PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 160, 302–311 (1979)

# Immunosuppressive Effects of Fusarium Extracts and Trichothecenes: Blastogenic Response of Murine Splenic and Thymic Cells to Mitogens (40439)

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Trichothecenes are metabolites synthesized by *Fusarium* and related species of fungi (1) which contaminate cereal grain in many parts of the world. These toxins cause severe human and animal diseases, such as Alimentary Toxic Aleukia (ATA) and Stachybotriotoxicosis, affecting the mucosa and immune systems. Leucopenia, anemia and bone marrow aplasia are the most important pathological symptoms of these diseases. Very serious secondary infection often occurs; Joffe (2) has reported that in most cases, death is caused by infection and necrosis of oral and laryngeal tissues.

Experimental leucopenia has been observed in most animal species tested: rabbit (3), mouse (4), dog, and cat (5, 6) after mycelial cultures or extracts were ingested, at doses allowing prolonged survival.

Trichothecenes purified from *Fusarium* species were shown to be as active as crude extracts. It was demonstrated that diacetoxyscirpenol, nivalenol and fusarenon X caused lymphoid tissues depletion (7–10). Lafont *et al.* in our laboratory observed a similar effect on the lymphoid system with extracts of a *Fusarium poae* strain isolated from mouldy cereals incriminated in ATA. They observed an early atrophy of the thymus and spleen, and leucopenia; bone marrow aplasia occurred only after longer treatment (10).

Trichothecenes administered *in vivo* induce widespread cellular death in actively dividing populations. *In vitro* experiments have shown that trichothecenes inhibit protein and DNA synthesis (11, 12). As stimulated lymphoid cells actively synthesize DNA and proteins, this inhibition is probably responsible for the severe depression of the immune system observed after treatment with *Fusarium* extracts (15), and for the subsequent sensitivity of treated mice to infectious diseases.

Our aim was to study: (a) the ability of mice treated with *Fusarium* extracts to synthesize anti-sheep red blood cell antibodies. (b) The blastogenic response of the thymic and splenic lymphocytes from treated animals to PHA (T cell mitogen) and LPS (B cell mitogen). (c) The *in vitro* action of crude extracts and purified toxins upon splenic and thymic lymphocytes of control mice stimulated *in vitro* by mitogens, and a murine fibrosarcoma cells in culture.

*Materials and methods.* I. *Fungal strains. Mycotoxin extraction and purification. Fusarium sporotrichioides* Sherb (strain no. 738) and *Fusarium poae* Peck Wr (strain no. 958) were provided by A. Z. Joffe (Lab. of Mycology and Mycotoxicology, Department of Botany, the Hebrew University, Jerusalem, Israel). The two strains were inoculated by the Lafont and Lafont procedure (13) and were grown on Czapek modified Bryan *et al.* (14) synthetic medium. Crude extract, $T_2$-toxin, diacetoxyscirpenol and butenolide were prepared as previously described (10).

II. *Animals and treatments.* Male IC (Institut du Cancer) mice, 4–6 weeks old, maintained in inbred colonies, were obtained from the Institut de Recherches Scientifiques sur le Cancer, Villejuif.

(a) *Treatment of mice with Fusarium extract.* Total extracts in Dimethylsulfoxide–phosphate buffered saline (DMSO-PBS) solution were administered by intraperitoneal injections. Controls were treated with the same quantity of DMSO-PBS solution.

Two types of treatment were performed. A short term treatment with high doses: one injection of 1/2 LD50 and a second with 1/4 LD50 24 hr later, and a 15-day treatment with lower doses: 1/12 LD50 daily.

(b) *Immunization with sheep red blood cells*

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0037-9727/79/030302-10$01.00/0

*(SRBC)*. Mice treated with *Fusarium* extracts as well as controls received an intraperitoneal injection of 0.2 ml 10% SRBC in PBS. Animals were bled 3, 4 or 5 days later and the complement inactivated by heating at 56° for 30 min.

