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NECESSITY OF ANTIBODY RESPONSE IN THE TREATMENT OF AFRICAN TRYPANOSOMIASIS WITH α-DIFLUOROMETHYLORNITHINE

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Abstract—The role of the immune system in the clearance of *Trypanosoma brucei brucei* from the bloodstream during α-difluoromethylornithine (DFMO) treatment was studied by determining the effects of dexamethasone on immune and therapeutic responses in rats infected with *T.b. brucei*. Normal DFMO-treated animals exhibited strong antibody responses to trypanosomes and were cured of *T. b. brucei* infection by a 7-day regimen of 2% DFMO in drinking water. Animals pretreated with dexamethasone were not cured by the same level of DFMO treatment. Nonetheless, in rats pretreated with dexamethasone, trypanosomes were cleared from blood during treatment with DFMO, but all immunocompromised animals eventually succumbed to relapses of the infection. Athymic (nude) mice were cured of *T. b. brucei* infection by a 72-hr course of treatment with 2% DFMO in their drinking water. These findings suggest that relatively low levels of T-cell-independent, antitrypanosomal antibodies are adequate to clear the bloodstream of parasites during DFMO therapy but that an intact immune response is necessary for cures of the disease to be obtained.

α-Difluoromethylornithine (DFMO), a catalytic irreversible inhibitor of ornithine decarboxylase [1, 2], has been shown to rapidly deplete the intracellular polyamines putrescine and spermidine in trypanosomes and to inhibit trypanosomal DNA synthesis and proliferation [3], as it does in a number of other cell types undergoing rapid proliferation [4]. Administration of DFMO effects cures of African trypanosomiasis in both experimental murine infections [5] and natural human infections [6]. The effects of DFMO on trypanosomes are thought to be primarily cytostatic rather than cytolytic [5, 7] and are clearly related to polyamine depletion since cures of murine trypanosomiasis by DFMO can be blocked by the coadministration of the polyamines putrescine or spermidine [8]. If DFMO is a cytostatic agent, there must be other factors involved in the clearance of trypanosome infections by the drug. One factor which could augment or supplement the effects of DFMO is an immune response to the trypanosome since antibody responses are known to be involved in the development of immunity to and clearance of trypanosomal infections [7, 9–11]. In fact, it was shown earlier that cyclophosphamide-immunosuppressed mice, which produced no trypanosome-specific antibodies, did not completely clear *Trypanosoma rhodesiense* from their blood during treatment with DFMO [7].

Treatment with glucocorticoids enhances the severity of infections with a variety of bacteria, viruses and parasites [12]. Dexamethasone treatment also inhibits the production of trypanocidal antibodies in rats infected with *Trypanosoma lewisi*, resulting in a normally benign infection becoming lethal [13, 14]. In the present work we have examined the production of antibodies by trypanosome-infected rats immunosuppressed with dexamethasone, a synthetic glucocorticoid, which markedly depresses both humoral and cell-mediated immune response in susceptible species [15]. We have also studied the effects of DFMO on the long-term survival of similarly immunocompromised rats and on congenitally athymic (nude) mice infected with *Trypanosoma brucei brucei*.

MATERIALS AND METHODS

Trypanosomes. *Trypanosoma brucei brucei* (EATRO 110), a monomorphic strain causing acute infections, was obtained from Dr. Cyrus Bacchi of the Haskins Laboratories, New York, NY. It was maintained in our laboratory by syringe passage in male Sprague–Dawley rats and was used in all experiments described herein. For survival experiments rats were injected intraperitoneally with 5×10^6 trypanosomes; the ensuing infection was lethal in untreated rats within 4–6 days.

Drug treatment. Male Sprague–Dawley rats weighing approximately 225 g at the beginning of the experiment were administered tetracycline (1 g/l) or tetracycline (1 g/l) plus dexamethasone (1 mg/l) in their drinking water. Dexamethasone at 1 mg/l in drinking water has been shown previously to produce significant immunosuppression in rats [16]. Tetracycline was present to protect the rats against bacterial infections which could arise during immunosuppression. After 21 days of dexamethasone treatment the animals were infected with trypanosomes. After 24 hr of infection, one group of rats was given 2% DFMO in their drinking water in addition to the dexamethasone and tetracycline.

