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COMMENTARY

EFFECTS OF FLAVONOIDS ON IMMUNE AND INFLAMMATORY CELL FUNCTIONS

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The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals such as the flavonoids. Some effects of the flavonoids on the function of T cells, B cells, NK cells, macrophages, mast cells, basophils, neutrophils, eosinophils and platelets will be described. Each of these cell types is involved in immunity and inflammation, and the effects of the flavonoids on these cells will be considered in this broad context. This commentary is selective and does not cover the literature exhaustively.

The flavonoids are comprised of a large group of low molecular weight polyphenolic secondary plant metabolites which possibly are important to the health and maintenance of herbivorous animals including humans. Some basic flavonoid structures, including many discussed herein, are shown in Fig. 1. Dietary exposure to flavonoids is not insignificant. For instance, the average Western diet contains approximately 1 g/day of mixed flavonoids [1]. Ingestible flavonoids are found in fruits, vegetables, nuts, seeds, stems, flowers, roots, bark, tea, wine and coffee. They are prominent components of citrus fruits and other food sources [2]. The flavonoids have long been considered to possess antiallergic and antiinflammatory activities. Their effects on a variety of inflammatory processes have been reviewed [3, 4]. They are also potent antioxidants and possess significant vitamin C-sparing activity [5, 6].

The flavonoids display a remarkable array of biochemical and pharmacological actions [7–12], some of which suggest that certain members of this group of compounds significantly affect the function of the immune system. As will be described in greater detail, a number of flavonoids affect the function of enzyme systems critically involved in the immune response and the generation of inflammatory processes, namely, both tyrosine and serine-threonine protein kinases, phospholipase A_2, phospholipase C (PLC†), lipoxygenases and others. Recently, it has become evident that these enzymes are critically involved in signal transduction and cell activation processes involving cells of the immune system as well as other cells. Much of the information on flavonoid effects has been obtained with *in vitro* systems; more *in vivo* studies are very much warranted.

T lymphocytes

The convergence of immunologic research on the nature of T cell antigen recognition and investigations of signal transduction in T and B cells have led to new fundamental concepts. T cell proliferation follows the cooperative interaction of CD4, CD8 and the T cell receptor (TCR)–CD3 complex upon exposure to foreign antigens and in association with appropriate molecules of the major histocompatability complex. It is now understood that the proliferative signal is generated by members of a family of protein tyrosine kinases (PTKs) which catalyze the phosphorylation of cellular substrates which in turn is accompanied by T cell proliferation [13]. Tyrosine phosphatases dephosphorylate the phosphoproteins returning the cell toward baseline conditions [14]. Certain flavonoids affect the activity of PTKs but nothing is known about their possible effect on tyrosine phosphatases.

T lymphocyte stimulation through the antigen receptor causes early activation of a tyrosine kinase [15–17] and the generation of phosphatidylinositol (PI) bisphosphate (PIP_2)-derived second messengers, namely inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), via activation of phospholipase C [18, 19]. Several cellular substrates are phosphorylated including TCR-zeta. The T cell PTK, $p56^{lck}$, may be involved in this process. Trevillyan *et al.* [17] showed that the isoflavone, genistein, a selective PTK inhibitor [20], blocked the activity of $p56^{lck}$ in a concentration-dependent manner (IC_{50} =

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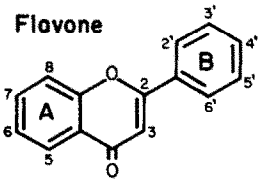
† Abbreviations: PLC, phospholipase C; TCR, T cell receptor; PTK, protein tyrosine kinase; PI, phosphatidylinositol; PIP_2, phosphatidylinositol bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; IL, interleukin; EGF, epidermal growth factor; PKC, protein kinase C; mAbs, monoclonal antibodies; TGP, tobacco glycoprotein; BSA, bovine serum albumin; SRBC, sheep red blood cells; PHA, phytohemagglutinin; DTH, delayed type hypersensitivity; DNFB, dinitrofluorobenzene; DMBA, dimethylbenzanthracene; TPA, tetradecanoyl phorbol acetate; EBV, Epstein–Barr virus; EA, early antigen; TNF, tumor necrosis factor; LT, leukotriene; PMA, phorbol 12-myristate 13-acetate; MPO, myeloperoxidase; and PAF, platelet-activating factor.

40 μM). Inhibition of enzyme activity correlated with reduced interleukin-2 (IL-2) secretion and IL-2R expression but not with TCR-mediated PI hydrolysis.

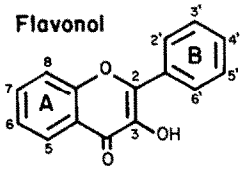
Recently it has been demonstrated that CD45 tyrosine phosphatase is essential for coupling the T cell antigen receptor to the PI pathway [18]. Experiments of Ledbetter *et al.* [19] and others demonstrated that CD45 tyrosine phosphatase can serve as a regulator of TCR complex-mediated PLC activation in human peripheral blood lymphocytes. CD45 inhibited the increase in cytoplasmic Ca^{2+} concentration, suggesting that PI hydrolysis is regulated by CD45. Also, ligation of CD45 inhibited phosphorylation of tyrosine on specific substrates during T cell activation. As well as an effect on PLC activation, CD45 may exert effects on cell activation through its enzymatic activity as a tyrosine phosphatase. It will be important to determine the effects of flavonoids on CD45 tyrosine phosphatase. Protein tyrosine phosphorylation and calcium mobilization are strongly augmented by cross-linking CD4 or CD8 with CD3; this finding has implications for positive and negative thymic selection [21]. Since protein tyrosine phosphorylation is known to be affected by at least two flavonoids, genistein [20] and quercetin [22, 23], it seems likely that this fundamental process determining thymic selection may be a flavonoid-sensitive mechanism.