III. *Hemagglutination.* Hemagglutination tests were performed in microtiter plates; 0.1 ml of serial dilutions of the inactivated sera were made in PBS. To each dilution, 0.1 ml of SRBC at 1% PBS was added, and the plates were incubated 2 hr at 37° and overnight at 4°. Patterns of agglutination were recorded, and the results expressed as reciprocal of the lowest dilution giving an unequivocal positive hemagglutination.

IV. *Lymphocyte cultures.* Spleens and thymuses were aseptically removed, minced with scissors, filtered through a 200 mesh stainless steel sieve, and washed once in Eagle's minimal essential medium (MEM). Single-cell suspensions were prepared in RPMI 1640 medium, supplemented with 2 m*M* glutamine (Gibco Grand Island, NY) and containing 5% fresh Human A B serum inactivated by heating at 56° for 30 min, 100 U Penicillin/ml and 100 μg streptomycin/ml. Aliquots of 100 μl containing $5 \times 10^5$ spleens cells or $1 \times 10^6$ thymic cells were placed in wells of Falcon 3040 microplates. To each well were added 100 μl of nutrient medium, and 50 μl of either nutrient medium or medium containing a mitogen. Phytohemagglutinin (PHA, HA 16, Research Laboratories Wellcome, England) was used at a final concentration of 2 μg/ml, and lipopolysaccharide (LPS, 055B5, Difco, Detroit, MI) at the final concentration of 20 μg/ml. All cultures were in triplicate. The plates were covered and incubated at 37° in an atmosphere of $CO_2$-air (5–95%) for 72 hr, 1 μCi of tritiated thymidine ($^3$H TdR) (TMM 48; CEA, France, specific activity 27 Ci/m*M*) was added to each well for 5 hr. Cultures were harvested with a multiple automated sample harvester (Microbiological Associates) on glass fiber filters (Reeve Angels). The discs were placed in Unisolve and the radioactivity was counted in an Intertechnique scintillation spectrometer. The results were expressed as mean counts per minute (cpm) of triplicate samples ± SE. The percent depression was calculated by the following formula:

$$\% \text{ depression} = \left(1 - \frac{\text{Mitogen cpm treated}}{\text{Mitogen cpm control}}\right) \times 100$$

V. *Activity of mycotoxins in vitro. Lymphocytes.* They were prepared and cultivated as previously described. The mycotoxins were added at the appropriate concentration in the presence or absence of PHA or LPS. The plates were then treated as described.

*Tumor cells.* A fibrosarcoma was induced by the subcutaneous injection of 1 mg methylcholanthrene in 0.1 ml olive oil in a female C3H mouse, and passaged in syngeneic mice. The tumor cells were cultivated in RPMI 1640 supplemented with fetal calf serum 10%, glutamine 2 m*M* Penicillin 100 U/ml and streptomycin 100 μg/ml. These cells were used as target cells between the 3rd and the 10th passage. The assay was performed in the same nutrient medium, with 2% fetal calf serum rather than 10%; aliquots of 100 μl containing $2.5 \times 10^3$ tumor cells were placed in wells of Falcon 3040 microplates, and to each well was added 100 μl of nutrient medium containing the mycotoxin at the desired concentration. All cultures were in triplicate. They were incubated, labelled, harvested and counted as described. The inhibition effect was calculated by the following formula

$$\% \text{ inhibition} = \left(1 - \frac{\text{cpm in test}}{\text{cpm in control}}\right) \times 100$$

The statistical significance between test groups was calculated using student's *t* test.

VI. *Histological observations.* Fragments of thymus, spleen and sternum were fixed in 95% alcoholic solution and the slices were stained by hematoxylin.

Different sections of the same organ were examined in order to compensate for variations in proportion of cortex and medulla in different sections of the same normal thymus.

