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MECHANISM OF ANTIVIRAL ACTION OF QUERCETIN AGAINST CARDIOVIRUS INFECTION IN MICE*

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Oral treatment with quercetin protected $ABD2F_1$/Jena mice significantly against intraperitoneal encephalomyocarditis, Col. SK, MM, $Mengo_{M,L}$ and $Mengo_M$ virus infections, but not against intracerebral challenge with $Mengo_M$ virus. Enhanced resistance to $Mengo_M$ virus were induced in the genetically different DBA 2/Jena, C57BL6/Jena, C57BL/Lati and $ABD2F_1$/Jena mice. C57BL6/Jena nu/nu mice were also protected, indicating that the thymus was non-essential to the protective effects of quercetin. In AB/Jena and Lati:CFLP mice the drug failed to be effective. Quercetin was not virucidal and did not interfere with Mengo virus replication in L cells. Interferon was not detected (< 1 : 8) in sera of $ABD2F_1$/Jena mice 1–48 h after oral administration of the drug. The virus spread from the site of injection to the local lymph nodes and other target organs were impaired. Silica treatment, to suppress macrophage function, did not evidently increase the susceptibility of $ABD2F_1$/Jena mice to Mengo virus. However, this treatment abolished the antiviral activity of quercetin, indicating the requirement for macrophages for quercetin to be effective. Virus replication could not be demonstrated in cultures of adherent peritoneal macrophages from either untreated or quercetin-treated $ABD2F_1$/Jena mice.

cardiovirus flavonoids antiviral activity mouse host resistance macrophage

INTRODUCTION

In previous studies antiviral activity of flavonoids and flavonoid-containing natural compounds was observed under in vitro [3–8,18,19,22,28,42,43] and in vivo conditions [10–12,16,38]. Recently, silymarin, a structural analogue of the flavonoids [36], and cyanidanol-3 (Catergen), a flavone derivative, have been shown to be effective against acute viral hepatitis in man [9,27]. The mechanism of antiviral action of flavonoids is unknown.

Previous studies have demonstrated that flavonoids after oral administration afford

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protection against lethal Mengo virus infection in mice [16,38] which is considered a good experimental model for the study of enterovirus infections [15]. As few compounds are known at present that suppress RNA virus-induced encephalitis in animal models, it was of interest to continue these studies using one of the effective drugs, namely quercetin, to investigate its action against cardiovirus infection in mice. (Part of this work was presented at the 4th International Symposium of Socialist Countries on 'Antiviral Substances' in Szeged, Hungary, June 23–25, 1980).

MATERIALS AND METHODS

Viruses

For the in vivo experiments four strains of cardiovirus were used. These were $Mengo_M$ virus [37,45], encephalomyocarditis (EMC) virus, Col. SK virus, and MM virus. After intracerebral (i.c.) infection of $ABD2F_1$ mice, stocks of virus were prepared from mouse brain extracts and stored above liquid nitrogen.

Stocks of the L cell-passaged Mengo virus variants $Mengo_L$ and $Mengo_{M,L}$, and vesicular stomatitis virus (VSV), Indiana strain, were prepared in L-929 cells.

Mice

Unless stated otherwise, male $ABD2F_1$ (AB/Jena × DBA 2/Jena) hybrids, 4–6 weeks old, and selected on 17–22 g body wt., were used. These as well as male mice of the inbred strains C57BL6/Jena, AB/Jena, DBA 2/Jena, and the congenitally athymic nude (nu/nu) mouse mutant of the C57BL6/Jena background, were obtained from the SPF Mouse Breeding Unit of the Central Institute of Microbiology and Experimental Therapy, Jena, G.D.R. Male outbred mice of the stock Lati:CFLP and C57BL/Lati inbred mice were obtained from the Breeding Farm Lati, Gödöllö, Hungary.

Animals were housed in plastic cages and maintained under conventional conditions with standardized pellet food and tap water ad libitum. The light–darkness cycle and atmospheric humidity were not regulated; mean temperatures were 22–24°C (Jena), and 24–26°C (Szeged).

Cells

L-929 monolayer cell cultures were grown in Eagle's minimal essential medium (MEM, State Institute of Immunopreparations and Nutritive Media, Berlin, G.D.R.) supplemented with 10% calf serum and antibiotics.