Flavone



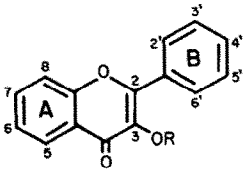
	5	6	7	8	3'	4'
Luteolin	OH	–	OH	–	OH	OH
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3	–	OCH_3
Apigenin	OH	–	OH	–	–	OH
Cirsiliol	OH	OCH_3	OCH_3	–	OH	OH

Flavonol



	5	7	2'	3'	4'	5'
Quercetin	OH	OH	–	OH	OH	–
Fisetin	–	OH	–	OH	OH	–
Kaempferol	OH	OH	–	–	OH	–
Myricetin	OH	OH	–	OH	OH	OH
Morin	OH	OH	OH	–	OH	–

Flavonol Glycosides

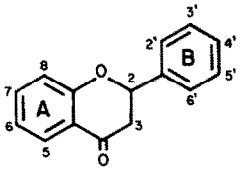


	3	5	7	3'	4'	5'
Rutin	R^1	OH	OH	OH	OH	–
Myricitrin	R^2	OH	OH	OH	OH	OH

R^1 = Rhamnosylglucoside

R^2 = Rhamnoside

Flavanone Glycosides

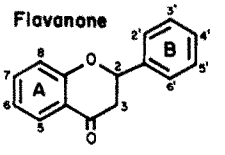


	5	7	3'	4'	5'
Naringin	OH	OR^1	–	OH	–
Plantagoside	OH	OH	OR^2	OH	OH

R^1 = Rhamnosylglucoside

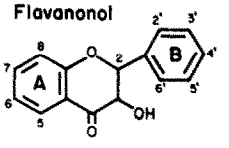
R^2 = β- D-glucopyranose

Flavanone



	5	7	3'	4'
Hesperetin	OH	OH	OH	OCH_3

Flavanonol



	5	7	3'	4'
Taxifolin	OH	OH	OH	OH

Silymarin

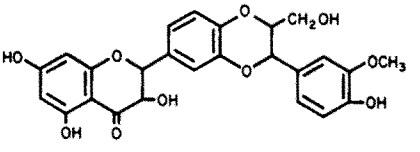
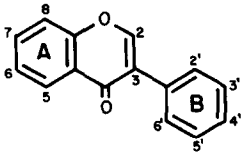


Fig. 1. Structures of various flavonoids.

Phosphatidylinositol turnover is a central phenomenon in intracellular signal transduction, occurring in response to neurotransmitters, growth factors and hormones [24, 25]. Oncogene-induced transformation by *ras*, *src*, *erb*, *fms*, and *fes* also augments cellular PI turnover [cf. Ref. 26]. An important enzyme in PI turnover is PI kinase which phosphorylates the inositol moiety of PI on the 4-position and is referred to as phosphatidylinositol 4-kinase. It is of interest, therefore, that Nishioka and co-workers [26] found that the isoflavone, orobol, was a potent inhibitor of PI kinase from *Streptomyces* with an IC_{50} of 0.25 μg/mL; quercetin had an IC_{50} value of 1.8 and fisetin of 2.0 μg/mL. Kinetic analysis revealed that orobol is competitive with respect to ATP and uncompetitive with respect to PI. Psitectorigenin, another isoflavonoid related to genistein and orobol, proved to be a more active inhibitor of epidermal growth factor (EGF)-induced PI turnover in A431 cells with an IC_{50} of approximately 1 μg/mL [27]. This compound inhibited PI turnover without affecting EGF receptor tyrosine kinase activity. Flavonoids with these biochemical properties should be useful probes in the functional analysis of PI turnover and its relationship to immune cell function.

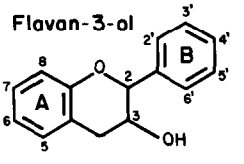
In addition to PTK, protein kinase C (PKC), the ubiquitous Ca^{2+} and phospholipid-dependent,

Isoflavone



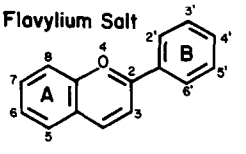
	5	6	7	4'	5'
Genistein	OH	–	OH	OH	–
Orobol	OH	–	OH	OH	OH
Tectorigenin	OH	OCH_3	OH	OH	–
Biochanin A	OH	–	OH	OCH_3	–
Formononetin	–	–	OH	OCH_3	–

Flavan-3-ol



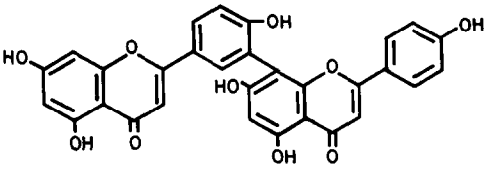
	5	7	3'	4'
Catechin	OH	OH	OH	OH

Flavylium Salt



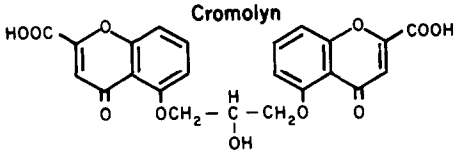
	3	5	7	3'	4'
Cyanidin	OH	OH	OH	OH	OH

Biflavonoid



Amentoflavone

Cromolyn



Flavone Acetic Acid

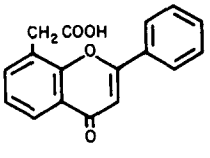


Fig. 1 (continued).

multifunctional serine-threonine phosphorylating enzyme, which is involved in a wide range of cellular activities including tumor promotion and T lymphocyte function [15, 28], has also been shown to be inhibited *in vitro* by certain flavonoids [29–31]. Fisetin, quercetin and luteolin were the most active compounds in the study of Ferriola *et al.* [31]. An isoflavone congener of genistein, formononetin, was inactive. Fisetin was shown to competitively block the ATP binding site on the catalytic unit of protein kinase C [31].