*Results. In vivo experiments. Short term treatment with Fusarium extracts.* Mice receiving 1/2 LD 50 on day 0 and 1/4 LD 50 on day 1 were killed at various intervals (1 to 33 days) after *Fusarium* extract administration. A control group was included in each experiment. The results are shown in Fig. 1. As early as 1 day after treatment with *Fusarium*

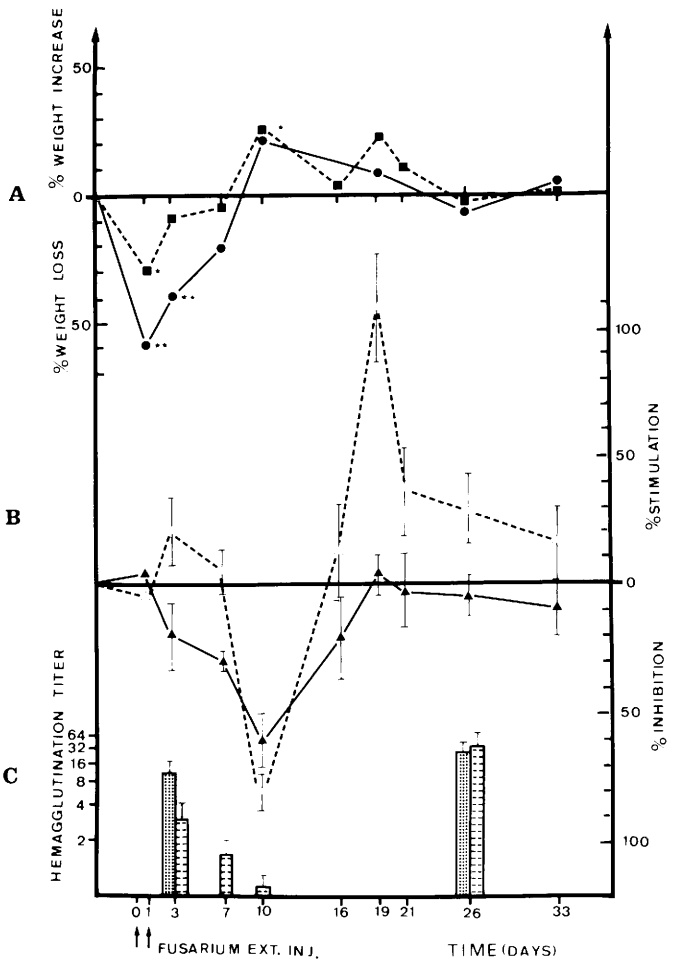


FIG. 1. Mice which received 1/2 LD50 on day 0 and 1/4 LD50 on day 1 were killed at various intervals of time, indicated in days on abscissa. Each result is the mean value for 3 experiments, and in each experiment, there were 5 subjects per point. (A) Evolution of spleen and thymus weight: weight loss or increase expressed as % of the control values. Significance of variations: $*P < 0.05$, $**P < 0.01$. ●——●, Thymus weight; ■- - -■, spleen weight. (B) Evolution of response of spleen cells to PHA ▲——▲ and LPS △- - -△ expressed as % of control value (right side). Each test was made in triplicate and the results are the mean value ± SE for 4 experiments. (C) Evolution of anti-SRBC antibodies in serum. For antibody titration on days 3, 7 and 10 mice were immunized with SRBC respectively on days 0, 4 and 7. For titration on day 26, the animals were immunized on day 22 and bled 4 days later. The controls are the same for days 3, 7 and 10. ⊡ anti-SRBC antibodies of controls, ⊠ anti-SRBC antibodies of treated mice.

extract, the weight of the spleen and thymus had decreased (Fig. 1A), the loss of weight of the thymus being greatest. Both organs rapidly returned to a normal weight, after a moderate but significant hypertrophy of the spleen, on day 10. The weight of other organs, such as liver and kidney, did not differ from the controls (results not shown).

For the response of spleen cells to PHA and LPS (Fig. 1B), the results are expressed as percent inhibition or stimulation as compared to controls. The PHA response was depressed as early as day 3 and this depression was maximum on day 10, progressively returning to normal levels by day 19. The LPS response was elevated on day 3 and was subsequently inhibited the depression being maximal on day 10. The LPS response then become higher than in control mice, and after a maximum on day 19, the value returned progressively to normal.

