Effects of Bioflavonoids on Phagocytosis, Intracellular Bactericidal Activity and Chemotaxis of Human Polymorphonuclear Cells*

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SUMMARY Three bioflavonoids (BF), Quercetin, Rutin and Rutosid were tested for their influence on phagocytosis, phagocytic index, intracellular bactericidal activity, chemotaxis and release of lysozyme from human polymorphonuclear cells. In the presence of serum, phagocytosis and the phagocytic index were markedly suppressed ($p < 0.01$) by Rutin in concentrations of 1.5 to 3.0 x 10^{-3} M/L. Rutin suppressed completely the enhancing activity of poly-L-lysine and poly-L-arginine on phagocytosis. Much less inhibition was observed without the serum. Two other BF had very little inhibiting influence on phagocytic activity. In the presence of human serum, intracellular bactericidal activity was markedly suppressed by Rutin and Quercetin ($p<0.01$) but not by Rutosid. Chemotactic activity toward N-formyl-L-methionyl-L-phenylalanine (FLMP) and *Escherichia coli* filtrate was significantly suppressed ($p < 0.01$) by Rutosid (6.7 x 10^{-4} M/L) and Quercetin (1.5 x 10^{-5} M/L) but less so by Rutin (1.5 x 10^{-3} M/L). Quercetin and Rutin suppressed chemo-attractant activity of FLMP in a dose-related fashion. With the design of the present study, it was impossible to assess whether lysozyme release was inhibited by these bioflavonoids. It may be concluded that BF exhibited previously unrecognized inhibitory effects on several aspects of phagocytic activity of human polymorphonuclear cells.

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Bioflavonoids (BF) are potent reducing agents which decrease capillary permeability, inhibit inflammatory response and protect against the effect of anaphylactic reaction.[1] The complete spectrum of their biological functions and pharmacological applications have not been firmly established. In rats, BF were found to stimulate the healing of wounds and to counteract the inhibitory effect of prednisone on the formation of granulation tissue.[2] It has been reported that BF inhibit histamine release from basophils[3,4] and exert a stabilizing effect on the lysosomes of some mammalian cells thus inhibiting the stimulated release of certain enzymes such as cathepsin or β-glucuronidase.[5-7] Since lysosomal stabilizers may have an inhibitory influence on phagocytosis, it was of interest to establish whether BF could modulate various aspects of phagocytic activity of human polymorphonuclear cells. It was found that they inhibit chemotactic responses, phagocytosis and intracellular bactericidal activity. This previously unrecognized effect of BF on various aspects of phagocytosis may require reassessment of their biological functions.

MATERIALS AND METHODS

Three types of BF were used, Quercetin (5,7,3',4' -tetrahydroxyflavone) lot 071487 MH, molecular weight 338 obtained from Aldrich Chemical Co. Inc., Milawaukee, Wisconsin; Rutin, hydrosoluble salt of rutin sulphate (3-rhamnoglucoside of 5,9,3',4', -tetrahydroxyflavonol), molecular weight 665, obtained from E. Merck Laboratories, Germany through BDH Chemicals; and Rutosid (vitamin P_4) - 0 - (β-hydroxyethyl) – rutoside, molecular weight 743, obtained from the Clinical Research Division, Zyma, Nyon, Switzerland. Quercetin was used in concentration from 1 μg/ml (2.9 x 10^{-6}M) to 300 μg/ml (8.9 x 10^{-4} M); Rutin, from 0.5mg/ml (7.5 x 10^{-4} M) to 3.0 mg/ml (4.5 x 10^{-3} M); and Rutosid, from 0.5 mg/ml (6.7 x 10^{-4} M) to 3.0 mg/ml (4.0 x 10^{-3} M).

Purification of polymorphonuclear phagocytes

Polymorphonuclear leukocytes (PMNs) were separated using the

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previously described method.[8] The cells were re-suspended in HBSS and counted in a haemocytometer. Wright's, myeloperoxidase and non-specific esterase stains were used for evaluation of contaminating cells. The viability of PMNs as assessed by the trypan blue dye exclusion test was 90-100 per cent. The yield of PMN exceeded 70 per cent. The purity of the PMN preparations was 98 ± 0.5 per cent (SEM).

Micro-organisms

Suspensions of viable *Staphylococcus aureus* and *Streptococcus faecalis* were washed three times at room temperature in phosphate-buffered sterile saline (PBS), pH 7.2 and centrifuged at 3,000 rpm for 20 minutes. Three ml aliquots of the suspensions were adjusted with PBS to an optical density of 0.3 at 525 nm and viable micro-organisms used at concentrations of 15-17 x 10^8/ml. The concentrations of BF used did not reduce the viability of these micro-organisms.

Phagocytic assays

PMN were mixed with viable micro-organisms in a proportion of 1:5 to 1:10. This proportion of bacteria to cells was found to be the best for assessment of phagocytosis.[9] Simultaneously, various concentrations of BF were added. Since Quercetin had to be dissolved in 0.032-0.16% dimethylsulphoxide (DMSO), appropriate adjustments were made by adding DMSO in the same concentrations to the controls. The mixtures were prepared in two parallel series, one with and one without 3% AB human serum. The mixtures were incubated for 60 minutes at 37°C in a shaking water bath. At the end of incubation, a drop of the mixture was removed and the viability of PMN was tested using trypan blue exclusion. Bioflavonoids did not reduce the viability of PMN. After centrifugation to exclude extracellular bacteria, three slides were prepared from each sample, dried and Gram stained. On each slide, 200 PMN were counted and the number of PMN that ingested bacteria (phagocytosis) and the number of bacteria per cell (the phagocytic index) were estimated. The controls included PMN with bacteria, PMN alone and PMN with BF without bacteria. Each experiment was repeated twice and done in duplicate. Means of the four experiments were calculated. Control values were as follows: phagocytosis of *Staphylococcus aureus* without serum, 51 per cent; with serum, 86 per cent; and phagocytic index, 2.5 and 2.7 respectively. For *Streptococcus faecalis* the correponding values were 51 per cent and 86 per cent and 2.9 and 5.9 respectively. In some experiments, in addition to BF, other additives were used such as poly-L-lysine 5 μg/ml, poly-L-arginine 5 μg/ml and poly-glutamic acid 10 μg/ml.

The intracellular survival of *Staphylococcus aureus* was tested by a modified lysostaphin method.[10] Briefly, after incubation of PMN with *Staphylococcus aureus* and appropriate additives, 1.0 millilitre of the mixture was mixed with 9 millilitres of sterile saline in siliconized tubes and spun at 200 xg for 5 minutes. The pellet was re-suspended in saline; lysostaphin 20 U/ml (Schwarz/Mann, Orangeburg, N.Y.) was added. The mixture was incubated at 37°C for 20 minutes in a shaking water-bath. Then 2.5% trypsin (Worthington Biochemical Corp., Freehold, N.J.) was added and the mixture was incubated for 10 more minutes. The final suspension was diluted with distilled water and cultured by a pour plate technique using 1.0 ml solution at 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions. As a control, PMN with bacteria were incubated, appropriately diluted in distilled water and poured over the culture media plates. Colonies were counted. Each experiment was performed in duplicate. The number of micro-organisms ingested by PMN was estimated in each sample by direct counting. The number of bacterial colonies obtained from 100 phagocytes was divided by the number of bacteria ingested by 100 cells in the corresponding sample. The resultant value was compared with similarly obtained control values and expressed as a percentage. This was called the Intracellular Bactericidal Activity (ICBA) index. Controls were as follows: the number of colonies (CFU) without serum at 10^{-3} dilution, 112; number of intracellular bacteria, 179, i.e., ICBA, 63 per cent; with the serum number of CFU, 252; and the number of intracellular bacteria, 421, i.e., ICBA, 60 per cent.

Chemotaxis

Chemotaxis was studied by the modified method of Nelson.[11-13] Agarose (Seakem, Marine Colloids, Rockland, Maine) 0.85% was mixed with 0.25% gelatin in M199 medium buffered with 1M stock HEPES solution. Each agarose plate consisted of six pairs of wells cut with a template in radial fashion. The cells were suspended in M199 medium buffered to pH 7.5 HEPES solution, with and without 50% inactivated AB human serum. The cells were placed in the centre well of the plates at a concentration of 2.5 x 10^5 cells/well (2.5 x 10^7 cells/ml). As chemotaxins, culture filtrates of *Escherichia coli* ECL3 (courtesy of Dr. W. Leers, Department of Microbiology, the Wellesley Hospital, Toronto) and of N-formyl-L-methionyl-L-phenylalanine (FLMP) were placed in the outer wells. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 2-2.5 hours. The plates were then stained with Wright's stain and the distance from the outer edge of the centre well to the leading border of migrating cells was measured with microprojector (Tri-simplex model, Bauch and Lomb Optics Canada, Ltd.) using a magnification of 40 x. The effect of BF on PMN chemotaxis was tested by pre-incubating the cells with various concentrations of appropriate agents or with medium

alone for 30 minutes at 37°C in a shaking water-bath. In other experiments BF were added to the chemotaxins and the mixture was incubated for 15 minutes at room temperature and placed in the outer wells. The potential of BF to act as chemotaxins or as chemotaxinogens was tested by placing them alone or in 90% human AB serum in the chemotaxin wells. Each experiment was repeated four times and each consisted of three parallel trials. The mean of 12 results was calculated. The distance was measured in millimetres subtracting the value of random migration of PMN.[11-13] Controls were as follows: without serum, migration of cells to FLMP, 12-36 mm, mean 22±6 (SD) (n=28); and with serum added, 47-85 mm, mean 65±10 (SD) (n=36).

The influence of BF on the release of lysozyme from PMN was tested as follows: aliquots were prepared of 250,000 cells in medium M199 at pH 7.4. Rutin was added to the cells in concentrations of 0.5, 1.5 and 3.0 mg/ml; Quercetin was added in concentrations of 50, 100, 200 and 300 μg/ml. The mixtures were placed in the wells of lysoplates which were incubated for 18 hours and the size of the lytic rings was calculated. Controls included PMN alone, standards of purified human lysozyme in concentrations of 5, 10 and 15 μg/ml, medium M199 alone and with DMSO 0.5%. Each experiment was done in duplicate or triplicate.

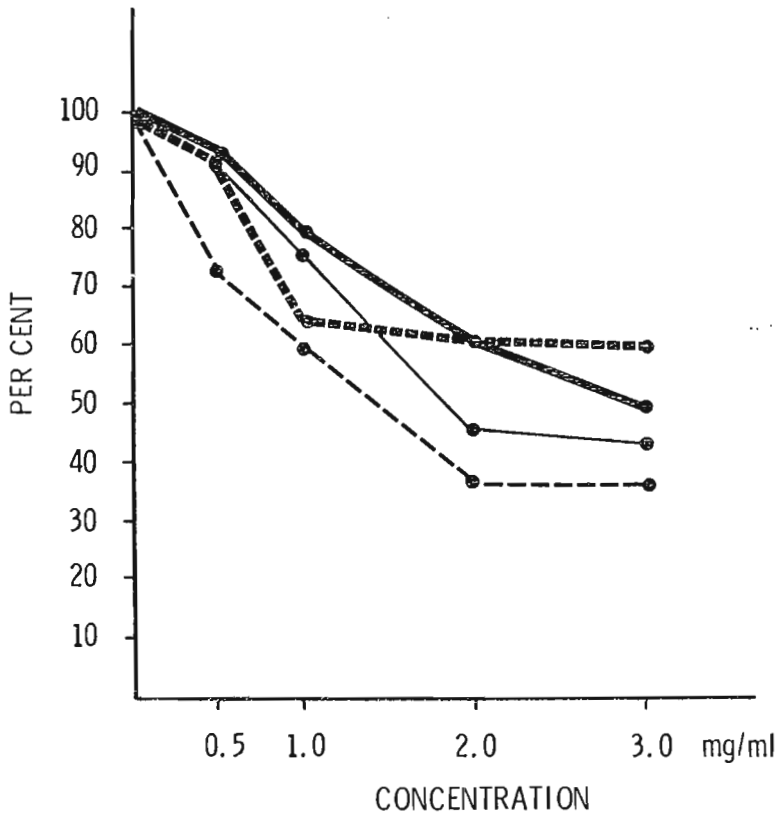

Fig. 1 Influence of Rutin on phagocytosis.
Thick solid line – phagocytosis (Ph) of viable *Staphylococcus aureus.*
Thick interrupted line – phagocytic index (PI) – viable *Staphylococcus aureus.*
Thin solid line – Ph of viable *Streptococcus faecalis.*
Thin interrupted line – PI – viable *Streptococcus faecalis.*
100 per cent corresponds to the control values determined in the presence of human serum but without Rutin. Control values were: *Staphylococcus aureus,* Ph – 80.3% and PI – 2.6; and *Streptococcus faecalis,* Ph – 93% and PI – 7.5.

To test whether BF influence directly the influence of lysozyme on the lysis of *Micrococcus lysodeikticus,* purified human lysozyme in concentration of 5, 10 and 15 μg/ml were mixed with Rutin 3.0 mg/ml or with Quercetin 300 μg/ml. The mixtures were applied to the wells of the lysoplates which were incubated for 18 hours. Lysozyme, Rutin, Quercetin and DMSO 0.5 % not mixed with each other served as controls. Lytic rings were estimated. Each experiment was done in duplicate.

RESULT

Phagocytosis and the phagocytic index

Phagocytosis and the phagocytic index of viable *Staphylococcus aureus* and *Streptococcus faecalis* were markedly suppressed by Rutin ($p<0.01$) when serum was present in the system (Fig. 1). Concentrations of 1 mg/ml and over ($\geq 1.5 \times 10^{-3}$ M/L) significantly inhibited the phagocytic index ($p<0.01$) of both micro-organisms. Significant ($p<0.01$) suppression of phagocytosis was achieved by using concentrations of 2 mg/ml (3×10^{-3} M/L). Without the serum, phagocytosis was only slightly suppressed (85% of the controls) even when tested at a concentration of 3.0 mg/ml. Rutosid at concentrations of 2.0 to 3.0 mg/ml reduced slightly the phagocytic index and phagocytosis of both micro-organisms to about 80 per cent of the control values. The influence of Quercetin was minimal; at a concentration of 25 μg/ml (7.4×10^{-5} M/L), it reduced the phagocytic index from 9.4 to 8.1 (86%) in the presence of human serum and from 9.2 to 7.0 (76%) without the serum. No influence on phagocytosis was detected within the range of 1.0 to 25μg/ml.

The influence of Rutin on enhancing the activity of polycationic substances was tested by adding Rutin 3.0 mg/ml (4.5×10^{-3} M/L) to a mixture of PMN and

bacteria to which poly-L-arginine (PLA) 5 μg/ml or poly-L-lysine (PLL) 5 μg/ml were added. Rutin abolished the enhancing activity of polycations regardless of whether serum was added or not. In the presence of serum, Rutin reduced PLA-enhanced phagocytosis from 82 per cent to 52 per cent and the phagocytic index from 9.5 to 2.7. Corresponding values without the serum were 59 per cent to 51 per cent; 4.3 to 2.3; 62 per cent to 49 per cent; and 5.2 to 3.6 respectively. As expected, neutral polyglutamic acid 10 μg/ml did not influence phagocytosis when compared with the controls, but Rutin added to the mixture reduced phagocytosis from 79 per cent to 28 per cent and the phagocytic index from 5.2 to 2.2.

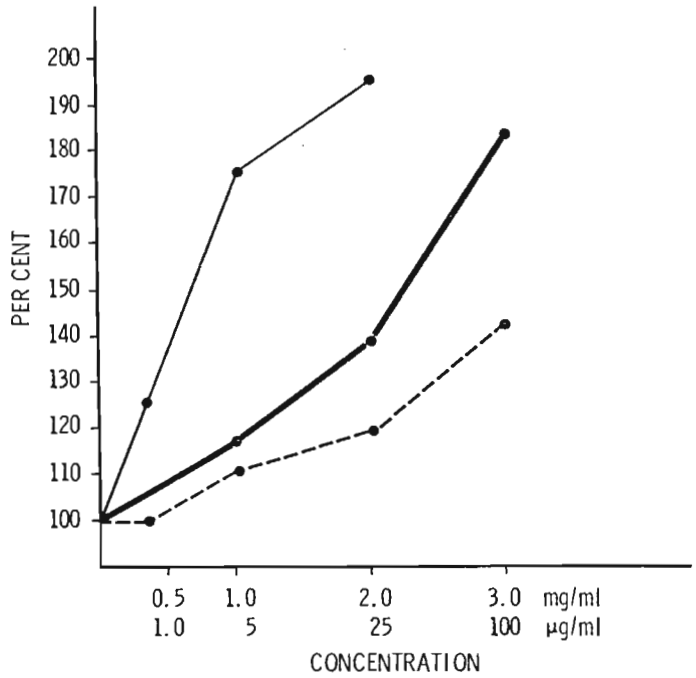

Fig. 2 Effect of bioflavonoids on intracellular bactericidal activity (ICBA) index
Thin solid line – Rutin; Thick solid line – Quercetin; Interrupted line – Rutosid
Rutin and Rutosid expressed in mg/ml. Quercetin expressed in μg/ml.
Increase in the ICBA index means an increase in the number of residual bacterial colonies, i.e., suppressed intracellular bactericidal activity.

Intracellular bactericidal activity (ICBA)

In the presence of human serum, BF markedly increased the ICBA index, i.e., they suppressed ICBA (Fig. 2). Rutin was the strongest inhibitor, inhibiting intracellular bactericidal activity significantly ($p<0.01$) in a concentration of 0.5 mg/ml (7.5×10^{-4} M/L). Quercetin over 1 μg/ml (3×10^{-6}M/L) and Rutosid over 2 mg/ml (2.7×10^{-3} M/L) were strong inhibitors ($p< 0.01$) as well. When no serum was added, BF did not influence ICBA significantly.

Chemotactic activity

Two systems were investigated. In one, PMN were pre-incubated with BF with and without human serum and then tested for chemotactic activity towards FLMP or *E. coli* filtrate. Marked suppression of chemotactic activity ($p<0.01$) was observed when Quercetin 5 μg/ml ($\sim 1.5 \times 10^{-5}$ M/L), Rutosid 0.5 mg/ml (6.7×10^{-4} ML/) or Rutin 1 mg/ml (1.5×10^{-3} M/L) were added to the cells (Fig. 3). When serum was added as well, higher concentrations of BF were required to suppress chemotactic activity, i.e., 25 μg/ml, 1.5 mg/ml and 5 mg/ml respectively. Similar concentrations were required when *E. coli* filtrate was used as a chemo-attractant.

When BF were added to the chemo-attractant FLMP, marked suppression of chemotactic activity was evident with Quercetin and Rutin (Fig. 4). When no serum was added to PMN, less than 5 μg/ml Quercetin and about 0.5 mg/ml Rutin were necessary to reduce the chemo-attractive influence of FLMP to 40-50 per cent. Rutosid had very little influence in reducing chemotaxis to 75 per cent at a concentration of 1.5 mg/ml. When serum was added to PMN, much more BF were ncessary to inhibit chemotaxis of PMN towards FLMP (Fig. 4). Bioflavonoids *per se* did not have chemo-attractant activity.

In a separate experiment, PMN were mixed with FLMP (10%) with and without human serum and the mixture was tested for chemotaxis towards FLMP. In such a situation, FLMP mixed with PMN should markedly inhibited chemotactic activity. Indeed, a drop to about 30 per cent of the control values was observed. When Rutin was added to the mixture, immediate but not complete restoration of chemotactic activity was observed (Fig. 5). Identical results were observed with Quercetin.

Release of lysozyme

Polymorphonuclear cells in aliquots of 250,000 released in 18 hours sufficient quantities of

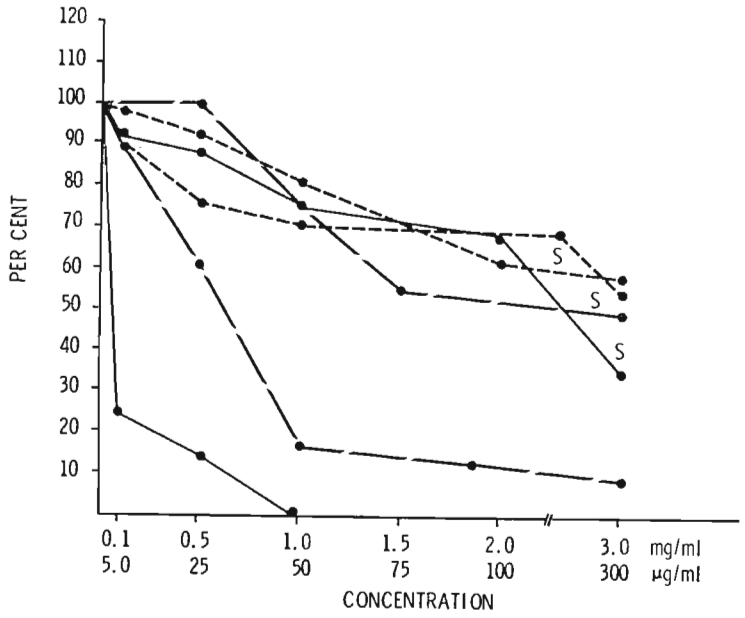

Fig. 3 Influence of bioflavonoids on chemotaxis
Bioflavonoids with or without serum added to the cells.
Chemo-attractant – FLMP.
Solid line – Quercetin (µg/ml). Interrupted (long) line – Rutosid (mg/ml).
Interrupted (short) line – Rutin (mg/ml)
S = serum added

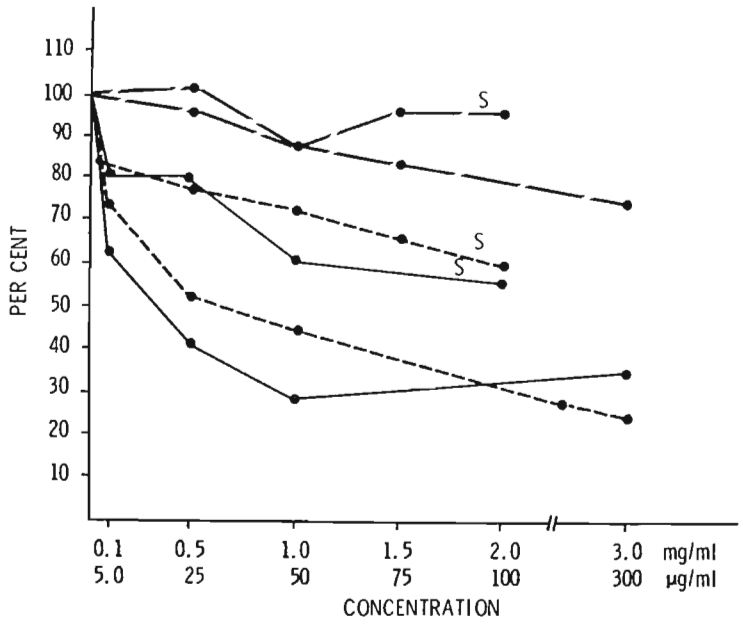

Fig. 4 Influence of bioflavonoids on chemotaxis
Bioflavonoids were added to FLMP.
Solid line – Quercetin (µ g/ml).
Interrupted (lone) line – Rutosid (mg/ml).
Interrupted (short) line – Rutin (mg/ml).
S – Serum added to the cells.

lysozyme to produce lytic rings corresponding to 7.3-7.5 µg/ml of lysozyme. When Rutin was added to the cells in concentrations of 0.5 to 3.0 mg/ml, smaller rings were observed corresponding to 6.2 µg/ml. Quercetin added in concentrations of 50 µg, 100 µg, 200 µg and 300 µg/ml caused marked diminution of the lytic rings corresponding to 2.9, 1.4, 0.6 and 0.5 µg/ml of lysozyme standard, respectively. In order to distinguish between inhibition of release from the cells and inhibition of interaction of lysozyme with its substrate, *Micrococcus lysodeikticus,* pure human lysozyme was mixed with BF; the mixture was applied to the lysoplate wells and incubated for 18 hours. Under these conditions Quercetin markedly inhibited lytic reaction whereas Rutin was much less inhibitory (Fig. 6). Thus, BF inhibit enzyme/substrate interaction. This inhibition makes it difficult to assess their inhibitory activity on enzyme release from the cells.

DISCUSSION

Interest in Bioflavonoids has recently been stimulated by detection of their activity on various cells. BF inhibit histamine release from antigen-stimulated human basophils.[3,4] It was noted that the biologically most active compound, Quercetin, is chemically related to the anti-allergic drug cromolyn, thus their action may be similar in affecting the microtubule function of cells.[3] BF exhibited potent viricidal activity[14] and were found to be mutagenic to *Salmonella typhi murium.*[15] The inhibitory influence of BF on the growth of tumour cells was observed *in vitro* and *in vivo.*[16,17] BF inhibited the glycolysis of Ehrlich ascites tumour cells by interference with the generation of adenosine diphosphate and inorganic phosphate[16] and increased the rate of survival of mice inoculated with tumour cells.[17] The influence of BF on normal cells was

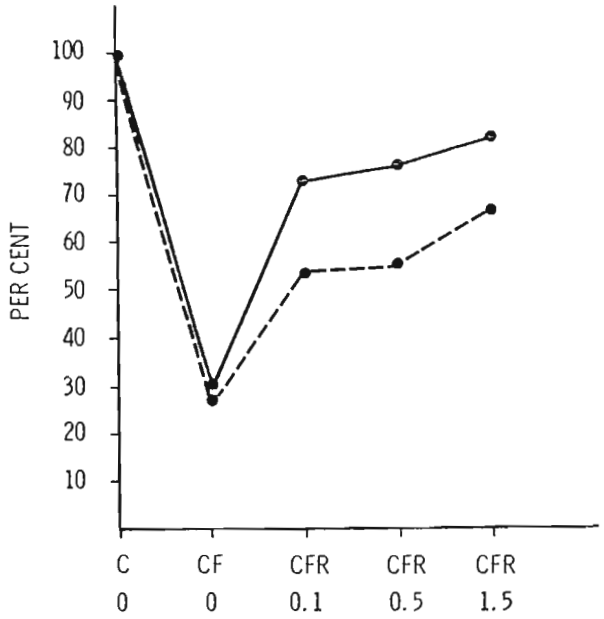

Fig. 5 Influence of Rutin on inhibitory activity of FLMP added to polymorphonuclears. Chemotactic activity expressed as a percentage of control cells.
C – Cells without FLMP (Control). CF – Cells with FLMP.
CFR – Cells with FLMP and Rutin added in concentrations of 0.1, 0.5 and 1.5 mg/ml.

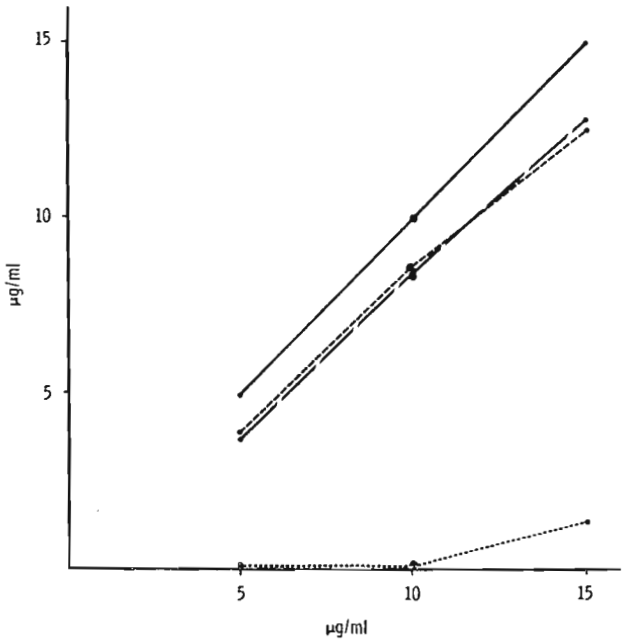

Fig. 6 Influence of bioflavonoids on interaction of lysozyme with the substrate
Solid line – Standards of lysozyme 5, 10 and 15 μg/ml.
Dotted line – Quercetin 300 μg/ml added to lysozyme standards.
Interrupted (lone) line – Rutin 3.0 mg/ml added to lysozyme standards.
Interrupted (short) line – DMSO 0.5% added to lysozyme standards.
Actual readings (on abscissa) are expressed as μg/ml of lysozyme standards.

documented by observing that Rutin and Quercetin inhibited PHA induced ^{3}H-thymidine incorporation into the DNA of human peripheral blood lymphocytes.[14] Furthermore, Quercetin inhibited *in vitro* cytotoxic T lymphocyte generation and effector functions, blocking CON-A induced DNA synthesis.[18] It has also been shown that Rutin an Rutosid stabilized lysosomes of Wistar rat's liver cells and blocked the release of cathepsins from the cells.[5] A recent study demonstrated that, in a concentration-dependent manner, Quercetin inhibits the release of β-glucuronidase from human polymorphonuclears stimulated by zymosan-activated serum.[7] It was suggested as well that Quercetin inhibits phospholipase A_2 since it has been shown to inhibit the release of ^{3}H-arachidonic acid from prelabelled PMN stimulated by zymosan-activated serum.[7] Thus, it was of interest to determine whether BF are capable of influencing various phagocytic functions of human PMN.

Our study shows that BF exert a marked inhibitory influence on various aspects of phagocytic activity. The observation of particular interest was that various BF had a different influence on PMN and that the presence of serum generally enhanced the inhibitory activity of BF. Of the three BF tested, Rutin was the strongest inhibitor of phagocytosis (Ph), phagocytic index (PI) and intracellular bactericidal activity (ICBA) of human PMN. Its influence on chemotaxis was moderate. Rutin inhibited the activity of FLMP as a chemo-attractant and reduced FLMP's inhibitory activity when it was added to the PMN. Rutosid had no marked influence on Ph, PI or ICBA but it suppressed markedly the chemotaxis of PMN. In contrast to Rutin, Rutosid had no influence on the chemotactic activity of FLMP. Quercetin had little influence on Ph and PI, but in the presence of serum it markedly suppressed ICBA. It also markedly suppressed chemotaxis of PMN and

inhibited the chemotactic activity of FLMP. Quercetin seemed to influence markedly the release of lysozyme from polymorphonuclear cells. However, it was found that Quercetin inhibits *in vitro* lysis of *Micrococcus lysodeikticus* by lysozyme. Such interference in the interaction of the enzyme with its substrate made it impossible to assess accurately the release of lysozyme from the cells.

Different action of various BF has already been noticed in the past. For instance, Quercetin but not Rutin was capable of inhibiting histamine release,[3] killing viruses[14] or inducing mutation in Gram-negative micro-organisms.[15] On the other hand, both Rutin and Quercetin inhibited tumour cells' growth *in vivo*[17] and both inhibited thymidine incorporation into DNA.[14] In the latter assay, 2½ times more Rutin than Quercetin was necessary to obtain significant results.[14] Both Rutin and Rutosid were capable of stabilizing lysosomes.[4] Variability in the influence of various BF most probably depends on differences in their chemical structure. It was suggested that compounds with a C_4-keto group but without A or B ring hydroxyls or alternatively compounds with A and B ring hydroxyls but without the C_4-keto group are inactive.[4] In our study, Quercetin and Rutin but not Rutosid had the most pronounced influence on biological functions of PMN. However, Rutosid was capable of inhibiting quite markedly the chemotactic activity of PMN. Much lower concentrations of Quercetin were necessary to obtain inhibitory activity. The concentrations of BF used in the present study conformed with those by other investigators.[3,4,14-18] To our knowledge, plasma concentrations of BF in humans have not been estimated. The average diet contains about 1 gram per day of mixed flavonoids. It was suggested that this amount is sufficient to achieve concentration which affects immunological and other cellular functions.[4] Pharmacological dosage of various BF vary. Rutosid has been given orally up to 1,000 mg/day;[19] Rutin, 180-400 mg/day; and Quercetin, 60-400 mg/day.[20] Since neither the biological level nor pharmacological concentrations of BF are known, one cannot extrapolate results obtained *in vitro* and apply them to *in vivo* situations. It is obvious, however, that BF exert a profound, usually inhibitory, influence on various functions of mammalian cells, tumour cells, bacteria and viruses. Such an influence may require reassessment of biological functions of BF and may possibly have clinical applications.

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