Protective and immunostimulating activity of a low dose of cyclophosphamide in the experimental infection of mice with foot-and-mouth disease virus

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Summary. Administration to mice of a low, non-immunosuppressive dose of cyclophosphamide 4 days before infection with foot-and-mouth disease virus decreases viral replication, enhances the immune response against the virus and prevents pancreatic damage.
Key words. Foot-and-mouth disease virus; cyclophosphamide; pancreatitis; immune response.

The immunosuppressive activity of cyclophosphamide (Cy) is currently used to explore the putative immune pathogenesis of tissue damage in viral infections [1-3]. It is usually held that if lethality or tissue alterations are prevented or decreased by treatment with the drug immune mechanisms would probably be involved in the production of alterations [3]. However, when it is administered at a low dose and before antigenic challenge, Cy can display an immunomodulating activity, enhancing antibody synthesis [4]. On this basis we decided to study the effect of a low dose of this drug on the evolution of the experimental infection of mice with foot-and-mouth disease virus (FMDV).

We present herein evidence that when a low dose of Cy is administered before infection with FMDV, viral replication and tissue damage can be prevented and the immune response against the virus is enhanced.

Materials and methods. Experimental design: Adult 3-month-old male and female outbred Swiss mice were infected by intraperitoneal route (i.p.) with 1×10^4, 5×10^4 or 1×10^5 PFU of FMDV, strain 029. Cyclophosphamide (50 mg/kg) was administered i.p. 4 days before infection. Groups of 3 animals each were sacrificed at 1,2,3,7,21,28,60 and 90 days post-infection (p.i.). Pancreas and blood were collected for virus isolation and for searching of antibodies against FMDV. Controls were animals infected with FMDV but not pulsed with Cy.

Virological studies: The 029 strain FMDV grown in BHK 21 monolayers was used for all the experiments. Virus concentration in supernatants of BHK 21 infected cells and from blood or pancreas was determined by the plaque-forming assay on methyl-cellulose covered BHK 21 monolayers. For quantitation of FMDV in pancreas the organ was homogenized in MEM Eagle's (1/10 W/V). Suspensions were centrifuged at 25,000 g for 30 min at 4 °C and tenfold dilutions of the supernatant was absorbed on BHK 21 monolayers for 1 h at 37 °C. Titer was expressed as PFU/ml for samples of BHK 21 supernatants and blood or PFU/g for samples of pancreas.

Neutralization assay: Serum was separated from the blood samples and kept at −70 °C. Neutralizing activity against FMDV was assayed by the plaque-forming test. Titer was expressed as the reciprocal of a base 2 maximal dilution which gave a 80% reduction of the number of plaques on BHK 21 monolayers.

Immune-complexes detection: In order to determine the presence of immune-complexes in the sera of the Cy-treated and non-treated mice the polyethyleneglycol (PEG) precipitation technique developed by Creighton et al.[5] was carried out. Briefly, serum (1 ml diluted 1/10 in borate buffer) was mixed with 1 ml of 7% PEG in borate buffer, pH 8.6 and incubated at 4 °C for 18 h. The mixture was then centrifuged at 20,000 g for 30 min at 4 °C. The supernatant was discarded and the precipitate was washed, centrifuged with 2 ml of 3.5% PEG in borate buffer at 20,000 g for 30 min at 4 °C, resuspended in 2 ml of 0.1 M NaOH and read in a spectrophotometer at 280 nm. The statistical analysis of the results was done according to Fisher's method [6].

Histopathological studies: Samples of pancreas, spleen, liver, tongue, heart, lungs and kidney were fixed in Bouin's fluid, embedded in paraffin, sectioned at 7 µm and stained with hematoxylin and eosin.

Results. Histopathological findings: The only lesion found in the infected mice not treated with Cy was a marked pancreatic necrosis with polymorphonuclear cell infiltrates. Lesions were evident at 24 h p.i. with 1×10^4 PFU, when small foci of acinar cells showed pyknosis and cytoplasmic eosinophilia with disappearance of secretory granules. Polymorphonuclear margination in the small blood vessels and a marked interstitial edema were also present at that time. Between 48 h and 72 h p.i. most of the acini appeared necrotic, and the interstitial space was infiltrated with polymorphonuclear leukocytes (fig. 1). At 4 days p.i. lesions were less intense and by day 5 p.i. no inflammatory infiltrates were present and numerous mitoses were seen in the acini. At 7 days p.i. the pancreas looked normal. In no case were the islets of Langerhans damaged.