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DFMO was administered for 7 days and then removed. The animals were then observed daily for mortality and periodically for parasitemia. Since long-term dexamethasone treatment can also cause fatal *Pneumocystis carinii* infections in rats [16], we examined the lungs of a similarly-treated group of animals for the presence of *P. carinii* and found them to be uninfected even after 6 weeks of dexamethasone treatment.

Body, spleen and thymus weights as well as hematological variables were also determined after 21 days of treatment with dexamethasone. Erythrocytes, leukocytes and platelets were all counted with an automated hematology analyzer. Lymphocyte percentages were counted in thin blood smears stained with Giemsa.

In experiments designed to measure antibody responses, rats were administered drugs and infected with trypanosomes, as above; sera were collected from rats after 5 days of DFMO (2% in drinking water) treatment, at a time when no intact trypanosomes were found in blood.

Antibody reponses. Antibody responses of infected rats to the variable surface glycoprotein (VSG) were measured by an indirect fluorescent antibody assay [7]. Blood was taken by cardiac puncture and allowed to clot in glass tubes. Serum was separated by centrifugation at 2500 rpm in a refrigerated centrifuge and then frozen at −20° until used for antibody assays. Viable trypanosomes were used as antigens in the indirect fluorescent assay. Organisms were separated from the blood of 48-hr infected rats using DEAE-cellulose chromatography as described [17]. Purified trypanosomes were washed twice with a solution of 10 mM Na_2HPO_4, 3 mM KH_2PO_4, 125 mM NaCl and 1% glucose (PBS/glucose) and then diluted in the same buffer to a concentration of 2×10^8 trypanosomes/ml. Serum was serially diluted with PBS/glucose, with 25 μl of each diluted serum sample pipetted into V-bottom wells of microtiter plates. To the wells was added an equal volume of buffer containing 5×10^6 trypanosomes and the mixture was incubated for 20 min on ice. The trypanosomes were washed twice with PBS/glucose by centrifugation, 50 μl of an appropriate concentration of FITC-labeled rabbit anti-rat immunoglobulin was added, and plates were incubated on ice for 15 min. After washing once, the trypanosomes were suspended in 50 μl PBS/glucose, and drops of the suspensions were examined by fluorescence microscopy. Control sera were obtained from uninfected rats. The reciprocal of the highest dilution of sera that resulted in fluorescence of the trypanosomes was determined as the titer of the antisera.

Athymic mice. Congenitally athymic (nu/nu) mice (20 g) were housed in sterile isolators and supplied with autoclaved food, water and bedding material. The mice were only removed from their isolators for injection of trypanosomes (2.5×10^5/mouse) and daily parasitemia checks, carried out on 3 μl of blood obtained from a tail vein. Beginning 24 hr post-infections, mice were administered 2% DFMO in their drinking water for 72 hr after which treatment was discontinued and daily checks were made for mortality.

Chemicals. Dexamethasone was purchased from Sigma, FITC-conjugated rabbit anti-rat immunoglobulins from Accurate Chemicals, and tetracycline from American Cyanamid. α-Difluoromethylornithine (DL-α-difluoromethylornithine hydrochloride monohydrate; MDL 71782) was synthesized in our laboratories [13].

RESULTS

Dexamethasone (1 mg/l) severely disrupted the normal physiology of the treated rats (Table 1). Three weeks of dexamethasone treatment led to a decrease in body weight and a general emaciated condition. Variables indicating immunodeficiency were decreased markedly: spleen weights, thymus weights and leukocyte counts, including the number of circulating lymphocytes. Erythrocyte counts were unchanged while platelets decreased approximately 50%.

Dexamethasone inhibited the production of trypanosome-specific antibodies by DFMO-treated rats. As shown by indirect immunofluorescence, normal DFMO-treated immunocompetent rats demonstrated a strong VSG-specific antibody response with average $\log_2$ serum titers of 7 (range = 5–9) (Table 2). Dexamethasone-treated rats responded to infection with trypanosomes with average $\log_2$ serum titers of VSG antibodies of only 4 (range = 0–6).