Drugs

Quercetin, cryst., mol. wt. 33,827 (batch No. 7521550) was obtained from Merck,

Darmstadt, F.R.G. For the animal experiments, drug suspensions were prepared shortly before oral administration as described recently [38]. For the studies in cell cultures, the drug was dissolved in 0.1 N NaOH and diluted with physiological saline. Tilorone · HCl (synthesized and kindly provided by Dr. W. Schulze from the Institute in Jena) was dissolved in physiological saline.

Crystal silica flour No. 12 (main part 0–10 μm) was obtained from Dörentruper Sand- und Tonwerke GmbH, Dörentrup, F.R.G. Freshly prepared suspensions of silica in phosphate-buffered saline (PBS) were vigorously shaken in a mini-shaker immediately before mice were inoculated intraperitoneally (i.p.) with 40 mg/kg body wt. 2 h before the virus infection. Under these conditions, the silica preparation was shown to deplete the macrophage population in mice [24].

In vivo assay of antiviral effectiveness

Unless stated otherwise, groups of 10 mice were injected i.p. with 10 LD_{50} of either virus strain, causing a mortality rate (q_C) of approximately 96%. Animals were treated orally according to approved schedules of treatment with quercetin [38], as indicated below. As a rule, administration was done at hours −12, −1, +8, +24, +36, +48, and +56, relative to infection, using placebo (drug diluent) or 20 mg quercetin/kg body wt., except the initial dose of 40 mg/kg, as described recently [38]. The number of mice in the drug-treated and the placebo-treated groups were equal ($n_T = n_C$). In the experiments with different mouse strains, five sham-infected toxicity control mice were included. Surviving animals were recorded daily for 14 days.

At the 14th day after infection, surviving animals in treatment and control groups, r_T and r_C, were counted for estimation of the 'rate of protection' $R = (r_T - r_C)/(n - r_C)$, and for statistical examination of its significance, according to a 'two-step procedure' [17,39,40).

In vitro assay of antiviral effectiveness

To test virucidal activity, equal volumes of quercetin solutions containing 300 μg/ml, or saline without drug, were mixed with $Mengo_L$ virus dilution containing approximately 10^6 $TCID_{50}$/0.1 ml, and incubated at 37°C for 2 h. Thereafter, yields of virus were determined by infectivity titrations in L-929 cells.

The inhibition of viral multiplication was tested by infecting cells with $Mengo_L$ virus at a multiplicity of 1 $TCID_{50}$ or 10 $TCID_{50}$. After an adsorption period of 30 min at 37°C, the virus was removed by washing the cultures twice. Thereafter, cells were replenished with maintenance medium and further incubated. Quercetin at concentrations of 20 μg/ml, or 30 μg/ml (maximal non-toxic dose for L-929 cells), was added to the cultures either simultaneously with the virus or at the end of the adsorption period. Cultures treated similarly with drug-free medium served as virus controls. At 8 h, the cultures were frozen and thawed three times, and total virus yields were determined.

Interferon assay

Groups of 12 $ABD2F_1$ mice, after a single oral treatment with 40 mg quercetin/kg body wt., were bled at intervals. Sera of mice, treated orally with tilorone·HCl (250 mg/kg body wt.) or placebo, bled after 24 h, served as controls. Pooled serum samples in aliquots of 1 ml were stored above in liquid nitrogen until assayed. L-929 cells in tubes were incubated with 1 ml of serum dilutions 1 : 8, 1 : 16, and 1 : 32 or, in the case of tilorone·HCl-treated mice, 1 : 100, overnight at 37°C before challenged with 10 $TCID_{50}$ of VSV. Interferon titres were determined by the cytopathic effect (CPE)-inhibition assay [14].

Assay of organs for virus

Blood, brain, spleen, and mediastinal lymph nodes, together with thymus, were collected aseptically and placed in PBS, containing 10% glycerol, and were frozen at −20°C until homogenized individually to a 10% suspension. In the case of the lymph nodes and thymus, the organs of two mice were pooled. After centrifugation at 5500 × *g* for 10 min, the supernatants were titrated in $ABD2F_1$ mice by i.c. inoculation. LD_{50} values were determined according to Reed and Muench [29].

Preparation of peritoneal macrophages

Mice were sacrificed and peritoneal cells (PC) were obtained by washing the peritoneal cavity of 10–14 week old mice with 4 ml warm Hanks' balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}. The pooled fluids were collected in 50 ml siliconized glass tubes, washed three times with HBSS, and resuspended in Eagle's MEM containing 10% calf serum. The number of macrophages in the cell suspension was counted after staining with neutral red [41], and 1×10^7 macrophages in 5 ml medium were allowed to adhere to the bottom (18 cm^3) of a 50 ml bottle.

