

Nifurtimox-induced alterations in the cell-mediated immune response to PPD in guinea-pigs

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SUMMARY

Positive skin reactions to PPD in guinea-pigs immunized with Freund's complete adjuvant (FCA) were reversed after treatment with 10 mg/kg/day nifurtimox for 12 days. The *in vitro* migration of peripheral blood leucocytes from FCA-immunized guinea-pigs was inhibited with PPD, but it returned to normal values after nifurtimox treatment. Furthermore, the cell-free supernatant from PPD-stimulated lymphocytes from FCA-immunized nifurtimox-treated guinea-pigs did not inhibit the migration of normal cells. Thus the administration of nifurtimox impaired the specific cell-mediated immune response to PPD both *in vivo* and *in vitro*.

INTRODUCTION

Chagas' disease (American trypanosomiasis) is one of the most important health problems in many South and Central American countries (Review Article, 1974). The treatment of Chagas' disease is carried out with trypanocidal drugs, mainly with a nitrofuranyl derivative, nifurtimox (3-methyl-4-(5'-nitrofururylidene-amino)-tetrahydro-4H-1,4-thiazine-1,1-dioxide), that is widely used in Argentina (Boccas Tourres, 1969; Wegner & Rohwedder, 1972). We have observed nifurtimox-induced alterations of the cell-mediated immunity in Chagas' disease after nifurtimox treatment (Lelchuk *et al.*, 1973). The *in vitro* migration of peripheral leucocytes from untreated chronic Chagas' disease patients was inhibited with *Trypanosoma cruzi*-specific antigens, but the migration of the cells from nifurtimox-treated Chagas' disease patients was not inhibited by the same antigens. In order to understand the mechanism involved in this process, an experimental model with an antigen unrelated to the *Trypanosoma cruzi*-specific antigens was developed. In this work, skin reactivity and leucocyte inhibitory activity of guinea-pigs immunized with Freund's complete adjuvant was studied before and after nifurtimox treatment.

MATERIALS AND METHODS

Animals. Guinea-pigs of either sex weighing 500-700 g were injected with 0.2 ml of Freund's complete adjuvant (FCA, Difco), twice, in the footpads, with a 10 days interval.

Nifurtimox-treated* guinea-pigs were given 10 mg/kg/day of the drug dissolved in 3% gelatine, by gastric intubation, for 12 days. The protocol of treatment is shown in Fig. 1.

Skin test. Skin tests were performed by inoculating 0.1 ml PPD† (10,000 iu/ml, 0.2 mg/ml) subcutaneously into the back thigh, 10 days after immunization with FCA and/or nifurtimox treatment (Fig. 1). Skin tests were read at 48 hr and areas of induration of 10 mm in diameter were considered positive.

Direct leucocyte migration inhibition test (LMI). Leucocytes were obtained from blood drawn by heart puncture at times shown in Fig. 1. Leucocyte migration inhibition tests were performed in agarose (Hoffman *et al.*, 1975) with PPD free of preservatives at a final concentration of 100 µg/ml (5000 iu/ml). The migration index (MI) was calculated as follows:

* 3-methyl-4-(5'-nitrofururylidene-amino)-tetrahydro-4H-1,4-thiazine-1,1-dioxide (Lampit®, powder, free of additions) was kindly given by Bayer Argentina Laboratories.

† PPD used in the present work was provided by Centro Panamericano de Zoonosis, Oficina Sanitaria Panamericana, Oficina Regional of the World Health Organization.

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$$MI = \frac{\text{migration area with PPD}}{\text{migration area without PPD}} \times 100.$$

The values used represented the average of triplicate tests.

Indirect leucocyte migration inhibition test. The indirect leucocyte migration inhibition test was performed at the times shown in Fig. 1. Peripheral blood lymphocytes (PBL) were purified in Ficoll-Hypaque gradients (Böyum, 1968). They were composed of 85–90% mononuclear cells. Viability, assessed by the trypan blue exclusion test, was 98–100%. 10^7 PBL per ml were incubated with PPD at a final concentration of 100 $\mu\text{g/ml}$ (5000 iu/ml) for 24 hr at 37°C. Then the cells were centrifuged at 400 g for 10 min, and the cell-free supernatants were frozen at -20°C (Lipsmeyer, 1974). Supernatants were assayed for migratory inhibitory factor in the agarose LMI test (Hoffman *et al.*, 1975). In this case they were used instead of the antigen with peripheral blood leucocytes from normal guinea-pigs. The indirect migration index (IMI) was calculated as follows:

$$IMI = \frac{\text{area of migration in supernatant with PPD} \times \text{area of migration in media without PPD}}{\text{area of migration in media with PPD} \times \text{area of migration in supernatant without PPD}} \times 100.$$

The measured area of migration was represented by the average of triplicate determinations.