Recently, Bagnasco *et al.* [32] studied transmembrane signaling by both CD3 and CD2 human T cell surface molecules and the involvement of protein kinase C translocation. T cell activation by monoclonal antibodies (mAbs) directed against both CD3/T cell receptor (CD3/TCR complex) and the CD2 molecule resulted in the rapid increase of intracellular ionized Ca^{2+}. Moreover, it was demonstrated in the human leukemic T cell line Jurkat that triggering with appropriate anti-CD2 mAbs induced the generation of IP_3 and DAG from the breakdown of PIP_2. The appearance of such second messengers suggests that the CD2 molecule, like the CD3/TCR complex, may be linked to PLC. The investigators demonstrated that activation of Jurkat cells by anti-CD2 mAbs is also accompanied by translocation of PKC activity to the cell membrane in association with increased intracellular Ca^{2+}. By analogy with the effects of flavonoids on PTK, each of the steps in these experiments is potentially flavonoid-sensitive.

An important question is whether PTK activation is a prerequisite for PLC activation or whether these two pathways of signal transduction are regulated independently. It appears from experiments of June *et al.* [33, 34] that rapidly increased PTK activity is measurable prior to PLC activation (as determined by the appearance of IP_3) after T cell receptor complex ligation with anti-CD3 mAb. The PTK activity is sensitive to the effects of herbimycin, a benzoquinonoid ansamycin antibiotic which blocks oncogenic transformation by $pp60^{v\text{-}src}$. Mustelin and coworkers [35] obtained similar results, but they used the isoflavone genistein as an inhibitor of PTK. At concentrations which inhibited tyrosine phosphorylation of the TCR-zeta subunit, but not PLC activity (IP_3 increase), genistein blocked TCR-CD3-mediated activation of PLC, T cell proliferation and expression of IL-2 receptors. The effects were not related to genistein toxicity.

While all of these results clearly demonstrate that both tyrosine and serine-threonine kinases and PI kinase can be inhibited *in vitro* by certain flavonoids, more *in vivo* experiments remain to be done which would clearly show an effect on some facet of immune function. (A note of caution to all immunologists using experimental animals: the animals' chow contains flavonoids which could affect the outcome of various experimental manipulations. An experimentally ideal but unnatural diet would be an elemental one such as Vivonex.) The reported effects of flavonoids on the enzyme systems described above clearly suggest that they influence immune cell function. Several reports support this contention. In initial experiments, Mookerjee and co-workers [36] demonstrated that both quercetin and tangeretin could depress the expression of class II histocompatibility (DR) antigens in human peripheral blood monocytes processing streptolysin O as antigen. The flavonoid effect was reversible. These investigators also observed that certain flavonoids reversibly inhibit lymphocyte proliferative responses to phytomitogens, soluble antigens and phorbol esters by blocking an early event (or events) that follows exposure to the stimulus. Furthermore, quercetin and tangeretin were found to inhibit thymidine transport in stimulated lymphocytes. The finding that a flavonoid such as quercetin inhibits lymphocyte uptake of thymidine confirmed earlier reports of Graziani and Chayoth [37] and raises an important point: studies of inhibitors of DNA synthesis using [^{3}H]thymidine uptake must have controls determining the effect of the inhibitor on thymidine uptake itself.

Ishikawa *et al.* [38] studied the possible involvement of quercetin in tumor cell immunity. After exposure of the metastatic tumor BMT-11 I-9 cells (a clone of BMT-11, a transplantable mouse fibrosarcoma) to quercetin and cloning, clones were obtained which spontaneously regressed in normal syngeneic hosts. Possible mechanisms of regression of the regressor clones were studied by measuring cytotoxic T lymphocyte activity generated during mixed lymphocyte/tumor cell culture of spleen cells obtained from tumor-bearing mice. These studies show the potential ability of flavonoids to cause enzymatic alterations (prostaglandin formation) that may result in the production of tumor variants exhibiting modified immunological responses. Quercetin has inhibitory activity toward phospholipase A_2 whose product, arachidonic acid, is the essential substrate for the synthesis of prostaglandins.

Other studies suggest effects of flavonoids on immune responses. For example, tobacco glycoprotein (TGP), a polyphenol-rich, rutin-containing substance, can be purified from cured tobacco leaves and tobacco smoke condensate. One third of normal humans exhibit IgE-mediated reactivity to TGP. In mice, intradermal TGP stimulates a prolonged IgE response [39]. Similarly, rutin-derivatized bovine serum albumin (BSA) stimulates an IgE response to BSA but without hemmagglutinating antibodies. The data suggested that rutin exerts a regulatory effect on isotype expression. Subsequently, it was shown that the polyphenol-containing substance stimulated IL-4 production by murine Th2 cells thus accounting for the augmented IgE formation [40].

In other experiments, Schwartz *et al.* [41, 42] described the effects of quercetin and several other flavonoids on the generation and effector function of cytotoxic lymphocytes. They showed that certain flavonoids inhibited the generation of cytotoxic lymphocytes in murine mixed spleen cell cultures and, as well, depressed their cytotoxic activity against P815 murine mastocytoma target cells. The addition of Cu^{2+} blocked the inhibition observed by certain flavonoids but not others, showing thereby that chelation of divalent cations such as Cu^{2+} cannot explain the action of all flavonoids in these systems. These experiments are of particular interest because

they demonstrate that two types of cognate cell–cell interaction can be affected by active flavonoids.

Several more reports indicate the capacity of selected flavonoids to affect immune responses. Yamada *et al.* [43] found that the flavanone glucoside, plantagoside, inhibited the *in vitro* immune response of mouse spleen cells to sheep RBC (SRBC) in a concentration-dependent manner. Plantagoside also inhibited the proliferative response of BALB/c spleen cells to the T cell mitogen concanavalin A but had no effect on the mitogenic activity of lipopolysaccharide or phytohemagglutinin (PHA). Also of interest is the fact that plantagoside is an α-mannosidase inhibitor; glycosidase inhibitors are useful probes in studies of inflammation, metastasis, transformation, and the immune response.