As normal adherent macrophages served those obtained from untreated mice. Macrophages were also obtained from mice treated orally with drug diluent (placebo) or quercetin at doses of 40 mg/kg and 20 mg/kg body wt. 12 and 2 h prior to harvesting PC. As a control, macrophages were elicited by i.p. injection of freshly prepared sterile 3% thioglycollate (Difco, Detroit, MI), 2 ml per animal, 5 days before PC collection.

Macrophage infection

At 2 h, PC monolayers were washed three times with 3 ml of Eagle's medium without serum to eliminate unattached lymphocytes and erythrocytes. The adherent cells were then inoculated with 10 $TCID_{50}$/cell of $Mengo_{M,L}$ virus in 0.5 ml, incubated at 37°C for 1 h, washed five times with 3 ml of Eagle's medium to remove unattached virus, and replenished with 3 ml of Eagle's MEM supplemented with 10% calf serum. This was considered as zero time (t_0).

Assay of macrophage infectivity

At the times indicated, the macrophages (approximately 10^7 cells in 3 ml) were disrupted by three cycles of freezing and thawing, and the suspension was tested for the presence of infectious virus by titration in L cells.

RESULTS

Examination of toxicity in different mouse strains

None of the various quercetin doses used in the following experiments caused death or obvious signs of illness in the genetically different non-infected toxicity control mice when administered orally seven or eight times (Table 1). In ABD2F$_1$/Jena mice or C57BL6/Jena mice, treated eight times with the drug at doses of 20 mg/kg body wt., the mean weight gain was not different from that of placebo-treated animals during a period of 12 days after the initiation of treatment. When treated in the same way with quercetin, the hybrid mice did not show any detectable histological lesions of the parenchymal organs, such as the liver, spleen, kidneys, brain, thymus, salivary glands, extraorbital

TABLE 1

Effect of oral quercetin treatment on different mouse strains

Mouse			Treatment[a]				
Strain	Age (weeks)	Initial weight (g)	Total dose of drug (mg/kg body wt.)	Survivors/total		Mean weight gain[b] (g)	
				Treated	Control	Treated	Control
DBA2/Jena	4–8	17–21	160	20/20	20/20		
AB/Jena	4–5	16–22	80	20/20	20/20		
			160	20/20	20/20		
			240	10/10	10/10		
ABD2F$_1$/Jena	4–5	17–22	160	20/20	20/20	4.30±1.86	4.20±1.08
C57BL6/Jena	5–7	19–22	160	20/20	20/20	4.35±1.32	4.30±1.00
C57BL6/Jena nu/nu	6–12	16–23	160	9/9	9/9		
C57BL/Lati	5–6	16–22	80	10/10	10/10		
Lati:CFLP	6–7	30–36	80	10/10	10/10		
			160	20/20	20/20		
			240	10/10	10/10		

[a] Seven (using a double initial dose) or eight times with drug or placebo at intervals of approximately 12 h. Animals were recorded for 14 days.

[b] Values represent mean weight gain ± standard deviation from 10 mice each in treated and in control groups 12 days after initiation of treament (8×).

lacrimal glands, or the mediastinal lymph nodes (J. Güttner, CIMET Jena, personal communication).

Effect on different cardiovirus infections

Since quercetin proved to be effective against lethal $Mengo_M$ virus-induced encephalitis in mice, we examined whether the drug also acted against infections caused by related viruses. The drug protected $ABD2F_1$/Jena mice significantly against lethal infections with different strains of cardiovirus when oral treatment was initiated 12 h prior to i.p. injection of virus and was continued twice daily for a period of three consecutive days. If $Mengo_M$ virus was inoculated i.c., quercetin proved not to be protective (Table 2).

Protection against Mengo virus in different mouse strains

Investigations were expanded to various mouse strains to verify whether quercetin was also effective in genetically different animals and, to obtain more insight into the mechanisms involved in its protective effect. The drug proved to be effective against $Mengo_M$ virus infection in different mice, including a nude mutant. However, in AB/Jena and Lati:CFLP mice, quercetin failed to show any protective effect (Table 3).

