, Drs. Dana Bovbjerg and Paul Szabo for critical review of the manuscript, and Mr. Tony DeBlasio for excellent technical help.

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