Delayed type hypersensitivity (DTH) reactions to dinitrofluorobenzene (DNFB), but not SRBC, were reduced in mice undergoing two-stage carcinogenesis: initiation with dimethylbenzanthracene (DMBA) followed by promotion with tetradecanoyl phorbol acetate (TPA). The reduced DTH response to DNFB appeared to be abrogated in mice treated with the flavonol glycosides mauritianin and myricitrin [44, 45]. A role for certain flavonoids in the maintenance of tumor immunity may be suggested by these experiments. Interestingly, the effects of flavonoid derivatives on TPA-induced inflammation [46] seem to be roughly parallel to their inhibitory activities on tumor promotion in mice [44].

A Hungarian team studied the hepatoprotective and immunomodulatory effects of systemically administered silymarin in patients with alcoholic cirrhosis. In addition to generally normalizing elevated liver enzymes, silymarin significantly increased the response of the peripheral blood lymphocytes of the subjects to stimulation with concanavalin A and PHA, while it decreased antibody-dependent cellular cytotoxicity and NK cell activity, and also reduced the percentage of T8+ cells in the peripheral blood [47]. More clinical investigations of this kind are badly needed in flavonoid research.

B lymphocytes

Cross-linking of B cell membrane immunoglobulin, the B cell antigen receptor, initiates the signal for B cell activation and maturation. B lymphocyte activation, like T cell activation, is accompanied by phosphorylation of tyrosine on particular B cell proteins [48–50]. In studies of human B cell precursors, Uckun *et al.* [51] found that IL-7 receptor ligation with rhIL-7 caused increased phosphorylation of tyrosine on multiple substrate proteins and stimulated phosphatidylinositol turnover with increased IP_3 generation (PLC activation) and also DNA synthesis. Genistein effectively abrogated the tyrosine kinase activity and the accompanying increase in IP_3.

An example of ongoing, concurrent phosphorylation and dephosphorylation is seen in the experiments of Carter *et al.* [52] who studied tyrosine phosphorylation of PLC-γ 1 in L4 B-lymphoblastoid cells. From 0 to 30 min there wac clear-cut evidence of phosphorylation followed by dephosphorylation of several cellular proteins. These investigators also studied the PTK inhibitors genistein, tyrphostin and herbimycin. They found that genistein reduced the rise in cytosolic Ca^{2+} in B lymphocytes following ligation of membrane IgM and also observed the PTK dependence of PLC activation. It has also been demonstrated that protein tyrosine phosphorylation is induced in the Burkitt's lymphoma cell line Akata after stimulation with anti-IgG [53]. At noncytotoxic concentrations, genistein inhibited Epstein–Barr virus (EBV) activation as determined by the induction of EBV early antigen (and other antigens) and EBV early BZFI mRNA and its protein product ZEBRA. Biochanin A, another isoflavone, was also an active inhibitor of early antigen (EA) induction. Tumor promoter-stimulated induction of EA expression in EBV genome-carrying lymphoblastoid cells (Raji Cells) and the effects of flavonoids were studied by Okamoto *et al.* [54]. Quercetin (and retinol) caused an effective inhibition of EA expression while α-naphthaflavone, a synthetic flavonoid, had a weaker effect. Several other naturally occurring flavonoids were inactive. Since, in many systems, flavonoids are potent inhibitors of tumor promoter effects, one can speculate that certain flavonoids would also inhibit the enhanced expression of IL-2 receptors and immunoglobulin secretion stimulated by TPA as, for example, on sublines of an EBV-immortalized human B cell line as described by Polke *et al.* [55] and in keeping with the observations of Trevillyan *et al.* [17] with T cells.

Immunoglobulin secretion from mitogen-stimulated human peripheral blood B lymphocytes is, in fact, affected by quercetin. Cumella *et al.* [56] found that quercetin, but not taxifolin (dihydroquercetin), inhibited mitogen-stimulated immunoglobulin secretion of IgG, IgM and IgA isotypes *in vitro* with an IC_{50} of approximately 30 μM for each isotype. Once again, in this secretory system, as in others (*vide infra*), quercetin was active while taxifolin was not.

Immunomodulatory effects of flavonoids: Interferon and NK cells

Recent reports indicate that flavone acetic acid, a synthetic flavonoid, exhibits dose-dependent *in vivo* antitumor activity against certain solid tumors in mice. The mechanism of action of flavone acetic acid is intriguing: the compound augments murine NK cell activity *in vivo* and this effect was found to be due to induction of interferon-α synthesis [57, 58]. Spleen cells of flavone acetic acid-treated mice demonstrated rapid expression of interferon-α mRNA [58]. Upregulation of interferon mRNA was also detected early following the administration of flavone acetic acid to mice. The flavone acetic acid effect was selective since no upregulation of splenic mRNA for interferon-β, IL-1α or -β or IL-2 was detected after administration of flavone acetic acid. These data clearly indicate that flavone acetic acid stimulated gene activation. Another dimension of flavone acetic acid antitumor activity is its ability to cause vascular shutdown in the tumors. This effect can be attributed to the rapid induction of tumor necrosis factor (TNF); pretreatment with anti-TNF antibody abrogated the drug effect [59, 60]. It is tempting to speculate that naturally occurring dietary

flavonoids may also induce interferon synthesis and thereby act as anti-tumor agents.

NK cell cytocidal activity against NK-sensitive tumor target cells K562 and U937 is accompanied by early increased incorporation of ^{32}P into PI, suggesting activation of phospholipase C [61]. A high quercetin concentration (100 μM) profoundly inhibited the increased PI metabolism and also inhibited killing activity. Other studies established that T lymphocyte and NK cell cytotoxic activity rely on extracellular Ca^{2+}. Ng *et al.* [62] studied the Ca^{2+} dependence using quercetin and Ca^{2+} channel antagonists. Quercetin inhibited Ca^{2+}-dependent killing. Cytolysis could be induced by simultaneous stimulation with TPA and ionophore A23187, suggesting that protein kinase C activation is involved. The inhibitory effect of quercetin could be through its action on PKC [31].

