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Effect of Fusidic Acid on the Immune Response in Mice

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The effect of fusidic acid on the immune response in mice was studied. At the nontoxic dose of 500 mg/kg per day, the cell-mediated immunity was strongly inhibited. A marked and significant prolonged survival of split-heart allografts in treated animals was detected. The survival time of allografts in mice receiving fusidic acid from the day of the transplantation until the grafts were rejected was 26.1 days compared with 14.5 days in untreated animals. In mice treated also before the transplantation, the mean survival of the allografts were even longer. The phytohemagglutinin response, as well as the mixed lymphocyte culture stimulation of spleen lymphocytes from mice given 500 mg of fusidic acid per kg daily for 1 week, were significantly inhibited. At the same dose there was also a significantly decreased primary antibody response to sheep erythrocytes, but it was of limited biological significance. The immunosuppressive effect in animals treated with a human therapeutic dose of fusidic acid (25 mg/kg per day) was less pronounced but significant. The relevance of these results is discussed.

Fusidic acid is a steroid antibiotic isolated from *Fusidium cocineum*. It inhibits bacteria by preventing translocation of messenger ribonucleic acid to ribosome (16) and has a high capacity to bind serum protein (10).

Recent in vitro studies in our laboratory have shown fusidic acid to suppress the chemotaxis of human leukocytes (6) and also the lympho-proliferative response of human lymphocytes stimulated with phytohemagglutinin (PHA) or *Staphylococcus aureus* Cowan I (5, 8). The observations prompted us to investigate the in vivo effect of fusidic acid on the cellular and on the humoral immune response in mice.

MATERIALS AND METHODS

Animals. Mice CBA H-2^k (Anticimex AB, Evelund 19171, Sollentuna, Sweden) strains were used. They were 8 weeks old when first treated. Donor hearts were obtained from 12- to 36-h-old DBA/2J (Gl. Blomholtgård Ltd., 8680 Ry, Denmark).

Antibiotic. Fusidic acid (Fucidin, batch no. P7 NAG, Lövens, Sweden) was administered via a gastric tube in the morning in a single daily dose of 500, 250, 100, 50, or 25 mg/kg.

Antigen, immunization, and antibody titration. Immunization of mice with sheep erythrocytes (SRBC) and determination of agglutinating and hemolytic antibodies have been described before (1).

Antibody-producing cells. The splenic production of antibodies to SRBC was measured with a direct plaque-forming cell assay by a modification of the Jerne-Nordin method (4, 11), as described previously (1).

Transplantation. Heart grafts were transplanted with a modification of the Judd and Trentin method (13, 18) as described elsewhere (1).

Prepurification of the spleen cells. Spleen cells from CBA or DBA mice were suspended in RPMI 1640 (Gibco Bio-Cult, Ltd., Edinburgh, Scotland) containing 10% fetal calf serum and placed on nylon-wool columns by the method of Greaves et al. (9): 5×10^7 to 1×10^8 spleen cells in 1 to 2 ml of medium were placed on columns containing 300 to 500 mg of nylon fiber (12). The cells were then run into the column bed and incubated at 37°C for 30 min. Nonadherent cells were eluted from the column with 10 to 20 ml of warm (37°C) RPMI 1640 + 10% fetal calf serum at a rate of approximately 2 ml/min. These cells were used for mitogen stimulation and mixed lymphocyte culture (MLC) assay.

Stimulation with PHA. Prepurified spleen cells from CBA mice were stimulated in microtiter plates (Linbro Scientific Inc., Hamden, Conn.) containing 2.5×10^5 lymphocytes per culture in 200 μl of culture medium (RPMI 1640, 200 mM L-glutamine, 10% fetal calf serum, 5×10^{-5} M 2 mercaptoethanol [ME], and gentamicin (12 μg/ml).

PHA (Wellcome Research Laboratories, Beckenham, England) was added in final concentrations ranging from 5 to 1.25 μg/ml. Triplicate samples were incubated in 5% CO_2 at 37°C for 3 days. At 24 h before harvesting of the culture, 0.5 μCi of [^{3}H]thymidine, specific activity 48 Ci/mmol, (Net-0272; New England Nuclear Corp. Boston, Mass.) in RPMI 1640 was added. The radioactivity was measured in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.).