The anti-SRBC antibodies were titrated on

days 3, 7, 10 and 26 respectively (Fig. 1C). For the titration on days 3, 7 and 10, the mice were immunized with SRBC 3 days before being bled, this rather short time explaining why the anti-SRBC titer was low in controls. The controls are the same for days 3, 7, 10. In animals treated with *Fusarium* extracts, the anti-SRBC titer was considerably lowered on day 3, and even more so by days 7 and 10. On day 10, when spleens and thymuses had recovered a normal weight, no antibody synthesis occurred. On day 26, animals which had been immunized with SRBC 4 days previously, were bled, thus the higher control antibody titer. At that time, animals had recovered their ability to synthesize anti-SRBC antibodies.

Liver, kidney and bone marrow histology were not affected by the treatment. In the spleen, during the early phase, the periarteriolar sheaths (T-dependent areas) appeared atrophic before the follicles (B-dependent areas), and during the phase of regeneration both T- and B-dependent areas appeared hypertrophic (Fig. 2).

Particular attention was paid to the thymus (Fig. 3). One day, after the treatment, the cortex was atrophic while the medulla was proliferative, with numerous isolated lymphoid cells. On day 3 no change was observed. On day 7, the situation was reversed, the cortex was repopulated with numerous lymphoid cells, while in contrast the medulla was reduced, with some PAS positive cystic formations of epithelial origin.

*Prolonged treatment with Fusarium extracts.* Animals were treated daily with 1/12 LD50 *Fusarium* extracts for fifteen days. In each experiment a group of 10 treated mice and a group of eight control mice were used. In each group, half the mice were exsanguinated one day after the last injection of *Fusarium* extract and 5 days after the injection of SRBC for anti-SRBC antibodies titration. The spleens and thymuses were weighed and serum anti-SRBC antibodies were individually titrated. For the blastogenic response, the remaining mice were killed the same day and thymic and spleens cells of each animal were tested for stimulation by mitogens. Results are indicated in Table I and show that under these experimental conditions (in contrast to short term, high dose treatment) the weight of spleens and thymuses from treated animals differ very slightly from the controls. However, the titer of anti-SRBC antibodies was very low and the response of spleen cells from treated animals to PHA and to LPS was elevated. ($^3$H) TdR incorporation by unstimulated spleens cells from treated mice was also increased. By contrast ($^3$H) TdR incorporation by thymic cells from treated mice was inhibited in the presence or absence of mitogens.

*In vitro assays.* The activity of the crude *Fusarium* extracts, of the two trichothecenes purified from this extract: $T_2$-toxin and diacetoxyscirpenol, and of butenolide fraction, were tested *in vitro* on spleen cells stimulated with PHA or LPS, on thymus cells stimulated with PHA, and on fibrosarcoma tumor cells.

Mycotoxins were added at the time lymphocyte cultures were initiated at a final concentration of 0.01–20 ng/ml for *Fusarium* extracts and the two trichothecenes, and of 0.1–100 ng for the butenolide fraction. Mycotoxins were dissolved in DMSO or acetone at a concentration of 1 mg/ml, further dilutions of toxins were performed in DMSO or acetone 1: 2000 in PBS. Controls received DMSO or acetone at the same concentration without mycotoxin, to give the same final solvent-concentration.

*Spleen cells.* The addition of *Fusarium* extracts at doses of 2–10 ng/ml inhibited the PHA (Fig. 4a), and LPS responses (Fig. 4b) of normal spleen cells. At lower doses, 0.05–1 ng/ml, the mitogenic responses were increased. $T_2$-toxin strongly inhibited the response of spleen cells to both PHA and LPS, although increased responses were observed at doses of 0.01–0.1 ng/ml. Diacetoxyscirpenol inhibited the response of spleen cells less severely than did $T_2$-toxin, and no significant increase was observed at low doses. Butenolide inhibited the response at doses of 20–100 ng/ml, and at lower doses increased the lymphocyte response to PHA and LPS.