Macrophages and monocytes

Few studies on the effects of flavonoids on macrophage function have appeared. Oxyradical generation by peripheral blood monocytes was suppressed by catechin as noted by Berg and Daniel [63]. A synthetic lipophilic derivative, 3-palmitoyl-(+)-catechin, enhanced the phagocytic activity of guinea pig Kupfer cells *in vivo* according to Piazza *et al.* [64]. As described earlier, Mookerjee and co-workers [36] demonstrated an effect of selected flavonoids on antigen presentation by human peripheral blood mononuclear cells. This observation should be confirmed as it has important implications for the earliest stages of the immune response.

The synthesis of IL-2 and leukotriene B (LTB_4) by human peripheral blood mononuclear cells was studied by Atluru *et al.* [65]. At a noncytotoxic concentration, genistein inhibited PHA-induced cell proliferation and IL-2 formation. The isoflavone also blocked LTB_4 generation in A23187-stimulated cells while H-7, a protein kinase C inhibitor, had no effect. LTB_4 formation in carragenin-induced intrapleural exudates in rats was reduced by intraperitoneal injection of quercetin and quercitrin but not by apigenin or luteolin, both of which lack a 3-position hydroxyl group (present in quercetin).

Studies of adhesion among human mononuclear leukocytes were reported by Patarroyo and Jondal [66]. The investigators studied the effect of phorbol ester-induced adhesion among human mononuclear leukocytes, the requirement for extracellular Mg^{2+}, and the effects of inhibitors of protein kinase C, lipoxygenase and ATPase. It was found that quercetin significantly inhibited phorbol 12,13-dibutyrate-induced cell aggregation/adhesion. The authors attributed the quercetin effect to inhibition of cellular ATPases but it is alternatively possible that the effect of quercetin could be due to its activity as an inhibitor of lipoxygenase and/or protein kinase C as described elsewhere in this report.

Mast cells and basophils

Mast cells play a central role in the pathogenesis of diseases such as allergic asthma, rhinoconjunctivitis, urticaria, anaphylaxis and systemic mastocytosis, and may well be important players in other chronic inflammatory disorders such as Crohn's disease, and other varieties of inflammatory bowel disease, vasculitis, rheumatoid arthritis and others. Basophils are perhaps emerging as an important cell in the pathogenesis of late phase allergic reactions [67].

Both mast cells and basophils possess high affinity receptors for IgE in their plasma membranes. Cross-linking of these receptors is essential to trigger the secretion of histamine and other preformed, granule-associated mediators and to initiate the generation of newly formed phospholipid-derived mediators. Various flavonoids have been shown in a number of systems to influence this secretory process, most frequently as inhibitors. Definitive evidence of flavonoid regulation of secretion was first provided by Fewtrell and Gomperts [68, 69] and Bennett *et al.* [70] in studies of the secretion of histamine from rat mast cells stimulated with antigen, mitogen, or the divalent cation ionophore A23187 and the release of β-glucuronidase from stimulated rabbit leukocytes. Quercetin, kaempferol and myricetin were found to inhibit the release of rat mast cell histamine. Following these reports, Middleton and co-workers [71, 72] undertook an examination of the effect of a number of naturally occurring flavonoids on the secretion of histamine from human basophils. Quercetin inhibition of antigen-stimulated human basophil histamine release [71] was found to be concentration-dependent, instantaneous in onset of action, antagonistic to the histamine release-augmenting effect of D_2O, not affected significantly by increased extracellular Ca^{2+} concentrations, and not enhanced by theophylline (suggesting that inhibition is not a cyclic AMP-dependent process). Subsequent experiments revealed critical structure–activity relationships governing the flavonoid effect on antigen-induced histamine release [72]. Inhibition of antigen-induced histamine release was associated with the following structural features: the presence of a C4 keto group, a reduced double bond at position C2–C3 in the γ-pyrone ring, and an appropriate pattern of hydroxylation in the B ring. Flavonoid glycosides, rutin and naringin, were inactive as were the flavanones (reduced C2–C3 bond), taxifolin and hesperitin. Morin, catechin and cyanidin were also inactive. Polymethoxylated compounds such as nobiletin and tangeretin showed less or no inhibitory activity against antigen-induced histamine release (as compared to their activity as inhibitors of lymphocyte activation) [36].

Further studies were undertaken to determine the effect of flavonoids on basophil histamine release stimulated by anti-IgE and concanavalin A (IgE-dependent histamine releasing agents); the chemo-attractant peptide, f-MetLeuPhe, and the tumor promoter phorbol ester, TPA (both f-MetLeuPhe and TPA are receptor-dependent histamine-releasing agents); and the divalent cation ionophore A23187 (bypasses receptor-dependent processes and carries Ca^{2+} directly into the cytoplasm). The results showed that the histamine-releasing effect of each of these secretogogues could be inhibited by some, but not all, of eleven flavonoids representing five different chemical classes [73]. The nature of the stimulus for histamine release and the structure of specific flavonoids appeared to determine whether a

particular compound would exert inhibitory activity. The results suggest that each of the secretogogues may utilize a different pathway of cell activation and that these pathways may be differentially sensitive to the action of particular flavonoids. It is not surprising, perhaps, to record that yet another stimulus of basophil histamine release, i.e. histamine releasing factor, can be inhibited by quercetin [74]. The effect of quercetin to uniformly inhibit basophil histamine secretion stimulated by a variety of agonists strongly suggests that there is a final common pathway utilized by each of these agonists which is sensitive to quercetin and other structurally appropriate flavonoids. A number of other investigators have also described inhibition of histamine release from mast cells by certain flavonoids [75–77], including some structurally unique flavonoid dimers such as amentoflavone (a biapigenin).