MLC. A one-way mixed lymphocyte culture (MLC) with stimulator cells from CBA mice and responder cells from DBA mice was used. Prepurified stimulator cells from DBA mice were suspended in serum-free RPMI medium, incubated in the presence of 25 μg of mitomycin C per ml, and washed twice at 37°C for 30 min. Responder and stimulator cells were thereafter treated with *N*-acyl-neuraminidase (Vibrio Comma

neuraminidase, Behringwerke, Marburg/Lahn, Germany). A 1-ml amount of cell suspension was incubated with 25 μl of neuraminidase (1 U/ml) at 37°C for 30 min and washed twice with serum-free RPMI medium (15).

Culture conditions for MLC. Responder and stimulator cells were suspended in RPMI 1640 supplemented with L-glutamine (200 mM) containing 5% heat-inactivated (56°C for 30 min) mouse serum, 5×10^{-5} M 2-ME, and gentamicin (12 μg/ml) and cultured in microtiter plates. Each microwell contained 2.5×10^5 responder cells and either 1.25×10^5, 2.5×10^5, 5×10^5, or 7.5×10^5 stimulator cells in 200 μl of medium. Triplicate samples were incubated during 5 days in 5% CO_2 at 37°C. At 24 h before harvesting, cells were pulsed with 0.5 μCi of [^{3}H]thymidine in RPMI 1640. The glass fiber filters containing the cells were dried and transferred to scintillation vials containing 4 ml of Instafluor solution, and the radioactivity was measured in a Tri-Carb liquid scintillation counter (Packard).

The MLC was performed according to two different protocols: (i) the first one was with spleen cells from CBA mice treated with 25 or 500 mg/kg per day 1 week before the MLC experiment; (ii) the second one was with spleen cells from untreated animals but with fusidic acid added to the cultures at the beginning of the experiment in a final concentration of, respectively, 1, 5, 10, 25, 50, and 100 μg/ml.

Acute toxicity. The evaluation of the 50% lethal dose of fusidic acid in CBA mice was performed according to the standard protocol described in detail elsewhere (17).

Statistics. Student's *t* test was used.

RESULTS

Toxicity of fusidic acid. The antibiotic in a dose of 500 mg/kg per day was apparently well tolerated by the mice and no signs of toxicity were observed. The 50% lethal dose of the antibiotic found in the CBA mice was 1,070.5 mg/kg + 21.9 mg/kg.

MLC. Figure 1 shows results obtained when spleen cells from untreated CBA mice and DBA mice were tested in MLC. Various concentrations of fusidic acid were added to the cultures. At an antibiotic concentration of 10 μg/ml, the stimulation index was less than 40% of the control, and at a concentration of 50 μg/ml, practically no activation occurred.

Table 1 demonstrates the effect of fusidic acid given to CBA mice for 1 week before isolation of spleen cells and in in vitro MLC. For mice treated with 500 mg/kg per day of fusidic acid, the blastogenic index in MLC-stimulated culture was 23.5 compared with 51.9 in the control, whereas lymphocytes from animals treated at human therapeutic dose (25 mg/kg per day) responded normally in the MLC.

Mitogenic response. CBA mice were treated daily 1 week before the in vitro experiments with 25 or 500 mg of fusidic acid per kg. The spleen cells were isolated and stimulated with PHA. No significant inhibition of the lymphocyte stimulation was observed for cells from animals given human therapeutic dose (25 mg/kg per day) (Table 2). Fusidic acid treatment in a dose of 500 mg/kg per day reduced the response to approximately 50% of the control.

