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Oral flavonoids delay recovery from experimental autoimmune encephalomyelitis in SJL mice

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Abstract

Flavonoids are food components that appear to have potential beneficial health effects. There is a range of in vitro studies supporting the anti-oxidant and anti-inflammatory properties of flavonoids. Previously, we demonstrated that in vitro flavonoids, including luteolin and apigenin, inhibit proliferation and IFN-γ production by murine and human autoimmune T cells. In the present study, we examined the effects of oral flavonoids as well as of curcumin on autoimmune T cell reactivity in mice and on the course of experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis.

Continuous oral administration of flavonoids significantly affected antigen-specific proliferation and IFN-γ production by lymph node-derived T cells following immunization with an EAE-inducing peptide. Both luteolin and apigenin suppress proliferative responses as they did in vitro, whereas IFN-γ production on the other hand was enhanced. Other flavonoids exerted differential effects on proliferation and IFN-γ production. The effects of flavonoids and curcumin on EAE were assessed using either passive transfer of autoimmune T cells or active disease induction. In passive EAE, flavonoids led to delayed recovery of clinical symptoms rather than to any reduction in disease. In active EAE, the effects were less pronounced but also, in this case, the flavonoid hesperitin delayed recovery. Oral curcumin had overall mild but beneficial effects. Our results indicate that oral flavonoids fail to beneficially influence the course of EAE in mice but, instead, suppress recovery from acute inflammatory damage.

Keywords: Flavonoids; Apigenin; Luteolin; T cells; EAE

1. Introduction

Flavonoids form a large group of naturally occurring nutritional components, abundantly present in vegetables, fruit and natural beverages. Based on variations in their basic structure, flavonoids are divided into nine subclasses, of which the major three are flavones, flavonols and flavanones [1,2]. Flavonoids are known for their strong anti-oxidant properties, protecting tissues against oxidative stress. Several reports have suggested that diseases associated with oxidative stress and inflammatory diseases may be beneficially influenced by flavonoids [3,4]. Flavonoids reduce the incidence of cerebrovascular disease in humans [5], exert protective effects in cardiovascular diseases and cancer [4] and upon intraperitoneal administration, inhibit inflammation in an animal model of arthritis [6,7].

Flavonoids influence a diverse range of intracellular signaling events depending on different features in the basic structure of flavonoids [8–10]. Considering the importance of intracellular signaling involved in activation, proliferation and differentiation of lymphocytes, these cells are potential targets for modulation by flavonoids. Indeed, recent data reveal that in vitro flavonoids inhibit antigen-specific memory T cell proliferation and pro-inflammatory IFN-γ production [11] and reduce the phagocytic activity by macrophages [12]. The interaction of flavonoids with several single cell populations in vitro has been described extensively. Yet, it remains to be fully clarified to what extent the in vitro effects of flavonoids also manifests themselves in vivo upon oral administration. Importantly, the systemic effects of oral compounds are critically influenced not only by absorption and distribution, but also by substantial structural modification of

Abbreviations: PLP, proteolipid protein; IFN, interferon; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis

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flavonoids that accompanies their uptake [3,13]. Thus, also potentially bioactive metabolites will appear.

Apart from flavonoids also the nutritional component curcumin, a derivative of the plant *Curcuma longa*, is well known for its anti-oxidant and anti-inflammatory properties [14,15]. In the mouse model of experimental autoimmune encephalomyelitis (EAE) intraperitoneal administration of curcumin strongly inhibits disease development [16]. Although the actions of curcumin are not yet fully understood and may differ from the actions of flavonoids, it has been reported that curcumin inhibits important transcription factors, such as NFκB, IκB and AP-1 that are involved in the induction of pro-inflammatory cytokines like TNF-α, IL-1β and IL-12 [17–21].

Beneficial effects of dietary components have previously been reported in both the chronic inflammatory disease multiple sclerosis (MS) and in EAE, an animal model for inflammation of the central nervous system [22–29]. The therapeutic potential of these food components is currently evaluated. Our previous data suggest that flavonoids could have beneficial effects during the pathogenesis of MS and/or EAE since some of them have a strong inhibitory effect in vitro on proliferation and IFN-γ production of auto-antigen-specific T cells [11,30].

In the present study, the effects of oral compounds were examined on the development of autoimmune T cell responses in vivo and on the clinical course of EAE induced either by T cell transfer or by active immunization. Our data indicate that at the dose used in this study, oral flavonoids can inhibit autoimmune antigen-specific T cell reactivity in vivo. Somewhat unexpectedly, however, the major effect of oral flavonoids in EAE and in particular in passive EAE was found to be an inhibition of recovery following acute inflammatory damage. With the exception of curcumin that did exert mild beneficial effects, oral flavonoids therefore do not exert uncomplicated beneficial effects in murine EAE.