It is important to emphasize that the effects of active flavonoids on basophil histamine secretion are reversible, to wit, a basophil-containing cell suspension can be exposed to quercetin (50 μM) for 30 min and then washed twice, resuspended, and found to respond normally to antigen. However, if the histamine secretory reaction is initiated and an active flavonoid such as quercetin is added at 2, 5, 10 or 15 min after addition of antigen, there is at each time point an immediate cessation of further release of histamine [71]. These observations indicate that antigen activation of basophils results in the generation of a flavonoid-sensitive substance(s), interaction of which with the flavonoid strikingly alters the outcome of the activation process, in this case histamine secretion from basophils. The nature of the flavonoid-reactive substance(s) is unknown.

Other evidence suggests that calmodulin may be involved in the mechanism of secretion of histamine from granules of mast cells and basophils [78]. It is of interest, therefore, that quercetin appears to interact with the Ca^{2+}–calmodulin complex with resultant inactivation of Ca^{2+}–calmodulin-dependent activities, including the effects of tumor promoters [79–81].

A number of flavonoids possess lipoxygenase inhibitory activity [4, 82]. Marone *et al.* [83] showed that basophil histamine release was inhibited by eicosatetraynoic acid, a chemically unique lipoxygenase inhibitor, and suggested that a lipoxygenase-derived product of arachidonic acid metabolism may be required for basophil histamine release. Interestingly, many flavonoid inhibitors of histamine release are also good lipoxygenase inhibitors. Several flavonoids are relatively selective inhibitors of 5-lipoxygenase which initiates the biosynthesis of leukotrienes, compounds considered to be of importance in mediator release, inflammation and immediate hypersensitivity pathophysiology in tissues undergoing allergic reactions [84]. Cirsiliol (3′,4′,5-trihydroxy-6,7-dimethoxy flavone) is a potent inhibitor and caused 97% inhibition of the enzyme partially purified from rat basophilic leukemia cells. At 10 μM the compound caused 99% suppression of immunologic release of leukotrienes (SRS-A) from passively sensitized guinea pig lung (IC_{50} approximately 0.4 μM) [82].

Stimulation of Ca^{2+}-dependent protein phosphorylation during secretogogue-induced exocytosis in rat mast cells was described by Sieghart and coworkers in 1978 [85]. Purified rat peritoneal mast cells which had been labeled with ^{32}P and then stimulated by addition of compound 48/80 or ionophore A23187 resulted in the phosphorylation of four proteins of apparent molecular weights 78,000, 68,000, 59,000 and 42,000. Phosphorylation of the proteins with apparent molecular weights of 68,000, 59,000 and 42,000 was evident within 10 sec after addition of 48/80 whereas phosphorylation of the 78,000 molecular weight protein was not evident until 30–60 sec after addition of the secretogogue. The experiments clearly indicated that the secretory response of the mast cell is associated with protein phosphorylation and that regulation of exocytosis is related to protein phosphorylation. In subsequent experiments [86] the authors briefly noted that quercetin and kaempferol (10 μM), known inhibitors of rat mast cell histamine secretion, also increased the incorporation of radioactive phosphate into a single protein band with an apparent molecular weight of 78,000. The same group of investigators reported that the antiasthmatic drug cromolyn (chemically related to flavonoids) promotes the incorporation of radioactive phosphate into a single rat mast cell protein of apparent molecular weight 78,000 [87]. The finding provided an insight into the mechanism of inhibition by cromolyn of mast cell secretion triggered by an immunologic stimulus, anti-rat IgE. Whether the PTK family of kinase enzymes is involved in mast cell or basophil secretory responses has not been established, but Sagi-Eisenberg *et al.* [88] have demonstrated the involvement of PKC in rat basophilic leukemia cell histamine secretion.

A still unresolved question is just what cellular component in activated mast cells or basophils interacts with cromolyn (mast cells) or active flavonoids (mast cells and basophils) to inhibit the secretory process. Fewtrell and Gomperts [69] and Middleton *et al.* [71] demonstrated that only activated mast cells or activated basophils are affected by quercetin and other inhibitory flavonoids (i.e. the unstimulated cells can be exposed to the flavonoids, be washed, and subsequently shown to react normally to a secretogogue with histamine release). Fewtrell and Gomperts [69] also observed that pretreatment of rat mast cells with cromolyn (30 μM) for 30 min completely abolished the inhibition normally observed upon subsequent exposure to quercetin (30 μM) added together with antigen. This suggests that cromolyn and quercetin act at the same molecular site.

Another dimension of the exocytotic process and cromolyn/flavonoid activity is the possible relevance of the Ca^{2+}-ATPase membrane transport regulator. Certain flavonoids, notably quercetin, interfere with the activity of membrane transport ATPases including the Ca^{2+}-dependent ATPase which is one of the intrinsic cellular mechanisms that maintains low cytosolic Ca^{2+} concentrations. Fewtrell and Gomperts [68] found a very good correlation between the ability of certain flavonoids to inhibit rat mast cell histamine secretion and inhibition of Ca^{2+}-dependent ATPase activity. They suggested that the

effect of quercetin to inhibit secretion from stimulated cells was due to its inhibitory effect on plasma membrane Ca^{2+}-ATPase. Racker [89] has suggested that the transport ATPases of cell membranes with their associated ion flux pathways (channels) are separate structural entities which when properly coupled constitute the ATP-dependent ion pumps. Some flavonoids including quercetin inhibit aerobic glycolysis and growth of certain tumor cells by specifically modifying or "repairing" a defective control in the ATPase transport system [90].