Survival time of allografts. Figure 2 illustrates the effect of fusidic acid on the survival

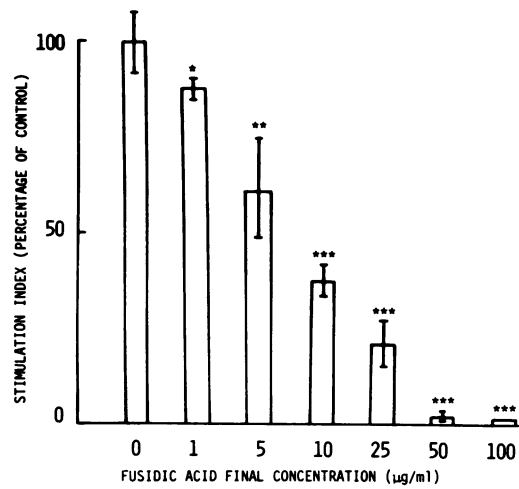

FIG. 1. *[^{3}H]thymidine incorporation in MLC-stimulated CBA lymphocytes in the presence of different concentrations of fusidic acid; 2.5×10^5 untreated CBA spleen lymphocytes served as responder and 5×10^5 mitomycin-treated lymphocytes from normal DBA mice as stimulator cells. The antibiotic was added at the first day of the experiment in final concentrations ranging from 1 to 100 μg/ml. The results are expressed as percentage (± standard error of the mean) of the stimulation index obtained in the absence of fusidic acid. The stimulation index in the reference group was 58.9 ± 5.8. Lymphocytes from five animals were tested in triplicate cultures at each antibiotic concentration. *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.*

TABLE 1. *MLC response of spleen lymphocytes from CBA mice treated with fusidic acid*[a]

Dose of treatment (mg/kg)	[^{3}H]thymidine uptake ± SE (cpm)	Stimulation index	Inhibition (%)
0	31,866 ± 3,721	51.9 ± 6.06	
25	32,191 ± 5,860	52.4 ± 9.5	
500	18,289 ± 4,614	23.5 ± 5.9[b]	44.8%[b]

[a] 2.5×10^5 responder lymphocytes from CBA mice were treated daily for 1 week before the experiment with 25 or 500 mg of fusidic acid per kg, and 5×10^5 mitomycin-treated stimulator lymphocytes from normal DBA mice were added to each culture. The present data are expressed as the mean ± standard error (SE) of counts per minute of [^{3}H]thymidine incorporation at the fifth day of triplicate cultures. Five animals were tested in each group. MLC response of age-matched untreated DBA mice served as reference.

[b] $P < 0.001$.

time of split-heart allografts from DBA mice in CBA mice when administered daily in a dose of 500 mg/kg from the day of transplantation until the day of rejection. Untreated transplanted mice were used as controls. The figure demonstrates a significantly ($P < 0.001$) delayed rejection of the grafts in antibiotic treated animals. When fusidic acid administration was started on the day of the transplantation, the allografts survived 26.1 ± 5 days compared with 14.5 ± 6.7 days in the reference group. In mice treated for 8 days or 7 weeks before the operation and from the day of transplantation until rejection, the mean survival time of the allografts, plus or minus standard deviation, was 27.5 ± 6.6 days and 33.3 ± 6.7 days, respectively.

Figure 3 shows the survival of allografts in mice given fusidic acid in a dose of 500 mg/kg per day only the last 20 days before the transplantation (day zero), or from day zero until day 7 after transplantation, or from day 9 after transplantation until the rejection. As shown in the figure, a notable ($P < 0.01$) delay occurred in the rejection of the grafts in the mice given fusidic acid from day 9 after transplantation until rejection. The mean survival time was 20.7 ± 6 days. In the two other groups of animals, the survival did not differ significantly from that in the untreated reference group.

Doses ranging from 25 to 250 mg/kg per day administered to the animals from the day of the

TABLE 2. *PHA response of spleen lymphocytes from CBA mice treated with fusidic acid*[a]

Dose of treatment (mg per kg per day)	[^{3}H]thymidine uptake ± SD (cpm)	Stimulation index	Inhibition (%)
0	53,715 ± 10,780	59.05	
25	48,805 ± 13,062	53.2	10[b]
500	29,285 ± 9,022	30.8	47.8[c]

[a] Spleen lymphocyte response to 1.25 μg of PHA per ml in CBA mice treated daily 1 week before the experiment with 25 or 500 mg of fusidic acid per kg. The data are expressed as the average counts per minute of [^{3}H]thymidine incorporation ± standard deviation (SD). Ten animals were tested in each group. Age-matched untreated mice served as reference.