The decreased antibody response following trypanosome infection in the DFMO plus dexamethasone treated rats was associated with an impaired ability of the immunosuppressed animals to eliminate the infection when given normally curative doses of DFMO. As shown in Table 3, normal animals were cured of their infections by treatment with 2% DFMO in drinking water for 7 days. Infected animals treated only with DFMO survived for longer than 60 days, whereas infected dexamethasone-

Table 1. Effects of dexamethasone treatment on rats

Drug treatment	Initial body weight (g)	Final body weight (g)	Spleen weight (g)	Thymus weight (g)	WBC ($\times 10^3$/mm^3)	Lymphocytes (% WBC)
Control	235 ± 11 (21)	353 ± 21 (22)	0.868 ± 0.212 (6)	0.450 ± 0.089 (6)	12.5 ± 1.1 (6)	83 ± 3.5 (6)
Dexamethasone	227 ± 13 (30)	196 ± 25 (29)	0.181 ± 0.038 (10)	0.041 ± 0.021 (10)	6 ± 2.6 (7)	12.1 ± 3.6 (7)

Values represent the mean ± S.D. for the number of animals shown in parentheses.

Table 2. Suppression of antibody responses to trypanosomes by dexamethasone treatment

Dexamethasone	Trypanosomes	Antitrypanosomal antibody titers in individual animals
0	0	0, 0
0	+	128, 64, 128, 32, 32, 128, >512, 64
+	0	0, 0
+	+	16, 2, 8, 0, 0, 2, 64

Table 3. Effects of dexamethasone and DFMO on the survival of trypanosome-infected rats

Drug treatments		Average survival time	
Dexamethasone	DFMO	(days)	No. cured/No. infected
0	0	5	0/6
0	+	>60	6/6
+	0	5	0/8
+	+	20	0/8

treated rats survived only an average of 20 days after treatment with DFMO. The latter animals died from a fulminating trypanosome infection which was confirmed by examining wet smears of blood taken from a tail vein. No immunosuppressed animals were totally cured with DFMO, although it was noted that after 7 days of DFMO treatment both normal and immunosuppressed animals were free of apparent parasitemia.

Athymic mice infected with *T. b. brucei* rapidly cleared their parasitemia and were cured (lived longer than 30 days past controls) by treatment with 2% DFMO in their drinking water (Fig. 1).

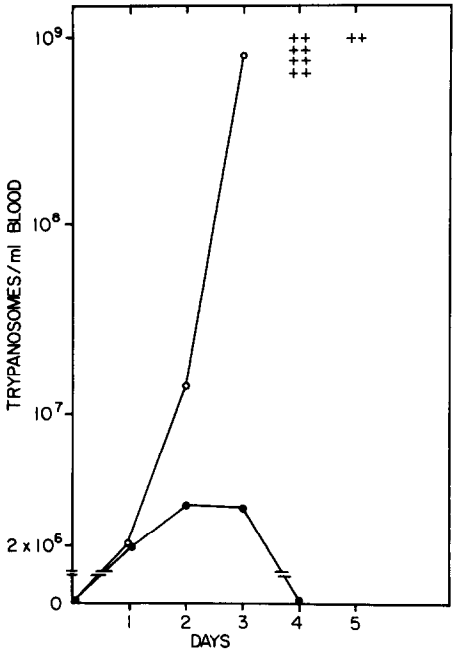

Fig. 1. Effects of DFMO treatment on the progress of *T. b. brucei* infections in athymic mice. Athymic mice were injected with 2.5×10^5 trypanosomes which were collected aspectically from 72-hr infected rats by cardiac puncture. DFMO treatment (●) was begun on 10 mice 24 hr after infection while ten mice served as untreated controls (○). Animal deaths in the untreated control group are signified with a + sign.