2. Materials and methods

2.1. Test compounds

The compounds apigenin, luteolin, quercetin and hesperitin were obtained from Kaden Biochemicals (Hamburg, Germany), morin and fisetin were acquired from Indofine Chemical Company (Belle Mead, NJ) and curcumin was purchased from Aldrich Chemie Co. (Milwaukee, WI). Each component was dissolved in 50 mM KOH at a final concentration of 2.5 mg/mL and supplemented with 1.5 mg/mL aspartame and 0.1% (w/v) white chocolate flavor (Numico Research, Wageningen, The Netherlands) to mask any taste difference and improve palatability. Of all solutions, the pH was set at pH 10 with 5N HCl. Mice had free and easy access to the solutions even when they developed clinical signs of EAE. The solution was replaced with a fresh solution every other day and average daily intake was monitored. Based on an average daily intake of 4 mL of the solution, each animal ingested a total amount of about 10 mg of the test component per day. Mice were fed with the test components, starting three days before immunization with $PLP_{139–151}$. In passive EAE the treatment continued until 10 days after immunization after which T cells were isolated and transferred into healthy recipients. In the case of active EAE, the treatment continued until day 21.

2.2. Induction of EAE

Female SJL/J mice were obtained from Janvier (Bioservices, Schuijk, The Netherlands) and kept under specific pathogen-free conditions. All mice were used between the ages of 8 and 12 weeks. Passive EAE was induced by immunizing SJL mice subcutaneously with 50 μg of the PLP peptide 139–151, emulsified in complete Freund's adjuvant containing 1 mg/mL *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI). Ten days after immunization, spleen, as well as auxiliary and inguinal lymph nodes, were isolated. Splenocytes and lymphocytes were seeded at 2×10^6 cells per mL in RPMI-1640 culture medium (BioWhittaker, Verviers, Belgium) supplemented with 100 U /mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercapthoetanol, 10 mM glutamax and 5% (v/v) FCS (BioWhittaker, Verviers, Belgium) and stimulated with 25 μg/mL $PLP_{139–151}$. T cells were harvested after three days of culture. The cells were washed twice with PBS and 2.5×10^6 T cell blasts were transferred intravenously into recipient mice. Recipient mice were injected intravenously on the day of transfer and again 72 h later with 9×10^{10} *Bordetella pertussis* bacteria (RIVM, Bilthoven, The Netherlands).

Active EAE was induced by immunizing SJL mice subcutaneously in the flank with 50 μg $PLP_{139–151}$ emulsified in complete Freund's adjuvant containing 1 mg/mL *M. tuberculosis* H37RA. On the day of immunization, and again 72 h later, mice were injected intravenously with 9×10^{10} *B. pertussis* bacteria. Mice were examined daily for clinical signs and were scored as follows: grade 0, no clinical signs; grade 0.5, partial tail paralysis; grade 1, complete tail paralysis; grade 2, paraparesis, limb weakness and complete tail paralysis; grade 3, complete hind or front limb paralysis; grade 3.5, paraplegia; grade 4, quadriplegia; grade 5, death.

2.3. T cell culture and cytokine assays

Splenocytes as well as cells isolated from the auxiliary and inguinal lymph nodes were seeded at 1×10^5 cells per well in the presence of 2×10^5 syngeneic irradiated (30 Gy) splenocytes from naive mice in 200 μL RPMI-1640 culture medium supplemented with 100 U/mL penicillin,

100 μg/mL streptomycin, 50 μM 2-mercapthoetanol, 10 mM glutamax and 5% (v/v) FCS. Varying doses of $PLP_{139–151}$ were added at the beginning of the culture. After 72 h of culture 100 μL of supernatant was removed for cytokine analysis and the cells were cultured for another 18 h in the presence of 20 Kbq [^{3}H]-thymidine. Incorporation of [^{3}H]-thymidine was measured using a betaplate counter (Perkin-Elmer, Turku, Finland).

IFN-γ in culture supernatants was determined using a commercially available ELISA kit (PharMingen, San Diego, CA) according to the protocol recommended by the manufacturer.