RESULTS

PPD skin tests in nifurtimox-treated guinea-pigs

Skin tests with PPD and *in vitro* assays for cell-mediated immunity (CMI) were performed on guinea-pigs immunized with Freund's complete adjuvant (FCA) before and after nifurtimox administration. The experimental scheme is shown in Fig. 1. Positive skin tests obtained after immunization became

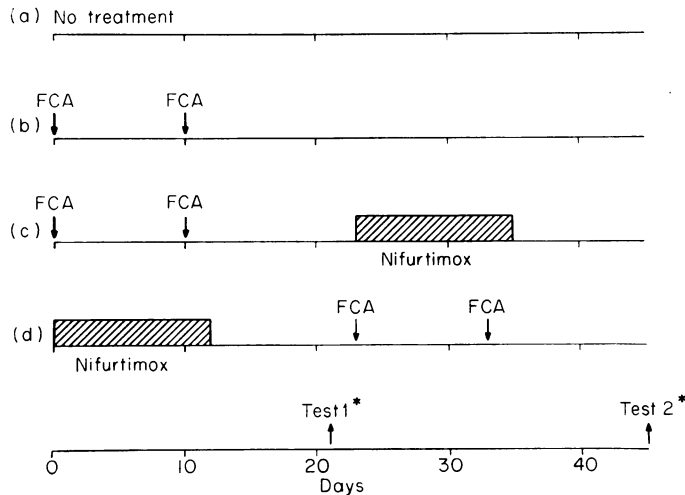


FIG. 1. Schematic diagram of FCA immunization and nifurtimox administration to guinea-pigs. (a) Group 1; (b) group 2; (c) group 3; (d) group 4. * Skin tests and direct and indirect leucocyte migration inhibition tests were performed.

negative after treatment with nifurtimox (Table 1, group 3, differences with group 2 after nifurtimox administration are significant: $P < 0.005$ by χ^2 test). When the drug was given before immunization, it did not alter the positive skin reaction (Table 1, group 4, differences with group 2 after FCA immunization are not significant). Skin tests were performed again 5 months after the suspension of the drug treatment. The nifurtimox-treated FCA-immunized guinea-pigs remained negative (group 3 = 0/5), while the untreated, FCA-immunized guinea-pigs yielded positive skin tests (group 2 = 5/6). Differences between them were significant ($P < 0.005$, χ^2 test).

TABLE 1. Effect of nifurtimox administration on PPD skin test of guinea-pigs immunized with FCA

Group	Guinea-pig treatment	Number of positive skin tests over	Diameter of induration areas (mm) (mean \pm s.e.m.)	Guinea-pig treatment	Number of positive skin tests over total tests	Diameter of induration areas (mm) (mean \pm s.e.m.)
(1)	—	0/14	1.0 \pm 0.2	—	0/12	1.3 \pm 0.4
(2)	FCA	9/11	8.7 \pm 1.6	—	9/11	10.3 \pm 1.6
(3)	FCA	12/14	9.6 \pm 1.1	Nifurtimox	1/13	1.3 \pm 0.6
(4)	Nifurtimox	0/12	1.0 \pm 0.4	FCA	11/12	11.5 \pm 1.1

FCA immunization and nifurtimox administration were performed as described in the Materials and Methods section.

Direct leucocyte migration inhibition tests in nifurtimox-treated guinea-pigs

Blood leucocytes from guinea-pigs of groups 1 and 3 were used in the direct migration inhibition test (LMI) with PPD. The mean values of the migration index (MI) are shown in Table 2. They were altered by nifurtimox administration in the same way as the skin test. The mean MI values of FCA-immunized guinea-pigs were lower than those of controls after FCA immunization, and fell within control values after nifurtimox treatment (group 3). The LMI tests with PPD was performed again 5 months after the suspension of the treatment. MI mean values of group 1 were 100.5 ± 9.5 ($n = 3$) and of group 3 were 100.8 ± 9.9 ($n = 4$), suggesting a long-lasting alteration after the suspension of the treatment. The migration areas of the cells from nifurtimox-treated guinea-pigs in the absence of antigen were similar to those of controls.