In mice treated with a low dose of Cy 4 days before infection lesions were almost absent. Only a few acini appeared necrotic at 48–72 h p.i. but the marked intercellular edema and the polymorphonuclear inflammatory infiltrate were not evident at any time p.i. (fig. 2).

In the remnant organs (e.g. spleen, liver, tongue, heart and kidney) no lesions were observed in Cy-treated and non-treated animals at any time p.i. No differences were observed when animals were inoculated with higher amounts of FMDV.

Virological studies: In the infected animals not treated with Cy virus could be recovered from pancreas as early as at 12 h p.i. Virus titer increased steadily until 24 h p.i. and decreased at 48 h p.i. At day 3 p.i. no virus could be recovered. In

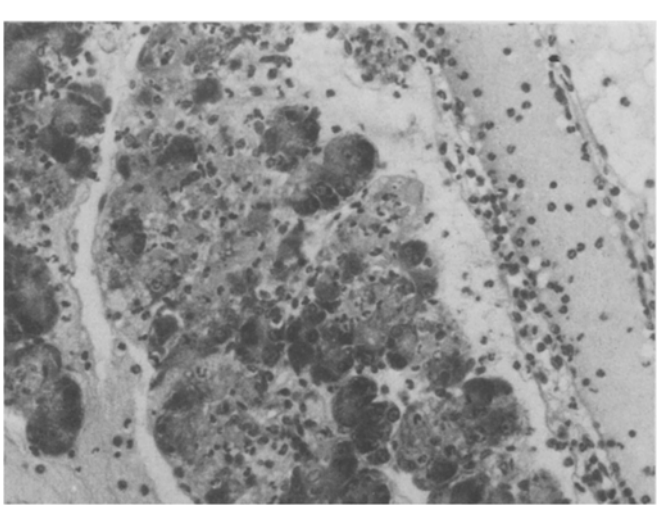

Figure 1. Pancreas of a mouse killed 48 h after infection with FMDV. Polymorphonuclear infiltrates, edema and acinar necrosis are evident. Hematoxylin and eosin. × 250.

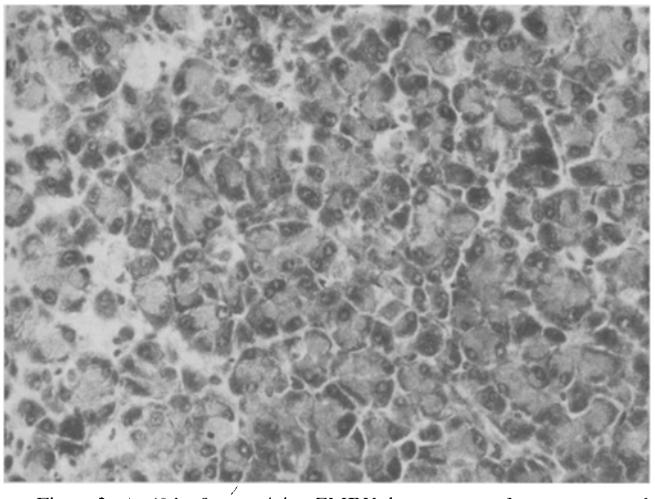

Figure 2. At 48 h after receiving FMDV the pancreas of a mouse treated with Cy 4 days before infection shows an almost normal appearance. Hematoxylin and eosin. × 250.

blood, virus was detected at 24 h p.i. and its concentration increased until 48 h. At day 3 p.i. no virus could be recovered from blood.

In animals pretreated with a low dose of Cy virus yields from pancreas was similar to the control non-treated group at 12 h p.i.; at 24 h p.i. virus concentration was more than 1 log lower than the control and at 48 h p.i. no virus could be recovered. In both groups, circulating virus was detected in blood at 24–48 h p.i. Although virus titer was lower in the Cy-treated animals, the difference between both groups was not significant. Results are summarized in tables 1 and 2. Attempts to recover virus from the other organs were negative at any time p.i.