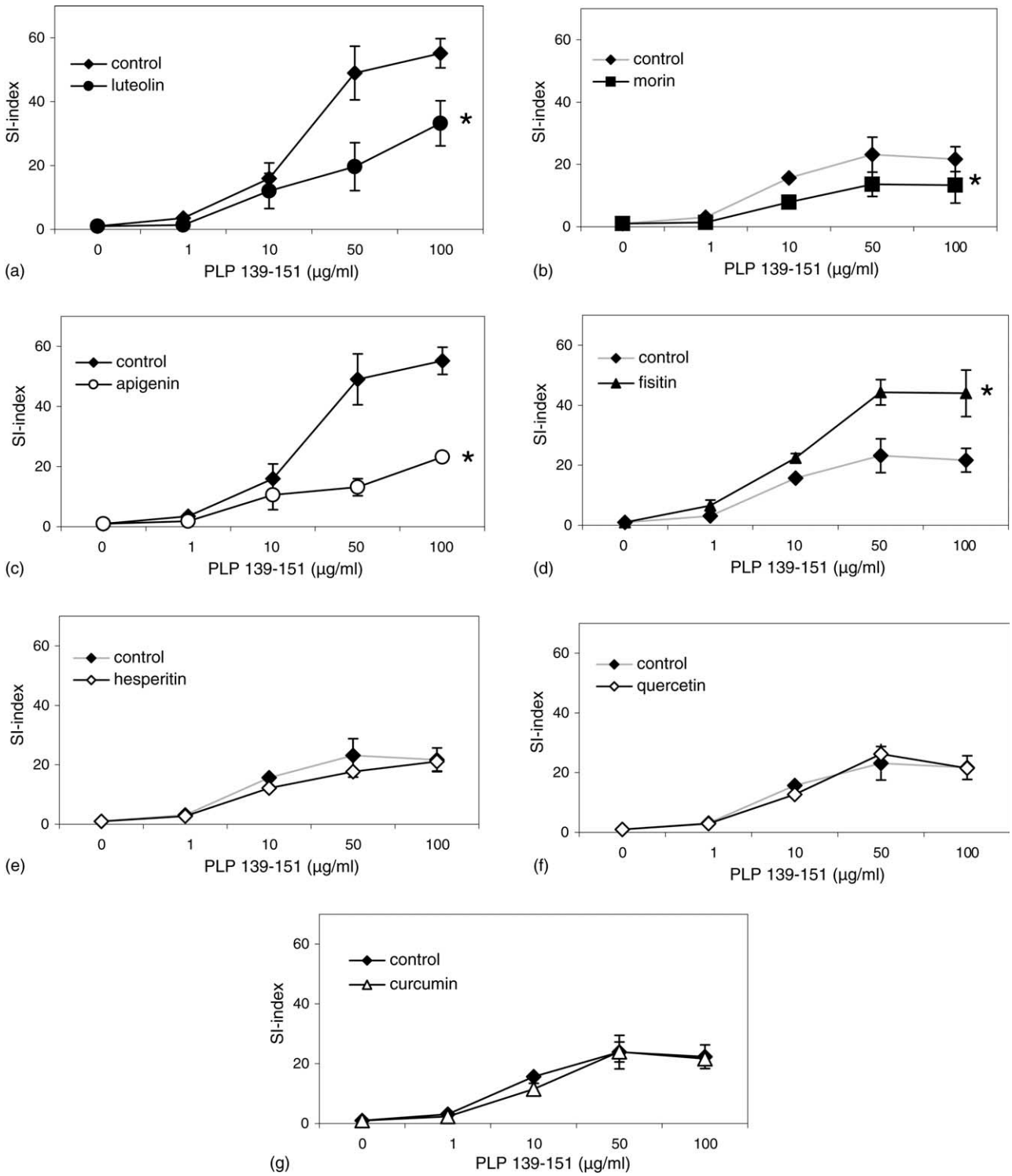

Fig. 1. The effects of oral compounds on T cell responses. Mice received 10 mg of compounds daily, starting three days before immunization with $PLP_{139–151}$ until ten days after immunization. At day 10, lymph node T cells were monitored for antigen-specific proliferation in vitro. Luteolin, apigenin and morin significantly ($p < 0.05$) reduced the response by 40–60% and fisitin stimulated the response 1.5–2-fold.

2.4. Statistical methods

Statistical analysis was performed using an SPSS statistical package. For statistical analysis of in vitro data, we used a univariate comparison to compare the influence of compounds. For analysis of clinical scores we used Mann–Whitney *U*-statistical analysis. In all cases a *p*-value of <0.05 was considered significant.