TABLE 2. Effect of nifurtimox administration on the migration index (MI) of guinea-pigs immunized with FCA

Group	Guinea-pig treatment	Mean MI (% \pm s.e.m.)	Guinea-pig treatment	Mean MI (% \pm s.e.m.)
(1)	—	105.4 \pm 3.5 ($n = 8$)*	—	110.7 \pm 4.2 ($n = 6$)
(3)	FCA	67.6 \pm 4.4 ($n = 8$)*	Nifurtimox	107.9 \pm 3.5 ($n = 8$)

FCA immunization and Nifurtimox treatment were performed as described in the Materials and Methods section.

* Significance of mean differences (Student's *t*-test): $P < 0.001$.

TABLE 3. Effect of nifurtimox administration on the indirect migration index (IMI) of guinea-pigs immunized with FCA

Group	Guinea-pig treatment	Mean IMI (% \pm s.e.m.)	Guinea-pig treatment	Mean IMI (% \pm s.e.m.)
(1)	—	100.1 \pm 5.0 ($n = 8$)	—	107.8 \pm 5.9 ($n = 5$)
(2)	FCA	70.0 \pm 9.0 ($n = 8$)	—	83.2 \pm 4.7 ($n = 5$)
(3)	FCA	71.5 \pm 7.7 ($n = 8$)	Nifurtimox	100.1 \pm 4.5 ($n = 8$)
(4)	Nifurtimox	104.7 \pm 8.6 ($n = 7$)	FCA	58.6 \pm 6.7 ($n = 4$)

FCA immunization and nifurtimox treatment were performed as described in the Materials and Methods section.

Indirect leucocyte migration inhibition tests in nifurtimox-treated guinea-pigs

Peripheral blood lymphocytes (PBL) from guinea-pigs of either group were assayed *in vitro* for the production of migration inhibition factor. Table 3 shows that the supernatants of PPD-stimulated PBL from FCA-immunized guinea-pigs contained migration inhibition activity. The supernatants of PBL from the same animals after treatment with nifurtimox were inactive (group 3). In contrast, PBL from FCA-immunized guinea-pigs produced migration inhibition activity *in vitro* (group 4) even when the animals were treated with nifurtimox before FCA immunization.

DISCUSSION

We have previously shown that the specific inhibition of Chagas patients' leucocytes with *Trypanosoma cruzi* antigens was impaired after treatment with a nitrofurantoin derivative, nifurtimox (Lelchuk *et al.*, 1973). This finding was important, since cell-mediated immunity (CMI) appears to be involved in the pathogenesis of chronic Chagas' disease (Cossio *et al.*, 1974; Santos-Buch & Teixeira, 1974; Teixeira, Teixeira & Santos-Buch, 1975), and this trypanocidal drug is widely used as a therapeutic agent (Cerisola, 1969; Levi & Neto, 1971).

The present study deals with the effects of nifurtimox treatment on the CMI response of guinea-pigs which had been immunized with Freund's complete adjuvant (FCA). Both the *in vivo* (skin test) and *in vitro* (LMI test) responses of the guinea-pigs to PPD were impaired after nifurtimox treatment (Tables 1 and 2). This effect persisted at least 5 months after the suspension of the treatment.

We were interested to determine which cell population was affected by nifurtimox treatment. Lymphocytes from treated or untreated immunized guinea-pigs were cultured with PPD and the cell-free supernatants were tested with normal peripheral leucocytes. The results shown in Table 3 clearly demonstrate the suppressor effect of the trypanocidal drug on the T-cell population or subpopulation involved in lymphokine production. Whether these cells fail to recognize antigens, are poor secretors of migration inhibition factor, produce defective factor or are inhibitors has not been ascertained with these experiments.

The sequence of treatment and immunization appears to be of extreme importance for the CMI impairment, as guinea-pigs that underwent nifurtimox treatment before immunization responded to antigen stimulation as well as controls (Tables 1, 2 and 3), both in the *in vivo* and *in vitro* direct or indirect LMI test. It is possible that cells sensitive to nifurtimox treatment appear at a definite time after immunization. The relationship between treatment and different times of immunization is currently being investigated in our laboratory.

Nifurtimox seems to be a specific cell suppressor. The fact that a commonly used pharmacological treatment can alter certain aspects of the cellular immune response in a parasitic disease and in an experimental model with unrelated antigen must be taken into account if changes in the immune response are used to judge the effectiveness of treatment.

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