Neutrophils

The inhibitory effect of flavonoids on secretory processes is not limited to basophils and mast cells. Bennett *et al.* [70] showed that a number of flavonoids were capable of inhibiting stimulated rabbit neutrophil lysosomal enzyme release. Also, Berton and co-workers [91] and Schneider *et al.* [92] reported that concanavalin A-induced secretion of lysosomal enzyme from polymorphonuclear leukocytes of albino guinea pigs and healthy human volunteers was inhibited by quercetin (which did not have any effect on the binding of concanavalin A to the cell membrane receptors). Rutin and morin were inactive, in keeping with the findings of the human basophil experiments.

Phagocytosis is accompanied by a dramatic increase in oxygen consumption (respiratory burst) with an attendant production of reactive oxygen intermediates such as the relatively less active superoxide anion and the various oxidizing species (hydrogen peroxide, hydroxyl radical and the hypohalites) by neutrophils [93]. These are generated sequentially starting with superoxide anion production by a membrane-bound NADPH oxidase activity [93]. The highly reactive oxygen metabolites along with other mediators elaborated by neutrophils and macrophages promote inflammation and cause tissue damage [94]. Oxidant release (as assayed by the production of luminol-dependent chemiluminescence) by human neutrophils was shown to be inhibited by certain flavonoids [95], an effect possibly related to inhibition of the 5-lipoxygenase pathway of arachidonic acid metabolism [96]. 'T Hart *et al.* [97] recently reported a similar inhibitory effect of different flavonoids on the production of reactive oxygen species by activated human neutrophils using the above chemiluminescence method. Four selected flavonoids inhibited myeloperoxidase (MPO) release, while two of these strongly inhibited this activity. Considering luminol-dependent chemiluminescence production by neutrophils to be an MPO-dependent process, these authors contend that these effects may mask the effects of flavonoids on oxidant production. Using the luminescent probe lucigenin for the exclusive detection of superoxide anion release, 'T Hart *et al.* [97] showed that the release of this species by human neutrophils was inhibited by flavonoids. Essential determinants for inhibition of superoxide anion release appear to be the OH groups located in the B-ring of the flavonoid molecule. The formation of superoxide anion is dependent on the activation of NADPH oxidase localized in the plasma membrane, which is subject to flavonoid inhibition [98].

In addition to inhibiting the activity of a purified human neutrophil MPO, quercetin was also found to depress this activity in a system employing intact human neutrophils [99]. In this case, in inhibiting the activity of the purified MPO, quercetin was significantly more potent than methiomazole, a known specific inhibitor of MPO [100]. In addition, quercetin was found to have an ability to directly scavenge hypochlorous acid (HOCl), a highly reactive chlorinated species generated by the MPO-H_2O_2-Cl system [99]. The inhibition of neutrophil MPO activity by flavonoids can result in the impairment of oxidant production. Such impairment could diminish the formation of highly toxic hypochlorous acid and hypochlorite ion (OCl^-). A consequence of this would be a decrease in the inactivation of α-antitrypsin [101], which could result in the progressive inactivation of neutrophil-derived and other tissue-damaging proteolytic enzymes.

Quercetin was found to be a potent inhibitor of human neutrophil degranulation and superoxide anion production induced by different secretogogues [102, 103]. Quercetin also inhibited the phosphorylation of neutrophil proteins accompanying neutrophil activation by PMA. Phosphorylation of a specific neutrophil protein (mol. wt 67,000) was reported to be particularly sensitive to quercetin at concentrations that also diminished neutrophil degranulation and superoxide production, suggesting thereby that the phosphorylation of this particular protein is an important intracellular event associated with neutrophil activation [102].

In other studies, Lee *et al.* [104] examined the effect of quercetin on the release of β-glucuronidase from human neutrophils stimulated with opsonized zymosan and found that quercetin inhibited the release of β-glucuronidase although the effect was not strong. However, it was also found that the release of [^{3}H]arachidonic acid from prelabeled neutrophils was also inhibited by quercetin, strongly suggesting an inhibitory effect of the flavonoid on phospholipase A_2 in keeping with the findings of Lanni and Becker [105].

Experiments performed by Busse and co-workers [95] showed that quercetin and chalcone were slightly effective inhibitors of neutrophil β-glucuronidase secretion stimulated by opsonized zymosan. These investigators also described that quercetin and the other flavonoids were quite effective inhibitors of opsonized zymosan-stimulated generation of superoxide anion and the chemiluminescence phenomenon. Long and co-workers [106] found that quercetin had at least three separate effects on human polymorphonuclear leukocytes: (1) quercetin inhibited the Mg^{2+}-dependent ecto-ATPase in a noncompetitive fashion, (2) it inhibited O_2 consumption, glucose oxidation and protein iodination in cells exposed to opsonized zymosan and TPA, and (3) it inhibited transport of the nonmetabolizable glucose analog, [^{3}H]2-deoxyglucose.

Eosinophils

Ionophore A23187-induced eosinophil degranulation with release of Charcot–Leyden crystal protein and eosinophil cationic protein is inhibited by

quercetin, but not by taxifolin (dihydroquercetin), in a concentration-dependent manner [107]. Thus, the activated eosinophil responds to these flavonoids in the same fashion as other secretory cells, e.g. basophils and mast cells. Whether eosinophil degranulation stimulated by other immunologic or nonimmunologic stimuli such as allergen or platelet-activating factor (PAF), for example, will be inhibitable by selected flavonoids remains to be determined but it is of interest that PI hydrolysis accompanies stimulation of murine eosinophils with anti-IgG $F(ab')_2$ acting through an Fc-γRII mechanism. This process is neomycin sensitive (inhibition of phospholipase C) and is required for 5-lipoxygenase activation and LTC_4 generation [108].