TABLE 2

Effect of oral quercetin treatment[a] on different cardiovirus infections in $ABD2F_1$/Jena mice

Virus[b]		Survivors/total		Rate of protection and significance[c] (%)
Strain	Route of inoculation	Treated	Control	
EMC virus	i.p.	10/20	0/20	50.0 +
Col. SK virus	i.p.	7/20	1/20	31.6 +
MM virus	i.p.	8/20	1/20	36.8 +
$Mengo_{M,L}$ virus	i.p.	10/20	0/20	50.0 +
$Mengo_M$ virus	i.c.	0/20	0/20	0.0 –
$Mengo_M$ virus[d]	i.p.	12/20	1/20	57.9 +

[a] At hours −12, −1, +8, +24, +32, +48, and +56, relative to virus infection. Initial dose of drug was 40 mg/kg body wt.; treatment was continued with doses of 20 mg/kg body wt.

[b] Doses of virus were 10 LD_{50} calculated from the specific titres after i.p. or i.c. titrations.

[c] 'Rate of protection' was estimated [39,40] and its significance was evaluated by the one-sided fourfold table test at the 95% level. 'Size of mesh' in screening was regulated by the rules of decision of a 'two-step procedure' with $R_0 = 30\%$, $q_C = 94\%$, and $n_T = n_C = 20$ [17].

[d] Used as a control [38].

TABLE 3

Protection of oral quercetin treatment[a] against lethal $Mengo_M$ virus encephalitis in different mouse strains

Mouse[b]		Survivors/total		Rate of protection and significance[c] (%)
Strain	H-2 genotype	Treated	Control	
DBA2/Jena	d/d	8/20	1/20	36.8 +
AB/Jena	o/o	0/20	0/20	0.0 –[d]
$ABD2F_1$/Jena	o/d	10/20	0/20	50.0 +
C57BL6/Jena	b/b	9/20	0/20	45.0 +
C57BL6/Jena nu/nu	b/b	10/20	1/20	45.0 +
C57BL/Lati	b/b[e]	9/20	0/20	45.0 +[f]
Lati:CFLP	Unknown	0/20	0/20	0.0 –[d]

[a] cf. Table 2.
[b] For age and initial weight cf. Table 1.
[c] cf. Table 2.
[d] No significant protective effect was obtained with doses of drug of 5, 10, 20 or 30 mg/kg body wt. per injection.
[e] Not confirmed.
[f] Initial dose of drug was 20 mg/kg body wt.; treatment was continued with doses of 10 mg/kg body wt.

In vitro antiviral effects against Mengo virus

To determine whether quercetin inactivated the virus or interfered with its multiplication, in vitro investigations were performed. No virus-inactivating effect was seen after exposure to 150 μg/ml of quercetin for 2 h at 37°C. At concentrations of 20 or 30 μg/ml the drug exerted no virustatic effect in L-929 cells at input multiplicities of infection of approximately 1 $TCID_{50}$ or 10 $TCID_{50}$. Inhibition of adsorption was not evident (data not shown).

Attempt to demonstrate interferon induction by quercetin in mice

Interferon induction was investigated in quercetin-treated $ABD2F_1$/Jena mice. The sera were tested for the presence of interferon at 1, 2, 3, 4, 6, 8, 10, 24 and 48 h. No virus-inhibitory effect was detected at serum dilutions of 1 : 8 or greater at any of the times indicated. Serum of tilorone·HCl-treated mice contained 10^3 units/ml interferon at 24 h (data not shown).

Effect on spread of Mengo virus to various organs

The influence of orally administered quercetin on the spread of $Mengo_M$ virus through

the body was studied early during the course of the infection. The virus content in the blood, spleen and brain of individual mice, and the pooled mediastinal lymph nodes and thymus of two mice, were determined in quercetin and placebo-treated mice at several intervals after infection (Fig. 1).

In placebo-treated mice, the virus was first detected in the mediastinal lymph nodes in nine out of 10 animals as early as 6 h after infection. At 8 h, low levels of virus were also found in the blood and in the brain of a few mice. After 24 h, the tissues of most, if not all, mice contained high virus titres.

Quercetin treatment prevented, in most mice, the spread of virus to the local lymph nodes, spleen and brain during the first 24 h after infection. The earliest protective effect could be seen at the level of the virus present in the mediastinal lymph nodes and thymus.