Anti-FMDV immune response: In the control Cy non-treated mice neutralizing antibodies in serum could be detected as early as 3 days p.i. Figure 3 shows that maximum titer was present at day 21 and decreased at day 28. No neutralizing antibodies were detected in the sera collected at days 60 and 90 p.i.

In the animals injected with Cy, a several-fold higher titer of neutralizing antibodies, as compared with the non-treated group was present from day 3 p.i. and remained high until day 28 p.i. At 60 and 90 days p.i., when no antibodies could be detected in the group not treated with Cy, titers as high as 1/640 and 1/320, respectively, were still present. Previous experiments had shown that the same titer of neutralizing antibodies were obtained until day 30 p.i. with different doses of FMDV.

Immune-complexes detection: In all cases, the concentrations of immune-complexes in the sera of Cy-treated and non-treated mice were similar. No significant differences were found between both groups at any time p.i.

Discussion. Foot and mouth disease is a major, although rarely fatal disease of cattle and other domestic animals[7]. Infection of adult mice with FMDV produces a self-limiting disease with no mortality. The only lesion found is a severe pancreatic necrosis, established as early as 24 h p.i.[8], associated with a polymorphonuclear inflammatory infiltrate. Virus replicates in the pancreas until day 2 p.i. and a mild viremia is also observed. Neither histological lesions, nor virus replication can be detected in other organs. The pancreatic lesion and viral replication are ephemeral, and by day 7 p.i. the pancreas regains its normal appearance and no virus can be isolated. This is associated with the synthesis of anti-

Table 1. Virus yield from the pancreas in cyclophosphamide-treated and non-treated mice infected with FMDV. Viral titer was expressed as PFU/g tissue. NVD: no virus detected.

Hours p.i.	Cy-treated	Non-treated
12	$2.6 \pm 0.10 \times 10^3$	$5 \pm 0.15 \times 10^3$
24	$0.9 \pm 0.05 \times 10^3$	$1.6 \pm 0.05 \times 10^4$
48	NVD	$3 \pm 0.45 \times 10^3$
72	NVD	NVD

Table 2. Virus yield from blood in cyclophosphamide-treated and non-treated mice infected with FMDV. Viral titer was expressed as PFU/ml. NVD: no virus detected.

Hours p.i.	Cy-treated	Non-treated
12	NVD	NVD
24	$1.5 \pm 0.04 \times 10^3$	$6.5 \pm 0.13 \times 10^3$
48	$1.3 \pm 0.05 \times 10^3$	$5.5 \pm 0.09 \times 10^3$
72	NVD	NVD

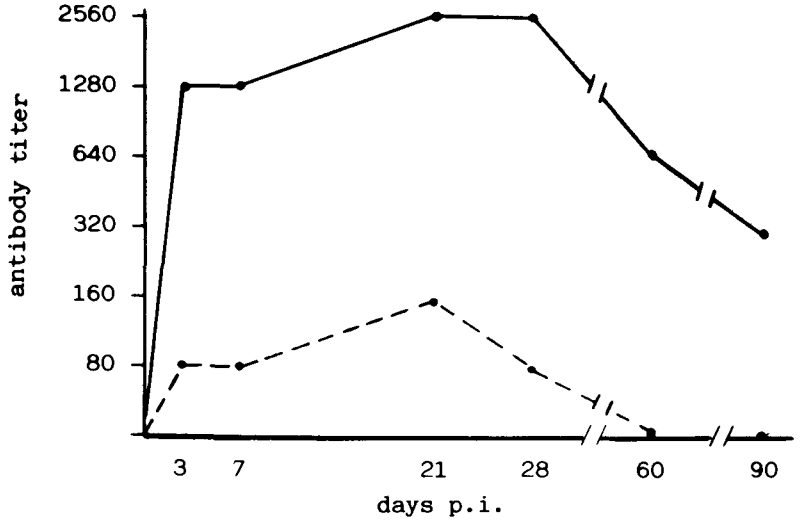

Figure 3. Titer of anti-FMDV neutralizing antibodies from non-treated (●----●) and Cy-treated (●—●) mice.