Platelets

In addition to their role in hemostasis and thrombosis, considerable evidence implicates platelets as inflammatory cellular elements [109]. Platelets are also key participants in atherogenesis. A number of proinflammatory mediators are derived from platelets including thromboxane A_2, PAF, and serotonin as well as transforming growth factor-β, platelet-derived growth factor and lipoxygenase metabolites. In light of the above, it is of interest that a number of flavonoids significantly affect platelet function. This subject has been reviewed in detail [110]. Flavonoids inhibit platelet adhesion, aggregation and secretion. Of the variety of compounds studied, the most active inhibitors of platelet function *in vitro* were effective in the 1–10 μM range. Certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase and this may be in part the explanation for their ability to inhibit platelet function. Flavonoids have been related to the inhibition of arachidonic acid metabolism by cyclooxygenase [111]. The inhibition by flavonoids of platelet activation by thromboxane-dependent stimuli may be related to this effect. The effect of selected flavonoids on platelet aggregation/adhesion is akin to their effect on mononuclear cell adhesion as described earlier and is another example of their potential capacity to regulate the activity of adhesion molecules [112].

Genistein apparently can affect cell function in ways possibly unrelated to protein tyrosine phosphorylation [113]. For example, platelet tyrosine phosphorylation stimulated by thrombin was only weakly affected by genistein but it inhibited platelet aggregation and serotonin secretion. On the other hand, this isoflavone suppressed platelet aggregation, serotonin secretion and protein tyrosine phosphorylation triggered by collagen and stable thromboxane A_2 analogs. These investigators also provided some insight into isoflavone structure–activity relationships: daidzein, an isoflavone lacking a 5-position hydroxyl group, is inactive as a PTK inhibitor but is capable, like genistein, of inhibiting binding of the stable thromboxane A_2 analog, U46619, to platelets with associated reduction in collagen- or U46619-induced platelet responses [113].

Gryglewski and coworkers [114] studied the mechanism of the antithrombotic action of flavonoids. Quercetin and rutin were capable of dispersing platelet thrombi adhering to rabbit aortic endothelium *in vitro* and prevented platelets from aggregating over a blood-superfused collagen strip (adhesion-related phenomena). The *in vivo* counterpart of these experiments involved the infusion of quercetin and rutin into an extracorporeal stream of blood. Quercetin and rutin inhibited the deposition of platelet thrombi on the blood-superfused collagen strip at calculated plasma concentrations of 0.05 and 0.03 μM. Analogously, in the model for studying platelet–endothelium interactions, quercetin and rutin, when infused into the stream of blood which superfused a rabbit aortic endothelial surface, caused the disaggregation of preformed platelet thrombi, again at low concentrations. Clearly the expression and/or activity of adhesion molecules are affected by the flavonoids. The authors concluded that flavonols are antithrombotic because they are bound selectively to mural platelet thrombi and, because of their free radical scavenging properties, modify damaged endothelial cells and permit normal prostacyclin and endothelium-derived relaxing factor synthesis [114]. The relevance of these experiments to immunologic and allergic tissue injury is once again related to the possibility that platelets may be activated and involved in some of these disorders.

PAF is a well recognized proinflammatory mediator derived from membrane phospholipids by the enzymatic activity of phospholipase A_2 and an acetyl transferase. PAF receptor-coupled activation of phosphoinositide-specific phospholipase C and phosphorylation of several cellular proteins has been reported. Dhar and colleagues [115] used the isoflavonoid, genistein, to investigate the possible involvement of tyrosine kinase in PAF-stimulated platelets and the relationship between protein phosphorylation and PLC activation. PAF alone stimulated PLC activity as measured by the production of IP_3 and genistein at the high concentration of 0.5 mM decreased PAF-stimulated PLC activity to control levels. At this concentration genistein also blocked PAF-stimulated platelet aggregation. In addition, genistein also reduced PAF-induced phosphorylation of proteins of molecular weights 20,000 and 50,000. Moreover, polymorphonuclear leukocyte-induced 40,000 molecular weight protein phosphorylation was additionally affected by genistein. Taken together these results strongly suggest that genistein inhibited PTK at an early stage of signal transduction resulting in inhibition of (or associated with inhibition of) PLC which in turn would result in decreased activation of protein kinase C via reduced PLC-catalyzed formation of DAG. The combined effects would, therefore, result in a reduction of protein phosphorylation. Based on these and other experiments the authors finally concluded that tyrosine phosphorylation is involved in the PAF receptor-coupled activation of PLC and signal transduction mechanism. It is tempting to speculate that there may be other isoflavonoid or flavonoid compounds, both natural and synthetic, which could affect the outcome of PAF-stimulated pathological states.

Conclusion

In summary, no doubt can remain that the

flavonoids have profound effects on the function of immune and inflammatory cells as determined by a large number and variety of *in vitro* and some *in vivo* observations. That these ubiquitous dietary chemicals may have significant *in vivo* effects on homeostasis within the immune system and on the behavior of secondary cell systems comprising the inflammatory response seems highly likely but more work is required to strengthen this hypothesis.

Ample evidence indicates that selected flavonoids, depending on structure, can affect (usually inhibit) secretory processes, mitogenesis, and cell–cell interactions including possible effects on adhesion molecule expression and function. The possible action of flavonoids on the function of cytoskeletal elements is suggested by their effects on secretory processes. Moreover, evidence indicates that certain flavonoids may affect gene expression and the elaboration and effects of cytokines and cytokine receptors.

How all of these effects are mediated is not yet clear but one important mechanism may be the capacity of flavonoids to stimulate or inhibit protein phosphorylation and thereby regulate cell function. Perhaps the counterbalancing effect of cellular protein tyrosine phosphatases will also be found to be affected by flavonoids. Some flavonoid effects can certainly be attributed to their recognized antioxidant and radical scavenging properties. A potential mechanism of action that requires scrutiny, particularly in relation to enzyme inhibition, is the redox activity of appropriately configured flavonoids.

Finally, in a number of cell systems it seems that resting cells are not affected significantly by flavonoids but once a cell becomes activated by a physiological stimulus a flavonoid-sensitive substance is generated and interaction of flavonoids with that substance dramatically alters the outcome of the activation process.

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