[b] $P > 0.05$ $P < 0.2$.

[c] $P < 0.001$.

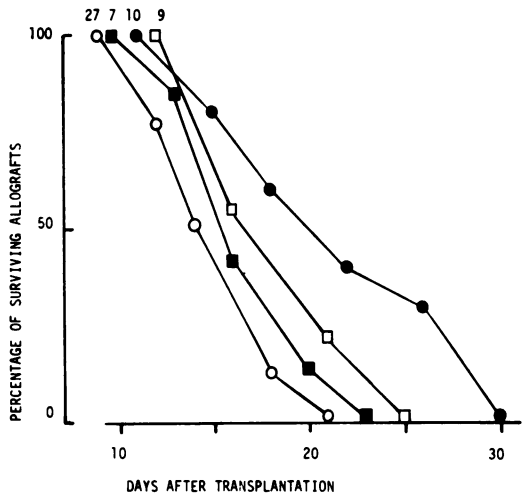

FIG. 3. *Percentage of surviving split-heart DBA allografts in CBA mice treated daily with fusidic acid (500 mg/kg per day) from the day of the transplantation until day 7 after transplantation (■), or from day 9 after transplantation until rejection of the grafts (●), or from D-20 until D-1 before the day of transplantation (□). Untreated transplanted mice (○) served as reference. Figures at the top of each curve denote the number of transplantation experiments in each group.*

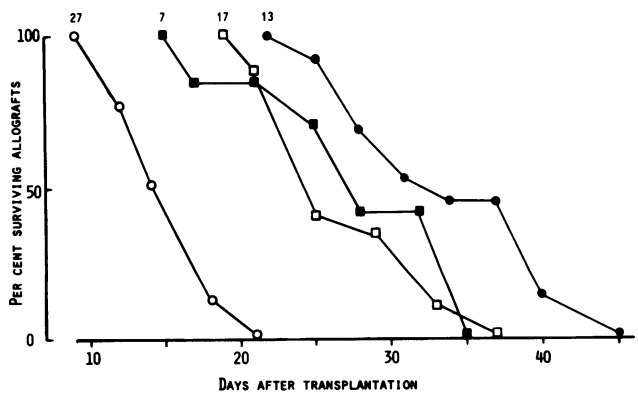

FIG. 2. *Percentage of surviving split-heart DBA allografts in CBA mice treated daily with fusidic acid (500 mg/kg per day) from the day of the transplantation until rejection of the grafts (□) and, in addition, for 8 days (■) or 7 weeks (●) before the transplantation. Untreated transplanted mice (○) served as reference. Figures at the top of each curve denote the number of transplantation experiments in each group.*

transplantation until the rejection of the grafts also gave a prolonged graft survival (Fig. 4). However, the effect was slighter than in animals receiving 500 mg/kg per day. In mice receiving a human therapeutic dose (25 mg/kg per day), the allografts survived 17.8 ± 2.3 days ($P < 0.02$) and in those receiving 250 mg/kg per day, a prolonged survival time of 20.6 ± 2.9 days ($P < 0.01$) was observed.

Antibody-producing cells. Direct plaque-forming cells from spleen of mice immunized with sheep erythrocytes and treated with 500 mg/kg per day of fusidic acid were evaluated. The animals were given the antibiotic daily from the day of immunization (day zero) until day 3, with or without pretreatment, or from day 10 to day 2 before the day of immunization (D-10 and D-2, respectively). Mice immunized but untreated were used as reference animals. As shown in Fig. 5, a marked ($P < 0.001$) inhibition of the primary antibody response to SRBC occurred in groups of mice treated from day zero to day 3. Treatment from day zero to day three was attended by a decrease of 55% in the number of plaque-forming cells, from D-30 to day 3 by a decrease of 51%, and treatment from D-7 to day 3 by a decrease of 57%. Figure 5 also shows that no decrease occurred in the number of plaques of hemolysis for spleen cells of mice which had only been pretreated with fusidic acid from D-10 to D-2 before immunization.