*Thymic cells. Fusarium* extracts inhibited the PHA response of thymic cells at doses of 2–10 ng/ml; at lower doses, a weak increase was observed. $T_2$-toxin was inhibitory at doses of 2–10 ng/ml, and lower doses caused strongly increased lymphocyte stimulation. Diacetoxyscirpenol was inhibitory at doses of 0.05–20 ng/ml. (Fig. 4c).

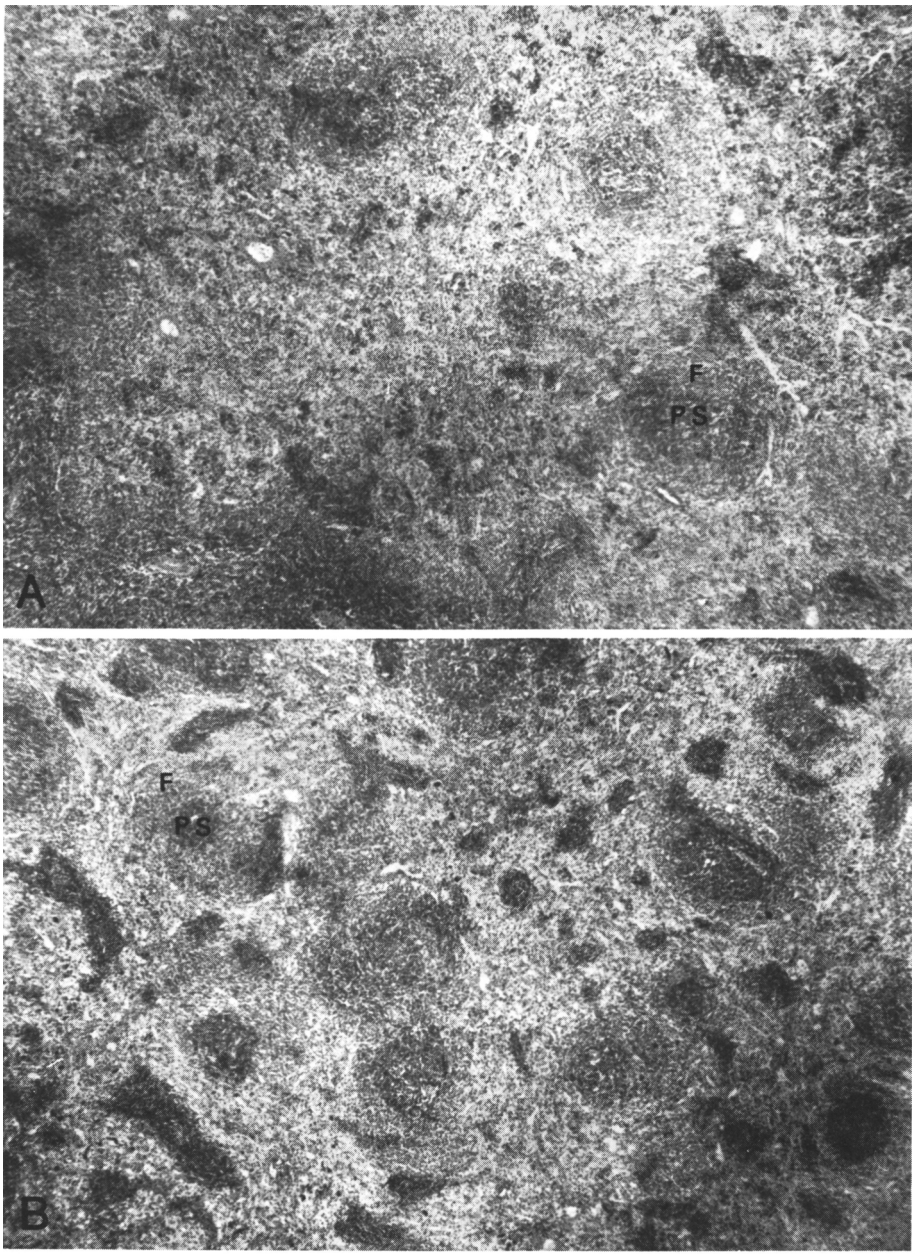


FIG. 2. Spleen (A) from Control. (B) 1 day after the end of treatment with *Fusarium* extract. Periarteriolar sheaths appeared atrophic before the follicles. (C) 15 days after the end of treatment. Regeneration of both T and B cells areas is observed. Hematoxylin (× 25) PS Periarteriolar Sheats (T-dependent areas); F follicles (B-dependent areas).