DISCUSSION

We have confirmed and extended the earlier observation [7] that there is a strong correlation between the production of trypanosome-specific antibodies and cure in infected DFMO-treated animals. Immunocompetent infected animals treated with DFMO rapidly cleared their bloodstream and, apparently, other tissues, of parasites and did not relapse for up to 60 days. Infected animals whose VSG-specific antibody responses were depressed by prior treatment with dexamethasone initially cleared parasites from the bloodstream, seemingly as well as the immunocompetent animals, but all of these immunosuppressed animals eventually relapsed and died from a fulminating trypanosome infection 20 days (on the average) after the initial inoculation. It seems, therefore, that the initial clearance of trypanosomes from the bloodstream during DFMO treatment requires only very low levels of antibodies, but the complete clearance of trypanosomes from the entire body requires a much stronger antibody response. This "sterile immunity" may be linked to penetration of antibodies to anatomically occult sites of infection.

The immune response to trypanosomes in DFMO-treated animals is probably mediated primarily by a T-independent B-cell response since we also found that DFMO will cure *T. b. brucei* infections in congenitally athymic (nude) mice. This finding is in general agreement with previous studies which demonstrated that congenitally athymic nude mice could be immunized against *T. rhodesiense* [10] but B-cell deficient mice did not develop immunity to *T. rhodesiense* [9], indicating that a T-cell-independent

antibody response is responsible for the development of immunity to *T. b. rhodesiense* infection in mice [9, 10].

Although there is a paucity of clinical studies, it is evident that one of the consequences of a natural trypanosome infection in humans [18], as well as experimental infections in animals [19], is suppression of both cell-mediated and humoral immunity. Parasite-induced immunosuppression almost certainly affects the well being of the sleeping sickness patient with regard to the possibility of secondary infection [20]. It is not clear, however, that the immunosuppressive effects of the trypanosome are important in determining the efficacy of chemotherapy with DFMO or other agents since it has been demonstrated that administration of Berenil (diaminazine aceturate), a trypanocidal drug, to cattle infected with *T. vivax* leads to restoration of host immune responses [19]. The same phenomenon may occur in sleeping sickness patients treated with DFMO since cures of late-stage trypanosomiasis have been obtained with DFMO in patients who would be expected to be maximally immunosuppressed by the trypanosomes [6] .

It is also significant that, in acquired immune deficiency syndrome (AIDS) patients who are severely immunosuppressed, DFMO has recently demonstrated therapeutic efficacy in the treatment of *Pneumocystis carinii* pneumonia [21, 22]. In the present work we have confirmed, in animals, that it is possible to obtain a therapeutic, although noncurative, effect (i.e. rapid clearance of bloodstream trypanosomes) in response to DFMO in the presence of almost complete immunosuppression.

Other modes of action of DFMO on the trypanosome may also exist. DFMO induces a morphological shift in monomorphic and plieomorphic trypanosome populations from the long slender trypomastigote form to short stumpy forms [3, 23]. Studies in our laboratory demonstrate that the short stumpy forms are no longer able to divide and therefore may be senescent or degenerate organisms which spontaneously lyse in the bloodstream of the host [24]. Thus, the depletion of polyamines following DFMO might not lead directly to cell death but rather to a cytological differentiation or transformation which, in turn, leads to death of the trypanosome.

The recent discovery of a spermidine-containing cofactor (trypanothione) for trypanosomal glutathione reductase [25, 26] has suggested yet another mode of action for DFMO in trypanosomes. Depletion of spermidine could lead to a reduction in trypanothione, a consequent reduction in glutathione reductase activity, and an increased susceptibility of the trypanosome to oxidative damage.

Although the data presented here clearly demonstrate that a well-developed antibody response is necessary for long-term cures of trypanosomiasis following DFMO treatment, the mechanism(s) by which the first wave of parasitemia is controlled during DFMO treatment is unclear. Factors contributing to destruction of the trypanosomes during DFMO treatment may include: (1) a low or nascent antibody response that may be sufficient for destruction, (2) DFMO-induced polyamine depletion leading to direct lysis of the long slender bloodstream forms of trypanosomes, or (3) DFMO-induced transformation of trypanosomes to degenerate short stumpy forms which spontaneously lyse in the bloodstream. We are currently investigating the contribution of DFMO-induced transformation to the overall effect of DFMO therapy.

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