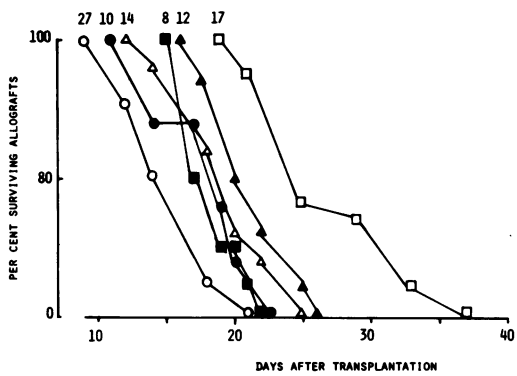

FIG. 4. *Percentage of surviving split-heart DBA allografts in CBA mice treated daily with fusidic acid: 25 mg/kg (■), 50 mg/kg (●), 100 mg/kg (△), 250 mg/kg (▲), or 500 mg/kg (□) from the day of transplantation until the rejection of the grafts. Untreated transplanted mice (○) served as reference. Numbers at the top of each curve denote the number of transplantation experiments in each group.*

Agglutinating and hemolytic antibodies. The agglutinating and hemolytic antibody titers in mice immunized with SRBC and treated with fusidic acid (500 mg/kg per day) were determined. Table 3 shows that at day 4 mice treated with fusidic acid from day zero until day 3 had an agglutinating antibody level ($P < 0.01$), as well as a hemolytic antibody level ($P < 0.01$), lower than the immunized but untreated reference mice. Mice treated for the same period, as well as from D-7 or D-30 before the immunization, also showed a striking decrease of both the agglutinating antibody titers ($P < 0.01$) and the hemolytic antibody titers ($P < 0.01$ and $P < 0.001$). In contrast, mice treated from D-10 to D-2 exhibited only a slightly and not significantly decreased primary antibody response to SRBC.

DISCUSSION

Only few reports are available on the interaction between fusidic acid and immunity. Forsgren and Schmeling (6) observed that fusidic

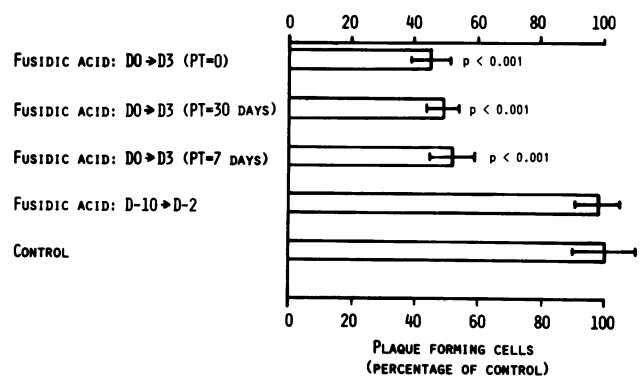

FIG. 5. *Direct plaque-forming cells from spleens of mice immunized with SRBC and treated with fusidic acid (500 mg/kg per day) from the day of immunization (D0) until day 3 (D3) with or without pretreatment (PT). Mice treated daily only from D-10 to D-2 before the immunization are included in the figure. The results denote the mean ± standard error expressed as percentage of the controls. Each group consisted of 10 mice. The number of plaque-forming cells in the control group was 101 ± 9 per 10^5 lymphocytes.*

TABLE 3. *Agglutinating and hemolytic antibodies from sera of mice immunized with SRBC*[a]

Status	Agglutinating titers ± SE	t	P	Hemolytic titers ± SE	t	P
Normal	102 ± 24			211 ± 49		
Fusidic acid: D0–D3 (PT = 0)	23 ± 4	3.246	<0.01	66 ± 13	2.860	<0.01
Fusidic acid: D0–D3 (PT = 7 days)	24 ± 5	3.181	<0.01	56 ± 13	3.057	<0.01
Fusidic acid: D0–D3 (PT = 30 days)	25 ± 7	3.080	<0.01	43 ± 11	3.345	<0.001
Fusidic acid: D-10–D-2	67 ± 10	1.346	<0.2	125 ± 24	1.576	<0.2