*Fibrosarcoma cells.* The crude extracts inhibited the tumor cell multiplication at doses of 5–10 ng/ml and stimulated multiplication at lower doses. $T_2$-toxin was inhibitory at doses of 0.1–10 ng/ml. Diacetoxyscirpenol was inhibitory only at high doses: 10–20 ng/ml (Fig. 4d).

*Discussion.* After experimental treatment of mice with *Fusarium* extracts, the liver and kidneys weights were not changed, while the thymus and the spleen were significantly reduced in weight. Also the stimulation of B and T cells by mitogens was depressed, as was the synthesis of anti SRBC antibodies.

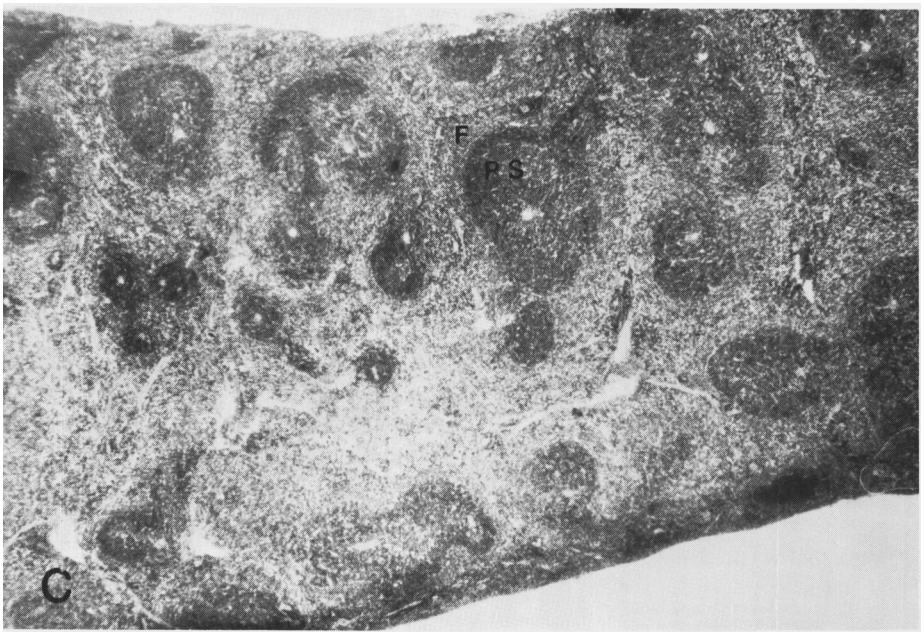


FIG. 2C

The crude fusarial extract was cytostatic *in vitro* at high concentration, but stimulated [$^3$H]TdR incorporation at low concentrations. Its activity was apparently the result of its different components: $T_2$-toxin and diacetoxyscirpenol are probably responsible for the cytostatic effect, while the stimulating effect at low concentrations was probably due to $T_2$-toxin and Butenolide. These substances act directly, without being previously metabolized and Butenolide is less inhibitory than the other substances.

T cell activity was profoundly impaired as early as 3 days after treatment, as shown by the depression of PHA responses and atrophy of the thymus. In the spleen, these changes were also observed early after treatment, while LPS responses were suppressed later. On day 10, the stimulation of spleen cells by both mitogens was considerably depressed, and the ability to synthesize anti-SRBC antibodies nearly abolished, despite the return to normal weight of the spleen and the thymus. As assessed by mitogen stimulation, the toxic effect on the animal was entirely reversible. The suppression of antibody synthesis might be due either to the impairment of antibody forming or T-helper activities. In order to resolve this question, work is in progress in our laboratory to test the ability of the mycotoxin-treated mice to synthesize antibodies against a thymus-independent antigen.