3. Results

3.1. Effects of oral components on priming of antigen-specific T cell responses in vivo

We first examined the effects of oral compounds on the developing T cell response against $PLP_{139–151}$, the peptide used to induce EAE in SJL mice. Compounds were administered daily at a dose of 10 mg, starting three days before immunization until 10 days after immunization. At that time T cells were isolated and tested for their antigen-specific responses in vitro.

T cells isolated from mice that received oral luteolin, apigenin or morin, showed a significant reduction in proliferation by 40–60% as compared to T cells obtained from control mice (Fig. 1). Fisitin, in contrast, significantly increased the proliferative response to $PLP_{139–151}$ (Fig. 1d). Lymph-node-derived T cells isolated from mice treated with fisitin reacted 1.5–2-fold stronger to $PLP_{139–151}$ than control lymph node T cells. The other compounds, viz. quercetin, hesperitin and curcumin did not affect T cell proliferation against $PLP_{139–151}$ (Fig. 1).

We also examined IFN-γ production by T cells under the influence of the flavonoids and curcumin. Oral luteolin and apigenin increased IFN-γ production in vitro, in contrast to their inhibitory effect on T cell proliferation (Fig. 2). Quercetin, while not affecting proliferation of T cells, did increase IFN-γ production. Morin reduced and fisitin increased both T cell proliferation and IFN-γ production. Hesperitin and curcumin did not significantly affect T cell proliferation nor IFN-γ production.

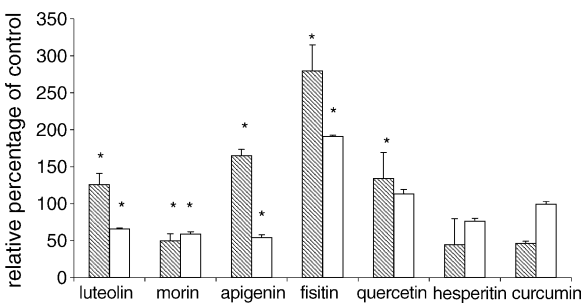

Fig. 2. The effects of oral compounds on IFN-γ production by T cells. Mice received 10 mg of compounds daily, starting three days before immunization with $PLP_{139–151}$ until ten days after immunization. At day 10, lymph node T cells were monitored for antigen-specific proliferation and IFN-γ production in the presence of 50 μg/mL $PLP_{139–151}$. Both IFN-γ production (dark bars) and T cell proliferation (white bars) are depicted as percentages of the control. Luteolin, quercetin and fisitin significantly ($p < 0.05$) increased IFN-γ production, while hesperitin, morin and curcumin significantly ($p < 0.05$) decreased IFN-γ production.

3.2. Effects of oral compounds on passive EAE

Since IFN-γ production as well as proliferation by antigen-specific T cells is relevant to the development of EAE [31,32], we examined whether the compounds affected autoreactive T cells in their capacity to transfer disease. Recipient mice that received T cells that had been treated with luteolin, apigenin, quercetin, morin, fisitin, hesperitin or curcumin in vivo, all developed clinical signs of EAE, the day of onset being either day 6 or 7.

In the first phase of EAE (day 1–11), mice that received T cells treated with luteolin, apigenin, morin, fisitin or curcumin did not show any difference in the development of clinical signs as compared to control mice (Fig. 3). The mean cumulative clinical scores in this first phase varied around a mean of 11 for all these groups (Table 1). Only two components influenced the mean cumulative clinical score during the first phase. Mice that received T cells treated with quercetin and hesperitin showed a significant increase in clinical signs as compared to control mice. The mean cumulative clinical score was increased by 25% with quercetin and by 21% with hesperitin (Fig. 3e and f).

During the second phase of the disease (day 12–21), control mice started to recover, whereas mice that received T cells treated with flavonoids all showed a significant delay in recovery from EAE. The most pronounced delayed recovery from disease was found with morin, quercetin and hesperitin. The mean cumulative scores of mice that received morin-treated T cells were increased 2-fold, while the response to quercetin- or hesperitin-treated T cells led to a 2.2-fold and 1.5-fold more severe disease, respectively (Table 1). In fact, 50% of the mice that received T cells treated with morin, and 65% of the quercetin group died (Fig. 3b–f). The remaining mice did not recover from clinical signs nearly as well as the control group did. Mice receiving T cells treated with luteolin, apigenin and fisitin also showed a reduced recovery from clinical symptoms albeit to a lesser extent (Fig. 3a, c and d). The mean cumulative clinical score was significantly increased by about 28% after transfer of luteolin-treated T cells, by 37% with apigenin-treated T cells, and by 20% with fisitin-treated T cells.

In contrast to mice that received T cells treated with flavonoids, mice that received T cells treated with curcumin showed improvement in the clinical signs during the recovery phase. Shortly after the peak of disease, these mice recovered more rapidly than the control group, as reflected by a reduction in the mean cumulative clinical score by 24% (Fig. 3g).

Clearly, T cells from mice treated with oral flavonoids did not lose their pathogenic capacity, but worsened disease development and impaired recovery with the exception of T cells that were obtained after treatment with curcumin.

3.3. Effects of oral compounds on active EAE

Next, we examined the effects of oral components in active EAE. Mice were fed with compounds again starting three days before immunization with $PLP_{139–151}$ until day 21. Mice received 10 mg of apigenin, luteolin, quercetin, hesperitin or curcumin daily. These components were selected for their previously observed effects on autoantigen-specific T cells in vitro and in vivo.

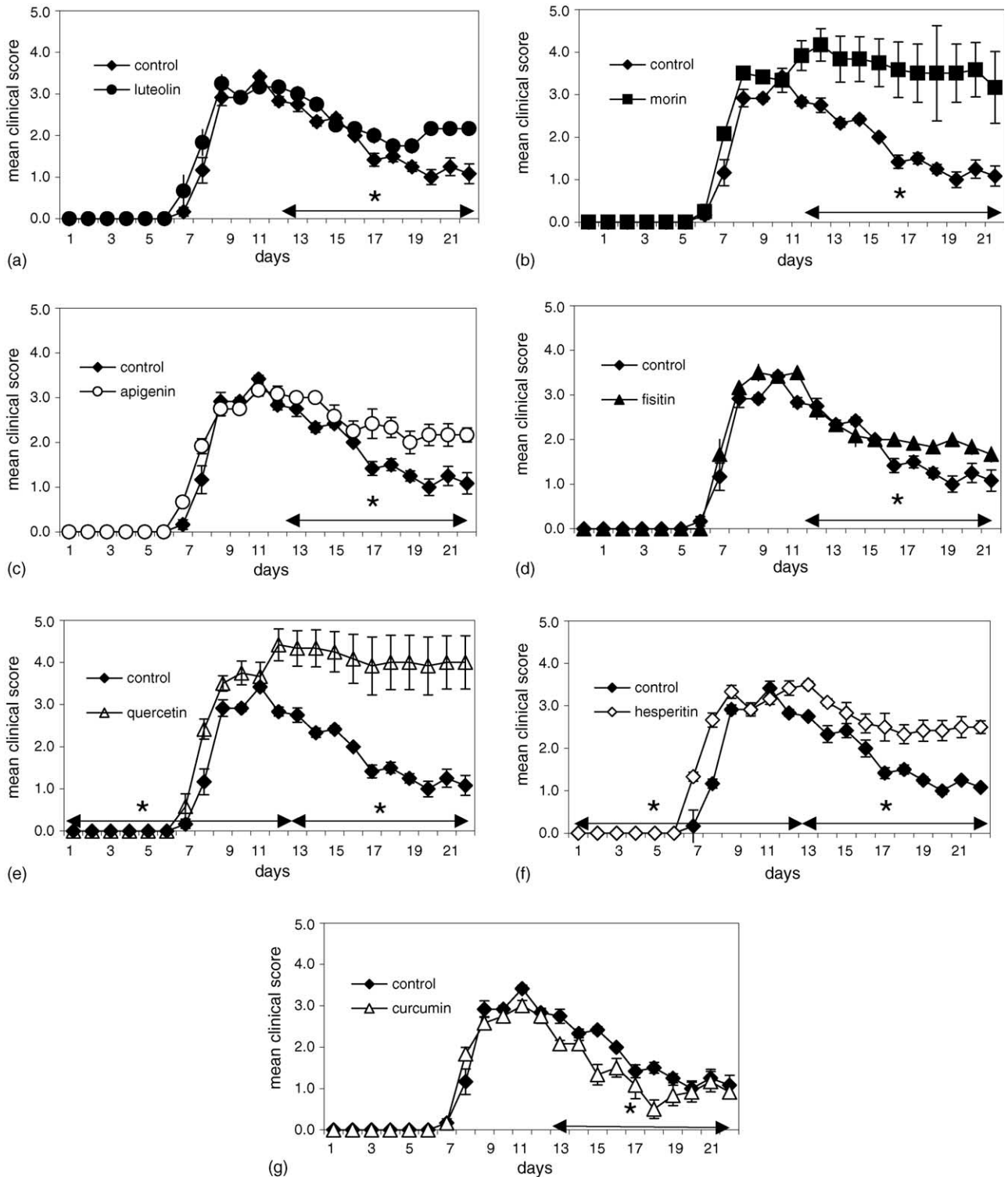

Fig. 3. The effects of compounds on passive EAE. Donor mice received 10 mg of compounds daily, starting three days before immunization with $PLP_{139–151}$ until ten days after immunization. At day 10, T cells were isolated and used for transfer into healthy recipients. Depicted are the mean clinical EAE scores. Groups consisting of at least six mice were used. Data represent mean ± S.D., $^{*}p < 0.05$ Mann–Whitney *U*-test.

Table 1
The effects of compounds on clinical score in passive EAE

	Incidence (%)	Day of onset[a]	Mean maximum score[b]	Days 1–11 mean cumulative score[c]	Days 12–21 mean cumulative score[c]
Control	100	6.8 ± 0.3	4.0 ± 0.0	10.3 ± 0.5	19.8 ± 0.9
Luteolin	100	6.3 ± 0.2	3.7 ± 0.2	11.8 ± 0.4	**25.3 ± 1.1**
Morin	100	6.6 ± 0.2	4.5 ± 0.2	12.6 ± 0.6	**39.3 ± 6.8**
Apigenin	100	6.3 ± 0.2	3.7 ± 0.2	11.3 ± 1.4	**27.2 ± 1.7**
Fisitin	100	7.0 ± 0.0	4.0 ± 0.0	11.8 ± 0.5	**23.8 ± 0.7**
Quercetin	100	6.3 ± 0.2	4.7 ± 0.2	**13.9 ± 1.2**	**45.3 ± 6.2**
Hesperitin	100	5.7 ± 0.3	4.0 ± 0.0	**13.4 ± 0.5**	**30.1 ± 0.8**
Curcumin	100	6.6 ± 0.2	3.3 ± 0.2	10.3 ± 0.5	**15.2 ± 1.9**

Significant differences $p < 0.05$ by Mann–Whitney U-statistical analysis, are depicted bold.
[a] Day of onset as mean day of onset ± S.D.
[b] Mean maximal score ± S.D.
[c] Mean cumulative score ± S.D.

Upon EAE induction, all mice developed clinical signs starting either at day 10 or 11 (Fig. 4). None of the compounds affected the day of onset. Mice treated with luteolin, quercetin or curcumin showed a trend towards a reduced mean maximal score (Fig. 4b, c and e). The most striking change was induced by oral hesperitin that significantly ($p < 0.02$) delayed recovery from EAE, in line with its similarly exacerbating effect on passive EAE.

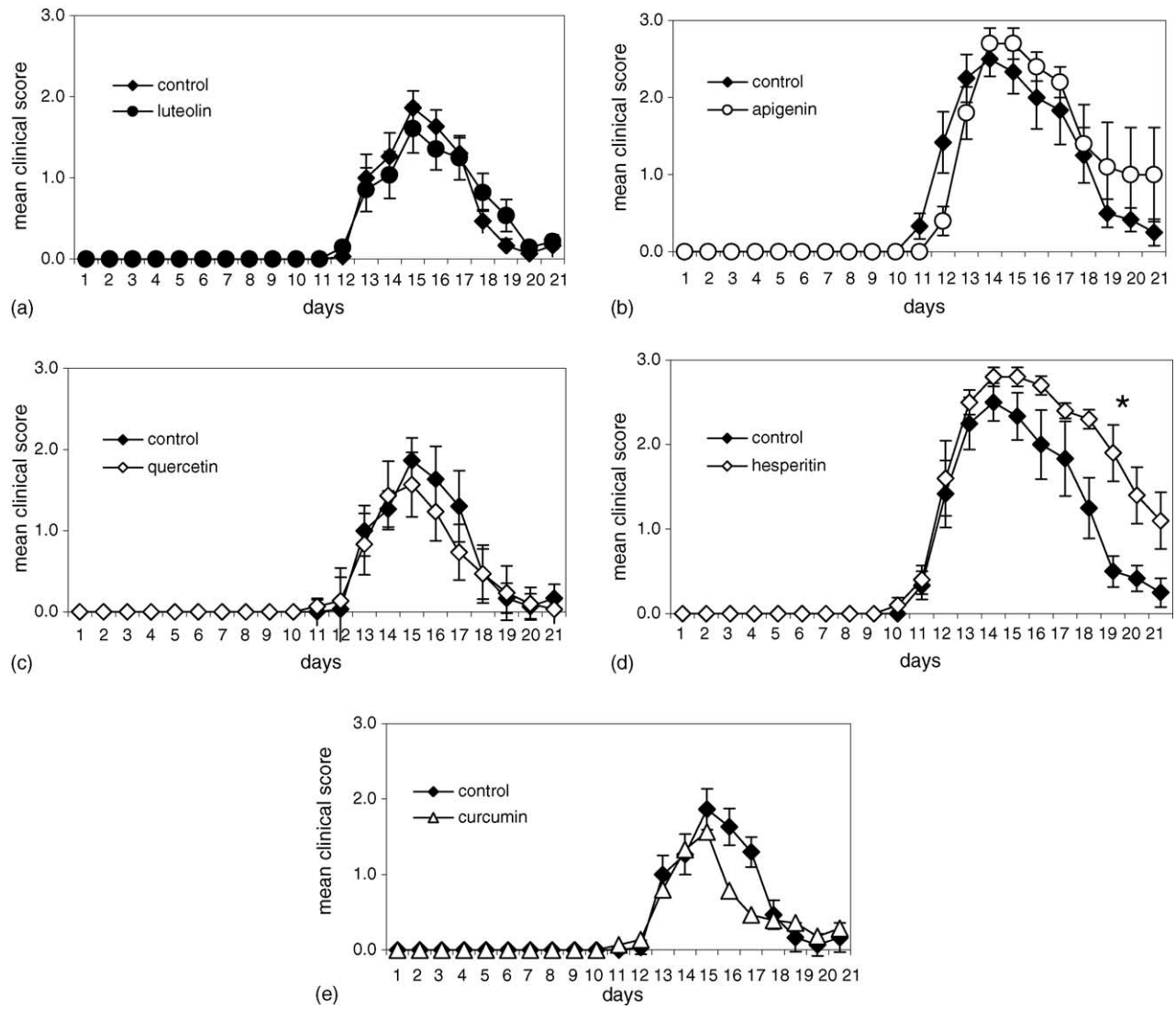

Fig. 4. The effects of compounds on active EAE. Mice received 10 mg of compounds daily, starting three days before immunization with $PLP_{139–151}$ until the end of the study at day 21. Shown are the mean clinical EAE scores in mice fed with apigenin (a), luteolin (b), quercetin (c), hesperitin (d) and curcumin (e). The group size varied between 8 and 15 mice. Data represent mean ± S.D., $^{*}p < 0.05$ Mann–Whitney U-test.

Hesperitin led to a 1.5-fold increase in the mean cumulative clinical score as compared to the control group (Fig. 4). A similar but less prominent effect was also triggered by oral apigenin, although this effect was not statistically significantly (Fig. 4). In contrast to the disease-enhancing effects of hesperitin and to a lesser extend apigenin, quercetin and curcumin tended to promote recovery from EAE, but the magnitude of these effects did not reach levels of statistical significance (Fig. 4). No effects on the course of EAE were observed upon oral administration of luteolin, despite its previously established ability to inhibit autoimmune T cell reactivity in vitro [11].

4. Discussion

Flavonoids and the plant-derived component curcumin are potential anti-inflammatory compounds, as has been extensively demonstrated in in vitro studies. This anti-inflammatory potential led to our selection of the flavonoids and curcumin used for this study. Evidence that anti-inflammatory effects also occur after oral administration of flavonoids, however, has not yet been reported. In fact, surprisingly few reports have addressed the effects of oral flavonoids in experimental models. In this study, we examined as a first step the effects of oral flavonoids and curcumin on the development of an experimentally induced T cell response, as judged by subsequent antigen-induced proliferation and IFN-γ production by these cells. Next, we evaluated the effect of continuous oral flavonoids and curcumin on disease development in both passive and active EAE.

The main result of the first part of the study is that oral luteolin, apigenin and morin reduced antigen-specific T cell proliferation, whereas fisitin increased T cell proliferation. These in vivo effects reflect in part the in vitro results we obtained in an earlier study [11]. In vitro luteolin and apigenin also inhibited antigen-specific proliferation of murine T cells against the same antigen. The concentrations of flavonoids we used in vitro ranged from 3.5 μM to 35 μM (∼1–50 μg/mL). At present, it is very difficult to relate this to serum levels that result from the oral administration regime used here. Not only are reliable analytical tools lacking to detect each of the flavonoids used in mouse serum, but the formation of potentially bioactive metabolites also has to be considered. Oral compounds are subjected to hydrolysis and degradation in the colon by the microflora. When absorbed by the gut barrier, they are extensively metabolized either by the tissue itself or by the liver [33–36]. As a rule, flavonoids are glucuronidated or sulphated and work by others has shown that only very small amounts of the unmodified original flavonoid are detectable in serum after oral administration [37,38]. Given the currently available tools and knowledge, it is essentially impossible to accurately establish serum levels of flavonoids along with their relevant bioactive metabolites after oral administration. The oral dose of luteolin, apigenin, morin and fisitin used in the present study is apparently sufficient to affect the development of a T cell response after immunization, as is apparent from the results of the first part of our study. In our view, this at least partly justifies the relevance of our current approach.

Interestingly, remarkable differences were seen in the effects of compounds on IFN-γ production between our previous in vitro and present in vivo studies. While in vitro antigen-specific IFN-γ production and proliferation of T cells were reduced by flavonoids in similar ways, opposite effects were frequently found in vivo. For example, while oral apigenin and luteolin reduced antigen-induced T cell proliferation, they stimulated IFN-γ production. After oral quercetin the proliferative response remained unaffected while IFN-γ production was stimulated. These apparent discrepancies could well relate to the fact that in vivo a complex mixture of systemic compounds along with their metabolites are present while in vitro the effects are seen only of the original, unmodified compound. Flavonoid-derived metabolites could well affect other signaling pathways that influence IFN-γ production. In response to antigen both proliferation and IFN-γ production require antigen activation through the T cell receptor [39]. Yet, several studies have shown that other pathways can also affect IFN-γ production [40]. By interfering with such alternative pathways for IFN-γ production flavonoid-derived metabolites could well influence not only IFN-γ production, but also in a more general sense disease progression in vivo [31,32,41,42].

Since flavonoids are generally seen as health-promoting substances one would expect them to exert beneficial effects also in experimental disease models including EAE. Surprisingly, however, upon transfer of T cells taken from mice treated with oral flavonoids, disease worsened in recipient mice and delayed recovery from EAE was especially evident. This effect was seen upon transfer of T cells from mice fed with all flavonoids, regardless of previously monitored effects on antigen-specific T cell responses. Flavonoids were also examined for their effects on active EAE and also in these experiments, flavonoids failed to exert beneficial effects. In fact, the only significant effect observed was a disease-exacerbating effect, in this case by hesperitin, again primarily caused by a delayed recovery from acute inflammatory damage. Even quercetin, one of the most extensively studied flavonoids, did not reduce the clinical signs of active EAE. The effects of oral flavonoids on active EAE were distinctly less prominent than those on passive EAE. Given the natural variation in active EAE, we evaluated the effects of oral flavonoids three times and in some cases even over four separate experiments. In all cases, the results were very similar and confirmed the lack of any significant beneficial effects of flavonoids. Only oral hesperitin showed an effect in delaying recovery from the acute phase. It remains difficult to explain why this is so, or by what mechanism flavonoids cause a delay in the recov-

ery phase. The compound curcumin should be distinguished from flavonoids both with regard to its chemical structure as well as its effects on EAE. Mice that received T cells pretreated with curcumin recovered from EAE more quickly than control mice. Also, oral curcumin during disease progression in active EAE led to a trend towards a more rapid recovery from EAE. These results are in line with a previous study that revealed strong inhibitory effects on murine EAE of curcumin, although in this case following intraperitoneal administration [16]. With regard to curcumin, therefore, our data are in line with previous studies and show the beneficial potential of this particular compound also after oral administration.

The number of animal model studies on flavonoids with which to compare our data is surprisingly limited. Quercetin and hesperidin, the glycolated form of hesperitin, have previously been reported to exert beneficial anti-inflammatory effects in an animal model of inflammatory arthritis [6]. A similarly beneficial effect of curcumin has been documented in murine EAE [16]. Yet, in both cases the compounds were administered intraperitoneally, which represents an essentially different route from the oral route, as explained above. Recently, a study by Hendriks et al. [43] showed a suppressive effect of oral luteolin in rat EAE. In this study, luteolin was administered intragastrically in a single bolus per day, bypassing the oral cavity. Apart from the species difference therefore, also systemic availability could be different from our approach.

Based on our current data, oral flavonoids appear to give rise to effects that are much more complicated than those suggested by in vitro studies on unmodified parental compounds only. While pathogenic processes may be affected by flavonoids, so may recovery processes. The application of high-dose oral flavonoids for therapeutic or even preventive purposes should therefore be taken with some caution.

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