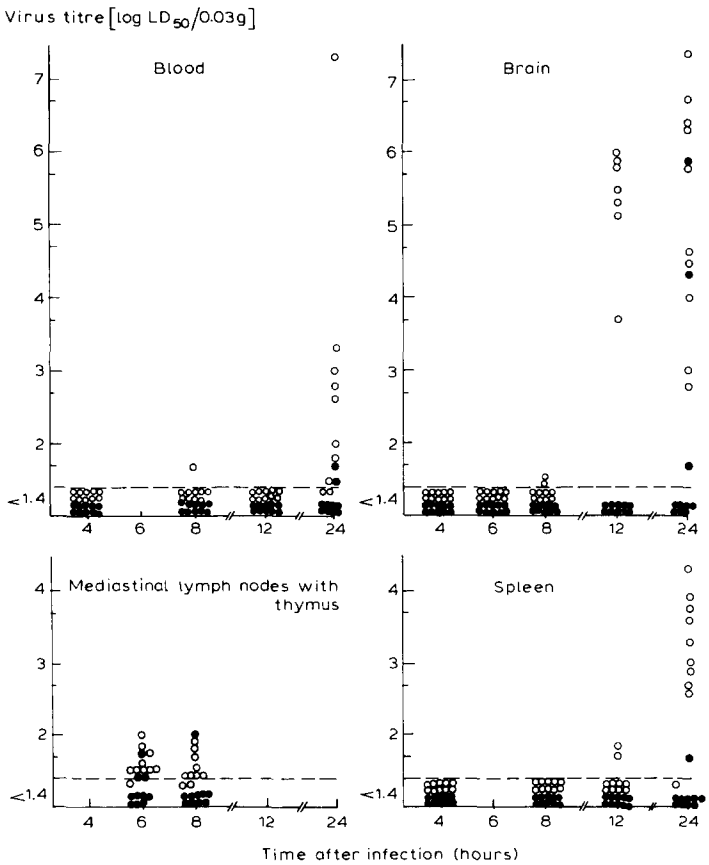

Fig. 1. Effect of orally administered quercetin on spread of Mengo$_M$ virus (10 LD_{50}, i.p.) to various organs. ○, Placebo-treated and infected; ●, quercetin-treated and infected.

Effect of suppression of macrophages on Mengo virus infection and protective activity of quercetin

ABD2F$_1$/Jena mice that received an i.p. injection of silica 2 h prior to i.p. infection with Mengo$_M$ virus (10 LD$_{50}$) did not show a markedly decreased resistance to the infection as compared to control animals (Fig. 2). However, the protective effects of quercetin could be abolished by silica. Mice were injected i.p. with silica 2 h before i.p. challenge with Mengo$_M$ virus (10 LD$_{50}$) and treated with quercetin at hours −1, +12, +24, +36, +48, +60, +72 and +84, relative to infection. The results show that silica completely abrogated the protective effect of quercetin (Fig. 2).

Interaction of mouse peritoneal macrophages from quercetin-treated mice with Mengo virus in vitro

There was no evidence of Mengo$_{M,L}$ virus replication in cultures of adherent peritoneal macrophages from ABD2F$_1$/Jena mice when incubated for a period of 72 h. Likewise, macrophages cultured from both drug-treated and placebo-treated mice after exposure to the virus showed no increase in virus titre upon incubation of 24 h. However, macrophages elicited by i.p. injection of thioglycollate were capable of supporting the replication of the virus in vitro within 8 h (Table 4).

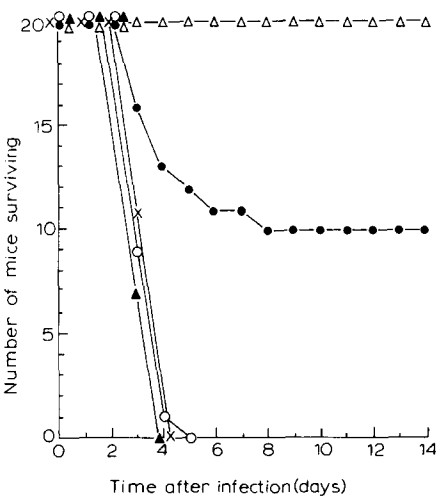

Fig. 2. Effect of silica (40 mg/kg body wt., i.p.) given 2 h before challenge with Mengo$_M$ virus (10 LD$_{50}$, i.p.) on the protective effect of quercetin in ABD2F$_1$/Jena mice. ○, Placebo-treated and infected; X, silica-treated and infected; ●, quercetin-treated and infected; ▲, silica and quercetin-treated and infected; △, silica-treated only.