A number of other metabolites are known to act preferentially on the immune system. Cyclosporin A inhibits antibody synthesis, delayed hypersensitivity, the capacity of spleen cells to respond to mitogens and cell-mediated cytotoxicity to tumor cells. This metabolite acts selectively on T cells (16). As for trichothecenes, cyclophosphamide (CY) is also toxic for both B and T cells and its effect on the animal is reversible. However, in contrast, CY acts first on B cells, and then on T cells (17, 18). The depressed response to PHA and LPS of lymphocytes from CY-treated animals is due, during the early phase, to the direct toxic activity of the drug. During the phase of spleen regeneration, the response to mitogens is still suppressed, but it was demonstrated that the depressed response was due to the presence of suppressor cells in the spleens of these animals. This mechanism does not appear to be involved in the *Fusarium* toxin-induced depression, since the response to mitogens was not depressed during the regeneration-phase and we failed to demonstrate the presence of suppressor cells (results not shown). Therefore, the mechanism of spleen regeneration appears to be different

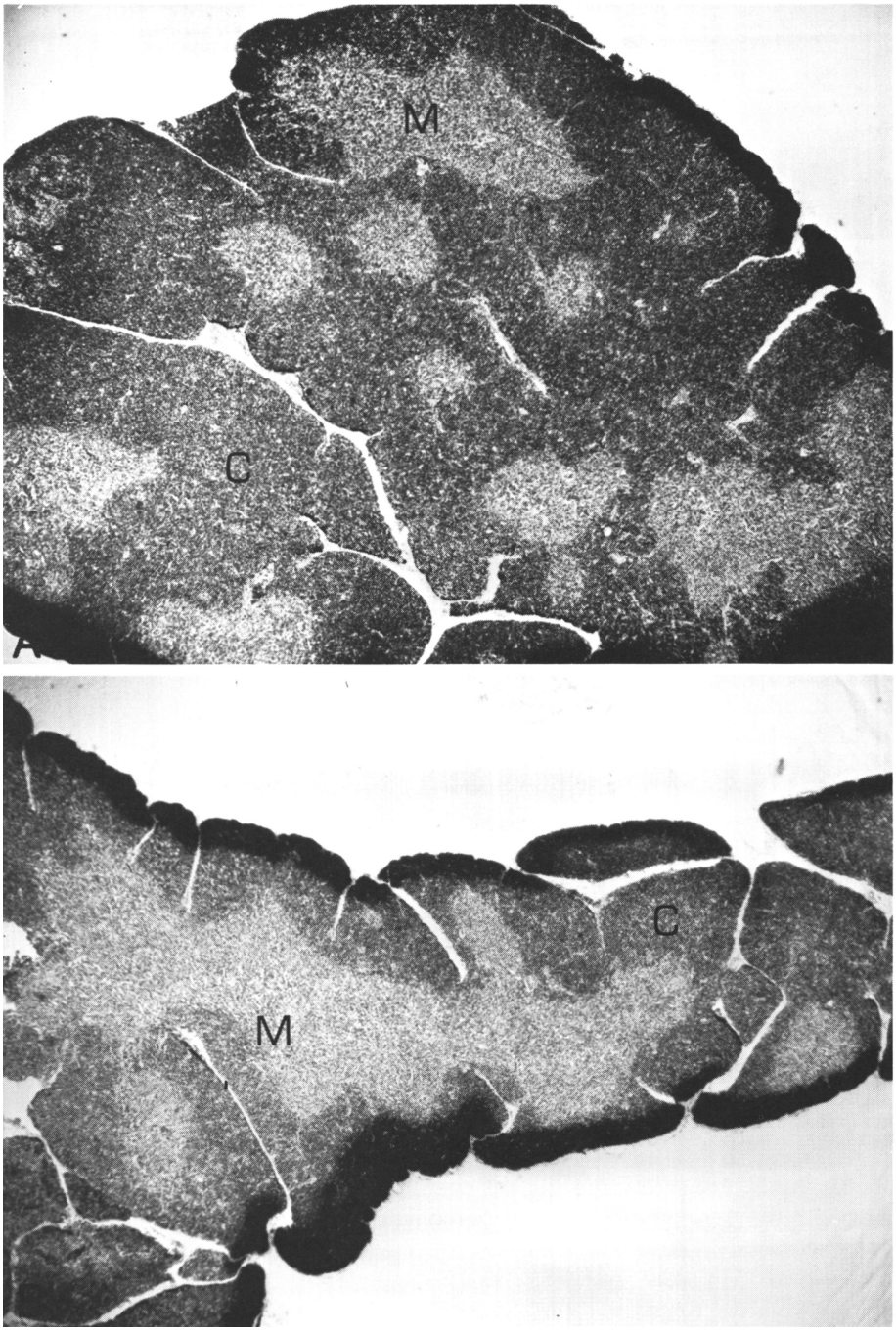


FIG. 3. Thymus (A) from Control. (B) 1 day after the end of treatment with *Fusarium* extract. Cortical atrophy can be seen. (C) 7 days after the end of treatment. Cortical regeneration is observed. Hematoxylin (× 25) C Cortex; M Medulla.

from that after treatment with CY.

The precise mechanism of action of these mycotoxins remains to be determined. Several authors have demonstrated that *Fusarium* extracts and trichothecenes inhibit DNA replication (11) and protein translation by blocking peptidyl transferase (12), or by destroying polysomes (19). These effects on

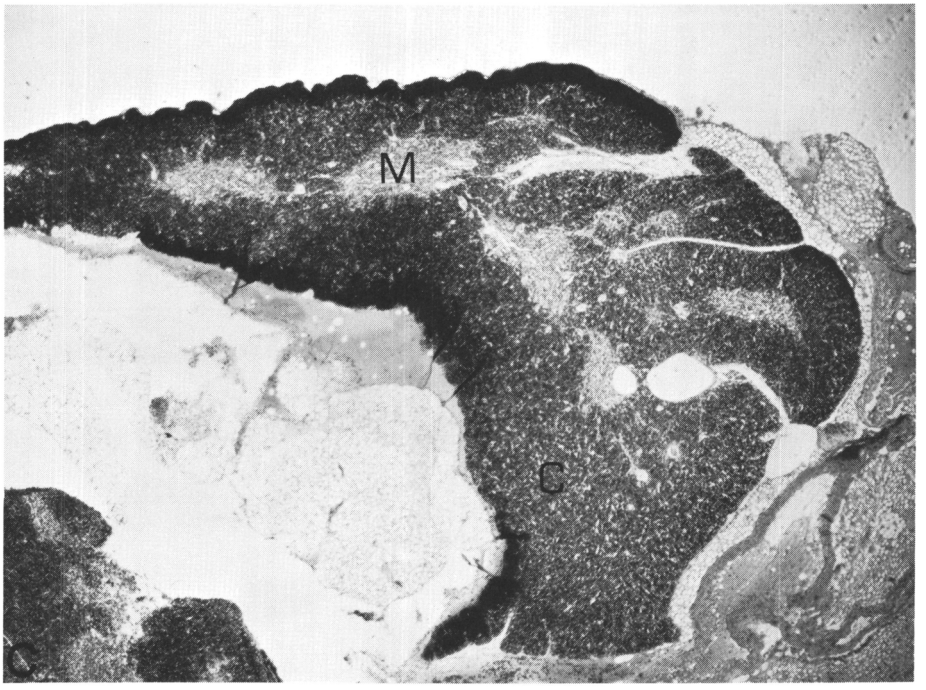


FIG. 3C

TABLE I. PROLONGED TREATMENT WITH FUSARIUM EXTRACT.[a]

| | Weight (mg) | | