MOLECULAR PHARMACOLOGY, 23:698-702

Interaction of Metal-Complexing Compounds with Lymphocytes and Lymphoid Cell Lines

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Received December 2, 1982; Accepted December 2, 1982

SUMMARY

A variety of metal-complexing flavonoids (β-hydroxyethyl rutoside, catechin, naringin, taxifolin, and quercetin) as well as several compounds which form mainly iron complexes (bathophenathroline, bathophenanthrolinebisulfonic acid, desferri-ferrioxamine B, ethylenediamine-di-*o*-hydroxyphenylacetic acid) were tested for their effects on mitogen-free and mitogen-activated mouse splenocyte cell cultures and on two human cell lines (Daudi, Bristol-8). The compounds strongly inhibited the incorporation of [^{3}H]thymidine into DNA at agent concentrations ranging from 10 to 500 μM. On the other hand, at subinhibitory concentrations, slightly enhancing or synergistic effects were observed. We conclude that immune cell behavior can be bidirectionally influenced by the presence of metal-complexing agents, depending on their concentration in the medium.

INTRODUCTION

There is evidence for an important role of divalent cations in the process of lymphocyte activation (1). The *Streptomyces* antibiotic A 23187, an ionophore specific for divalent cations, is a lymphocyte mitogen (2). The mitogenic lectin phytohemagglutinin initiates a slow accumulation of Ca^{2+} by lymphocytes (3, 4). Ca^{2+} is required for lymphocyte activation and proliferation (1). The requirement for Mg^{2+} was reported for the binding of sensitized lymphocytes to target cells (5, 6). It could also be demonstrated that complex-forming compounds such as citrate, EDTA (7), and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (8) impede cell activation. In this study we investigated the *in vitro* effects of a variety of metal-complexing compounds on resting and mitogen-activated mouse lymphocytes and on two human lymphoid cell lines.

MATERIALS AND METHODS

Lymphocytes. Lymphocytes from C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Me.) were prepared from spleens as described previously (9). Human lymphoid cell lines (Daudi, Bristol-8) were obtained from A. Ziegler (Tuebingen, Federal Republic of Germany).

Agents. Flavonoids (β-hydroxyethyl rutoside, catechin, naringin, taxifolin, and quercetin) were obtained from Zyma (Muenchen). The iron-complexing substances bathophenanthroline, bathophenanthrolinebisulfonic acid, desferri-ferrioxamine B, and ethylenediamine-di-*o*-hydroxyphenylacetic acid were kindly provided by K. Hantke (Tuebingen). Con A[4] was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Lipoprotein was prepared as described (9).

Cell cultures. Leukocyte cultures were carried out as described previously (9) in flat-bottom Falcon microtiter plates. In short, cultures were performed in 0.15-ml aliquots at a cell density of 3×10^6/ml in RPMI 1640 medium supplemented with 3.3% heat-inactivated human AB serum (Flow Laboratories), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (5×10^{-5} M). Cell proliferation was measured by the incorporation of [^{3}H]thymidine into DNA. Cultures were pulsed for 24 hr before harvesting by the addition to each well of 0.5 μCi of [^{3}H]thymidine (Amersham, Braunschweig), specific activity 40 Ci/mole.

RESULTS

In Fig. 1 the effects of the addition of metal-complexing flavonoids on [^{3}H]thymidine incorporation in LPS nonresponder C3H/HeJ mouse spleen cells are demonstrated. For catechin we found a weakly stimulatory effect at concentrations ranging from 50 to 100 μM; above 500 μM the compound exhibited a strongly inhibitory effect. For β-hydroxyethyl rutoside, inhibitory effects were observed at concentrations above 100 μM, and for taxifolin above 20 μM, for naringin above 200 μM, and for quercetin above 10 μM. Thus, all flavonoids tested showed inhibitory effects at concentrations ranging from 10 to 500 μM. At subinhibitory concentrations most of the substances were weakly stimulatory toward mouse splenocytes.

This work was supported by Grant SFB 76 from the Deutsche Forschungsgemeinschaft.

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[4] The abbreviation used is: con A, concanavalin A.

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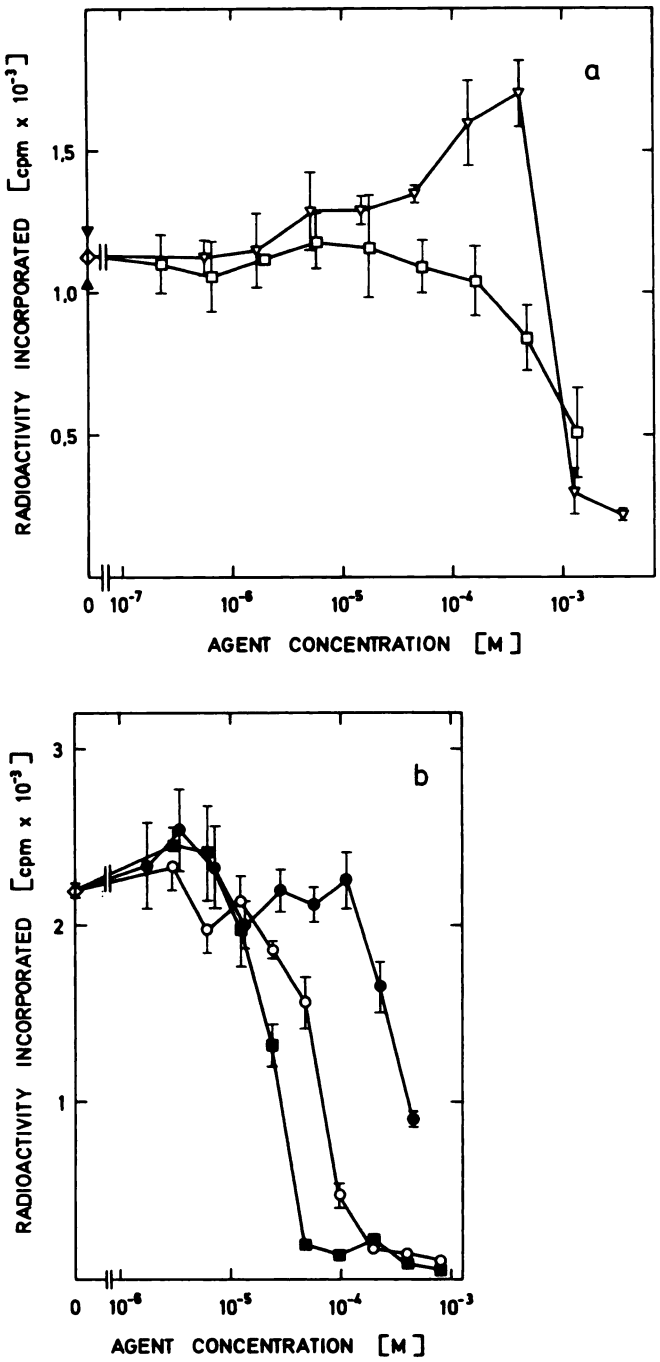

FIG. 1. *Thymidine incorporation in cultured C3H/HeJ mouse splenocytes*

Thymidine incorporation in splenocytes (4.5×10^5 cells) after the addition of catechin (▽) and β-hydroxyethyl rutoside (□) (a), and naringin (●), quercetin (■), and taxifolin (○) (b) to the cultures. ◇, Control, without the addition of agents. Duration of cultivation, 48 hr. Means of triplicate experiments ± standard deviations.

TABLE 1

Effect of several iron-complexing compounds on the incorporation of [³H]thymidine in C3H/HeJ mouse spleen cells

Splenocytes were cultured for 48 hr in serum-free medium (Iscove's modified Dulbecco's medium) in the presence of antibiotics as indicated under Materials and Methods. Means of triplicate experiments ± standard deviations.

Iron-complexing compounds	Drug concentration	[³H]Thymidine incorporation
	μM	cpm
Bathphenanthroline	0	1101 ± 84
	0.27	1036 ± 80
	1.33	993 ± 179
	6.64	382 ± 76
	33.2	109 ± 45
	166	35 ± 6
	830	78 ± 33
Bathophenanthrolinebisulfonic acid	0	1101 ± 84
	0.15	1113 ± 68
	0.75	1149 ± 150
	3.76	1127 ± 42
	18.8	1242 ± 141
	94	943 ± 28
	470	38 ± 6
Desferri-ferrioxamine B	0	1101 ± 84
	0.16	1324 ± 87
	0.78	1283 ± 134
	3.92	1295 ± 112
	19.6	1264 ± 137
	98	823 ± 67
	490	95 ± 21
Ethylenediamine-di-*O*-hydroxyphenylacetic acid	0	1101 ± 84
	0.24	1357 ± 136
	1.18	1383 ± 114
	5.92	1323 ± 151
	29.6	1286 ± 192
	148	889 ± 9
	740	34 ± 8

Several metal-complexing compounds which are mainly iron chelators were also tested for their effect on primary lymphocyte cultures (Table 1). The synthetic iron chelators ethylenediamine-di-*o*-hydroxyphenylacetic acid and desferri-ferrioxamine as well as the iron-chelator bathophenathrolinebisulfonic acid were inhibitory at concentrations above 100 μM. The more hydrophobic iron-complexing compound bathophenanthroline was by far more active, and strongly inhibited thymidine incorporation starting at about 10 μM. Most of the substances also exhibited a weakly enhancing effect at sub-inhibitory concentrations.

In order to test whether the inhibitory activity of the two most active compounds, quercetin and bathophenanthroline, could be reversed by the addition of divalent metal ions, we added magnesium, calcium, manganese, and iron ions at drug to metal molar ratios of 1:10 and 1:100 to drug-containing cultures. As seen from Table 2, the suppression induced by quercetin could be partially reversed by the addition of a 10- to 100-fold excess of these ions ($Ca^{2+} > Fe^{2+} > Mg^{2+} > Mn^{2+}$). For bathophenanthroline, a partial reversion of the inhibitory effect could be found after the addition of Mn^{2+} or Fe^{2+} to the cultures, whereas Mg^{2+} or Ca^{2+} had only a marginal effect.

We then investigated whether lymphocyte cultures carried out in the presence of T- or B-lymphocyte mitogens could be influenced by the addition of metal-complexing flavonoids. With catechin the stimulatory activity of the T-lymphocyte mitogen con A was strongly in-

TABLE 2

Partial reversal of the inhibitory effects induced by 830 μM bathophenanthroline or by 810 μM quercetin by the addition of divalent metal salts at different molar ratios to the cultures

Control cultures contained no additions. Values are means of triplicate determinations.

Metal	Inhibitory drug					
	Bathophenanthoroline (drug:metal molar ratio)			Quercetin (drug:metal molar ratio)		
	1:0	1:10	1:100	1:0	1:10	1:100
	% inhibition of control cultures					
Mg^{2+}	87.3	87.1	85.0	84.9	74.8	69.9
Ca^{2+}	87.3	81.5	86.0	84.9	60.0	41.2
Mn^{2+}	87.3	78.7	60.8	84.9	81.3	70.5
Fe^{2+}	87.3	78.2	57.1	84.9	81.3	59.3

hibited at concentrations above 100 μM (Fig. 2a). The stimulatory activity of the B-lymphocyte mitogen lipoprotein was markedly inhibited at concentrations above 400 μM, and a marginally synergistic effect was found at catechin concentrations around 10 μM (Fig. 2b). We also tested the effect of β-hydroxyethyl rutoside and found that the stimulatory effect of con A was slightly decreased at flavonoid concentrations above 100 μM (Fig. 2a). The stimulatory activity of lipoprotein (Fig. 2b) was marginally increased at concentrations around 10 μM, and at concentrations above 100 μM inhibitory activity was observed.

We also investigated the effects of flavonoids toward continuously proliferating cell lines (Daudi, Bristol-8), as shown in Fig. 3. Catechin inhibited the growth of the Bristol-8 cell line at concentrations above 50 μM, and at concentrations above 100 μM inhibitory activity toward the Daudi cell line was observed. Similar to the results obtained in primary cell cultures, at subinhibitory catechin concentrations, enhanced thymidine incorporation was seen. Also, the results obtained for β-hydroxyethyl rutoside closely resembled our findings obtained in the primary cell cultures. The compound had a very slight inhibitory effect on Daudi cells at high concentrations and caused enhanced thymidine incorporation at subinhibitory concentrations. Taxifolin inhibited the Bristol-8 line starting at concentrations around 10 μM. Naringin exhibited only at high concentrations (above 500 μM) an inhibitory effect on Bristol-8 cells, and Daudi cells were not affected. Quercetin was strongly inhibitory starting at concentrations around 5 μM (Bristol-8) and 10 μM (Daudi).

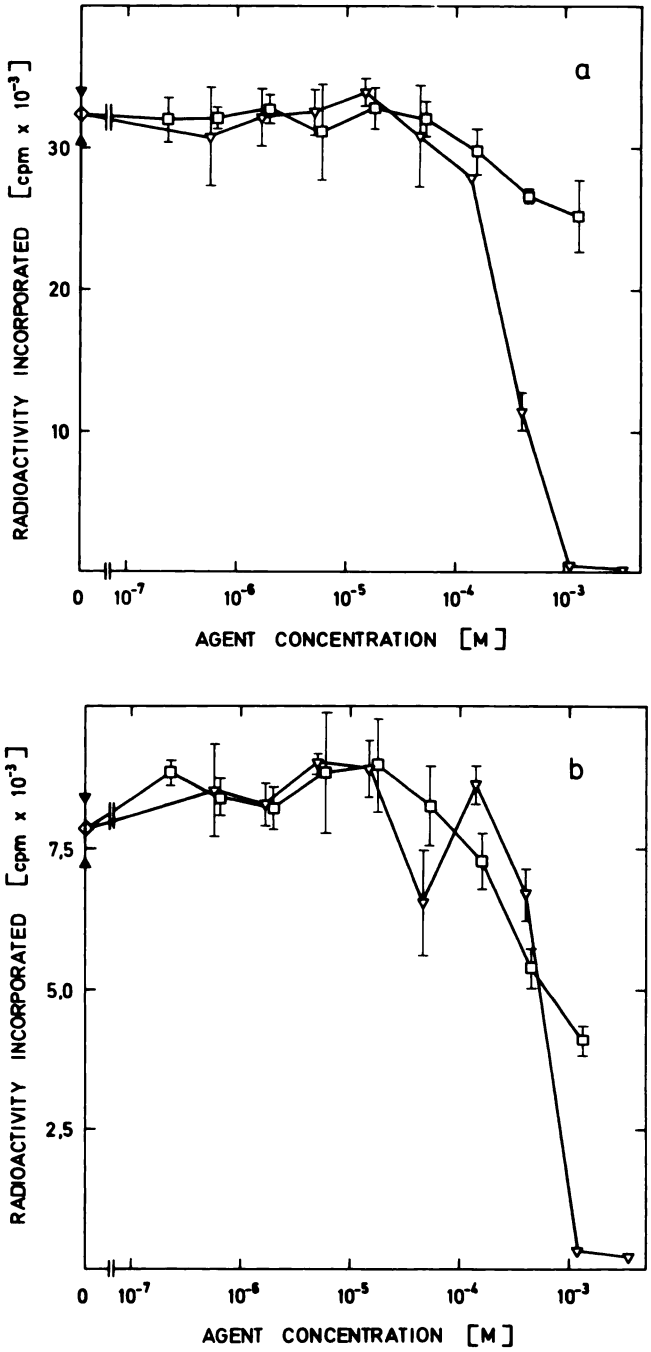

FIG. 2. *Thymidine incorporation in cultured C3H/HeJ mouse splenocytes (4.5 × 10⁵ cells)*

Cells were stimulated with con A (1 μg/ml) (a) or with lipoprotein (75 μg/ml) (b) in the presence of various concentrations of catechin (▽) or β-hydroxyethyl rutoside (□). Duration of cultivation, 48 hr. Means of triplicate experiments ± standard deviations. ◇, Control, without addition of flavonoids.

DISCUSSION

The metal-complexing properties of the substances tested in this study could be demonstrated by a variety of methods, including UV and VIS absorption spectroscopy (10). From CD studies of natural and modified flavonoids, the complexing behavior with bivalent cations was demonstrated for a variety of hydroxyethylated rutosides (11). This is in agreement with the proposal that flavonoids with hydroxyl functions at C-3 or C-5, and with an *ortho*-dihydroxy system, form metal complexes (12). An additional variety of compounds tested here are mainly chelators of iron. Bathophenanthrolinebisulfonic acid is a well-defined, water-soluble agent for the determination of iron in serum (13); bathophenanthroline exhibits increased hydrophobicity. Desferri-ferrioxamine B is an iron-complexing substance isolated from *Streptomyces* strains (14), and ethylenediamine-di-*o*-hydroxyphenylacetic acid represents a synthetic iron chelator (15).

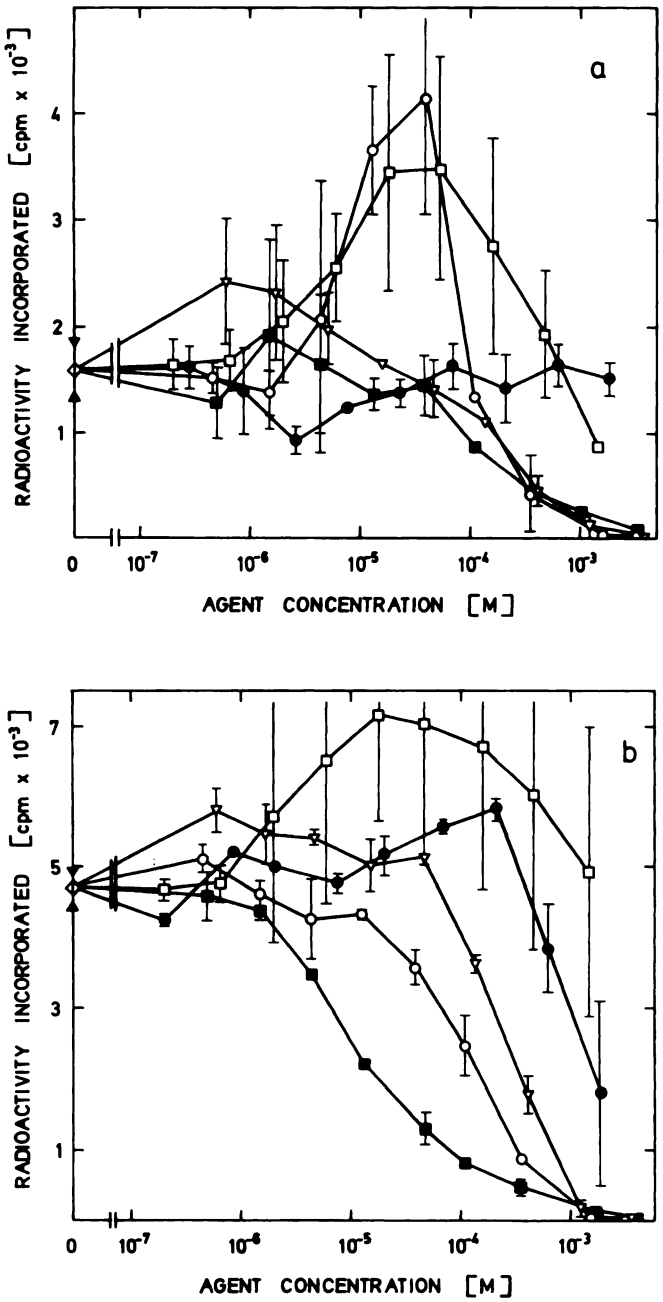

FIG. 3. *Thymidine incorporation in two cultured human lymphoid cell lines (4.5 × 10⁵ cells)*

Daudi cells (a) and Bristol-8 cells (b) in the presence of various concentrations of catechin (▽), β-hydroxyethyl rutoside (□), taxifolin (○), naringin (●), and quercetin (■). Cells were cultured for 42 hr in the presence of 10% inactivated fetal calf serum. Means of duplicate experiments ± standard deviations. ◇, Control, without addition of agents.

In our experiments a variety of flavonoids showed, at high concentrations, a strongly inhibitory effect on [^{3}H] thymidine incorporation. The effects found in mitogen-stimulated cell cultures or in cell lines closely resembled the effects in mitogen-free systems (Figs. 1–3). Similar inhibitory properties were found by us when testing the iron-complexing agents. These compounds, which possess activation constants for the ferric ion up to 10^{30}, markedly impeded lymphocyte growth at concentrations above 10–100 μM. The suppressive effects of the two most active substances, quercetin and bathophenanthroline, could be partially reversed by the addition of divalent cations (Fe^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}) to the cultures, suggesting that the inhibition is caused partially by the deprivation of cations from the medium. It remains to be determined whether the residual inhibitory effects are due to membrane-damaging or cell-toxic effects. The behavior of the metal-complexing compounds tested is similar to the action of a variety of other ionophorous and/or membrane-interacting substances. The Ca^{2+}-ionophore A 23187 (2), the membrane-modifying peptide antibiotics from *Trichoderma viride* (9), and a variety of B-cell mitogenic ionophores (16), also inhibited leukocyte growth at high concentrations. Our results for desferri-ferrioxamine B are in agreement with studies performed with this compound in both microbial and mammalian cells. Arcenaux and Byers (17) have reported the inhibition of DNA synthesis by desferri-ferrioxamine B (Desferal) in a mutant strain of *Bacillus subtilis*. Robbins and Pederson (18) and Robbins *et al.* (19) could show that desferrioxamine B decreases the uptake of [^{3}H]thymidine into HeLa cells, and Hoffbrand *et al.* (20) demonstrated a reduction of DNA synthesis in phytohemagglutinin-stimulated lymphocytes after the addition of ferrioxamine B. In the experiments reported, RNA or protein synthesis was not affected by the compound, and the inhibition could be reversed by the further addition of iron.

Remarkably, most of the complex-forming compounds exhibited, at subinhibitory concentrations, weakly stimulatory or synergistic effects. In both mitogen-free cultures and mitogen-containing cultures, and in cell lines, these enhancing effects could be observed. They might in part be due to facilitating the uptake of scarce amounts of iron into cells by the complex-forming agents, which could be stimulating ribonucleotide reductase (21). Accordingly, we also found[5] that subinhibitory concentrations of catechine and β-hydroxyethyl rutoside had a similar beneficial effect on serum-free cell line cultures, as it has been reported for transferrin (22). Regarding the flavonoids tested, the enhancing effects could also be due to the membrane-protecting and stabilizing properties of the compounds as described by Hennings (23). The immune-stimulating properties of the flavonoids may be of further interest, since these and similar substances are applied therapeutically in diseases accompanied by disorders of membrane architecture. Hydroxyethyl rutosides are used in the treatment of chronic venous insufficiency and also as radioprotective agents (24). Besides direct membrane-stabilizing properties catechin exhibits a strong inhibitory activity against substances and processes which induce lipoperoxydation and subsequent membrane lesions (25–27). This mechanistic view of the activity of catechin in various liver diseases has been reviewed elsewhere (23). Interestingly, in clinical studies an increase in elimination of the HB_sAg after flavonoid

[5] W. G. Bessler and C. Komischke, unpublished data.

treatment has been shown (28) which could be related to the enhancing effects reported here. Also, the efficiency of the plasma membrane Na^+/K^+ pump of Ehrlich ascites tumor cells is enhanced by quercetin (29). On the other hand, when applying metal-complexing agents to patients with iron-overload poisoning, or complexed iron to patients with deficiency diseases (30), the effect of the compounds on immune cells should be considered.

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