TABLE 4

Attempt to demonstrate $Mengo_{M,L}$ virus replication in peritoneal macrophages from $ABD2F_1$/Jena mice

Time after infection (h)	Virus titre[a] on L-929 cells (log $TCID_{50}$/0.1 ml)			
	Untreated	Placebo-treated[b]	Quercetin-treated[b]	Thioglycollate-treated[c]
0	2.02	3.28	2.97	2.79
8	2.17			5.59
24	2.05	2.71	2.38	
48	0.78			
72	0.69			

[a] 2 h cultures of peritoneal macrophages were inoculated with 10 $TCID_{50}$/cell of $Mengo_{M,L}$ virus, incubated at 37°C and, after washing, harvested at the times indicated. Mean values of two experiments.

[b] Donor mice were treated twice (cf. Table 2, footnote a) at an interval of 11 h. Peritoneal cells were collected approximately 2 h after last treatment.

[c] Donor mice were treated 5 days before peritoneal cell collection.

DISCUSSION

Quercetin-treated mice were protected against a lethal infection with either EMC, Col. SK, MM or $Mengo_{M,L}$ virus, confirming previous data with $Mengo_M$ virus [38]. Mice were protected against i.p. or intranasal [38] virus infections following oral treatment with quercetin. The drug was not effective against an i.c. $Mengo_M$ virus challenge.

Several strains of mice with different H-2 genotypes, as well as athymic nude mice, were protected equally well against Mengo virus by quercetin. However, in two mouse strains, AB/Jena and Lati:CFLP, the drug was not effective. Whether the major histocompatibility gene complex plays a role in the protection is not clear from these data. Resistance of mice against EMC virus induced by non-viable *Mycobacterium tuberculosis* was not correlated with H-2 genotype [21]. It is apparent from these findings that in tests for antiviral activity different mouse strains may produce contradictory results. There is a need, therefore, to use several genetically different mouse strains in such assay systems to avoid errors in interpretation [13,30].

In vitro Mengo virus was not inactivated by quercetin, and there was no evidence for inhibition of its adsorption to or replication in L cells, indicating a lack of correlation between the results of the in vivo and in vitro experiments. In other studies, quercetin also showed no virucidal effect on several types of polio- and adenovirus, whereas enveloped viruses were inactivated [8,25].

Since the in vivo antiviral effects of quercetin depended on its presence during the early phases of Mengo virus infection [38], and the drug did not interfere with virus replication in vitro, it would appear that non-specific host defence mechanisms are

involved in establishment of enhanced resistance, as reported for various agents [21,23, 44].

Interferon was not detected in sera of mice treated orally with quercetin. It therefore seems that protection did not occur as a result of systemic interferon production. The protection conferred by quercetin is reminiscent of the antiviral effects of certain immunomodulators, such as *Brucella* abortus, poly (C), poly (I), and a non-viable *Mycobacterium tuberculosis* vaccine which have been suggested to act through activation of macrophages rather than interferon induction [20,26,32,35].

Virus titrations ascertained that after i.p. infection $Mengo_M$ virus occurred first in the mediastinal lymph nodes which drain the peritoneal cavity of mice [1] and, thereafter, in the blood, brain and spleen. Early during the infection quercetin treatment prevented, in most mice, the spread of virus through the organism.

The thymus was not required for the antiviral resistance offered by quercetin, since athymic nude mice were equally well protected as normal mice. Resistance was also established in splenectomized mice treated with the drug [16]. Thus, T or B lymphocytes are not the critical determinants in the protective effect of quercetin. On the contrary, macrophages appear to play an important role in the antiviral protection of quercetin. The importance of the macrophage in our model was shown by the complete abrogation of quercetin-induced resistance to Mengo virus after i.p. injection of silica, which is selectively toxic for macrophages [2]. A requirement for macrophages has also been observed for polynucleotides [33,34] and interferon [31] to be effective against EMC virus infection of mice. The course of Mengo virus infection was not affected in macrophage-depleted $ABD2F_1$/Jena mice, implying that with the model used macrophages normally play a minor role in determining the outcome of the infection, as suggested by others for EMC virus infection of mice [31]. Replication of Mengo virus was not evident in adherent peritoneal macrophages from either normal or quercetin-treated $ABD2F_1$/Jena mice. Likewise, EMC virus failed to replicate in macrophages of mice (BK : W) [31].

Thus, it appears that macrophages are essential for quercetin to be effective in inducing early virus resistance by a mechanism that includes a restricted spread of virus through the organism. The role of macrophages in the enhanced resistance to cardiovirus infection deserves further investigation.

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