FMDV neutralizing antibodies which attain maximum titers in serum at about 3 weeks p.i.

Our results show that pretreatment of mice 4 days before infection with a low dose of Cy prevents the appearance of pancreatic lesions and decreases the virus yield. This was associated with an enhanced synthesis of anti-FMDV neutralizing antibodies, which not only reached titers in serum higher than those present in control mice, but also persisted for a longer time. This effect could be tentatively ascribed to the immunomodulating activity of Cy[4]. Given at a large dose Cy is a potent immunosuppressor agent[9-11]. However, it is known that Cy can also display an immunostimulating activity, probably by its action on the suppressor cascade[12]. Several reports claim that Cy, administered before antigenic challenge and in a low non-immunosuppressive dose, can enhance antibody formation[13].

In other systems, such as in implanted experimental tumors, a low dose of Cy can induce rejection and eventual cures, even when administered at a time where the tumors grow to a considerable size[4]. This phenomenon has also been associated to the activity of the drug on the T-suppressor cells[14]. Our results show that in Cy-treated mice the synthesis of anti-viral antibodies was markedly enhanced. This phenomenon could be responsible for the decrease in virus production in the pancreas and mildness of pathological alterations. Although the lower titer of neutralizing antibody in the serum of infected mice not treated with Cy could be ascribed to the presence of virus-antivirus complexes, this possibility seems unlikely since the level of circulating immune-complexes was similar both in the Cy-treated and non-treated mice.

The possibility that the drug can display a direct anti-viral effect seems also unlikely. The drug was administered 4 days before infection and according to its short half-life[15], a very small amount of Cy would be present at the time when virus replicates in the pancreas. Moreover, the fact that the first cycle of virus replication was not affected (at 12 h p.i. virus yields from pancreas of Cy-treated and non-treated mice were similar) supports the opinion that Cy, in the dose and schedule of administration we used, has no direct effect on virus replication.

Another alternative possibility for explaining the lack of pancreatic alterations is the well-known cytocidal effect of Cy on polymorphonuclear leukocytes[16]. Although leukopenia could be responsible for the absence of inflammatory infiltrates in the pancreas, other phenomena such as the acinar necrosis, which appears earlier than the polymorphonuclear infiltrate and the diminished virus replication, are unlikely to be related to the decrease in the leukocyte number.

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Effect of consumption of green and black tea on the level of various enzymes in rats

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Summary. Drinking of both green and black tea as the only liquid ingested resulted in significant decreases in the activity of transketolase in whole blood of rats both before and after the in vitro addition of thiamin diphosphate. Liver transketolase activity was decreased only by green tea. Mucosal transketolase activity was not affected by either type of tea. The activity of lactate dehydrogenase (LDH) was not affected by either type of tea, while whole blood LDH was decreased by both green and black tea. Neither tea had any affect on mucosal alkaline phosphatase, but thiamin diphosphatase activity was decreased by both teas. An increase in liver total thiamin resulted from the drinking of both types of tea.
Key words. Green tea; black tea; transketolase; lactate dehydrogenase.

Tea, both green (unprocessed) and black (processed), has been consumed as a beverage from time immemorial and in some societies fermented leaves are chewed as a stimulant. In some cultures, tea is the common drink in place of water. Many plants of nutritional value, including tea, have been shown to possess antithiamin activity[1-4]. Somogyi and coworkers[5,6] showed that the antithiamin activity was chiefly due to polyhydroxy phenols in the products. Tea is rich in such compounds, notably tannic acid, and has been shown to possess considerable antithiamin activity[3,7]. Hence studies have been made on the effects of tea consumption on thiamin nutrition and status in humans[8-10]. Heavy consumption of tea was shown to lead to a decrease in thiamin excretion in the urine and to an increase in the percent increase in the red cell transketolase activity after the in vitro addition of thiamin diphosphate (%TPP effect), which is considered diagnostic of thiamin deficiency if it is above 20%.