[a] Mice were treated daily with 500 mg of fusidic acid per kg from day zero (D0) to day 3 (D3) with or without pretreatment (PT). Mice treated daily with fusidic acid from D-10 to D-2 before the day of immunization are included. Agglutinating and hemolytic antibody titers from sera of untreated but immunized mice were used as references.

acid in a concentration of 10 μg/ml markedly suppressed the chemotaxis of human polymorphonuclear leukocytes and that this was due to an inhibition of protein synthesis (7). Forsgren and Banck (5) reported that at a fusidic acid concentration of 10 μg/ml, the incorporation of thymidine into PHA-stimulated human peripheral lymphocytes was 50% of that in control experiments and that at an antibiotic concentration of 50 μg/ml, the incorporation of thymidine was only 4% of that in controls. Similar results were obtained with *S. aureus* Cowan I as B lymphocyte mitogen and for [^{14}C]leucine incorporation in unstimulated lymphocytes. We observed a total inhibition of the mouse lymphocyte stimulation in MLC at an antibiotic concentration of 50 μg/ml which confirms the data mentioned above.

The observations in this study suggest that the in vitro results obtained by Forsgren et al. (5–7) may be relevant and may have in vivo implications. A substantially prolonged survival of allografts was observed in mice treated with fusidic acid. A very strong effect occurred at 500 mg/kg per day of the drug, whereas slighter but significant prolongation of the allografts was observed at a human therapeutic dose (25 mg/kg per day). Animals that were only pretreated with fusidic acid and not given the drug after transplantation showed insignificantly decreased immune response. This could indicate that the immunosuppressive effect of fusidic acid is reversible. The delayed rejection of the allografts when treatment was started at day 9 after transplantation might prove clinically useful in the prevention of graft rejection. We also observed a significantly decreased response of the spleen lymphocytes from mice treated daily with 500 mg/kg per day of the drug 1 week before PHA stimulation or MLC assay.

Our study also demonstrates a significant immunosuppressive effect on the primary antibody response to SRBC estimated from the number of plaque-forming cells detected and the agglutinating and hemolytic antibody titers. However, it might be possible to interpret that the effect of fusidic acid on humoral immune response is of more limited biological relevance than the effect on cellular immunity. It is of interest that the results in the present study on fusidic acid were almost identical with those obtained in a corresponding investigation of the immunosuppressive properties of rifampin (1).

The mechanism by which fusidic acid interferes with the animal cells is not properly understood, and only few tentative explanations have been offered. Malkin and Lipman (14) observed that the antibiotic inhibits the synthesis of protein by interfering with the ribosome-dependent guanosine 5'-triphosphatase activity of a supernatant fraction from reticulocytes. They also reported inhibition of incorporation of [^{14}C]valine into hemoglobin in reticulocytes, suggesting effective diffusion of the antibiotic into the cells and a mode of action in animal cells similar to that in bacteria. Inhibition of protein synthesis was also observed by Forsgren et al. (5, 7), an effect suggested to reduce chemotaxis and lymphocyte stimulation. Brown and Percival (3) reported a good diffusion of fusidic acid into human peripheral blood leukocytes and mouse macrophages. It would seem that the marked immunodepression caused by fusidic acid is due to high intracellular levels of the drug giving inhibition of protein synthesis in animal cells. This is in accordance with a report on a catabolic effect upon protein synthesis in clinical situations caused by fusidic acid (19). It is also known that fusidic acid is a surface active agent causing alterations of the cell membrane surface.

The rate of elimination of fusidic acid from the blood is rather slow, and accumulation of the drug occurs. A serum concentration of 100 μg/ml is normally obtained after a week of treatment at human therapeutic dose in clinical situations (16). However, the protein binding of

fusidic acid is high in human serum (10), but we do not know about the binding properties in mice. Therefore, no obvious extrapolation of our results to clinical situations can be done. However, we want to emphasize that mice tolerate usually 10 to 20 times as much immunosuppressive drugs as humans (2), which could make the present data of high clinical significance. Therefore, a better understanding of this process is certainly desirable, especially since it might have in some situations favorable and in other situations unwanted clinical consequences.

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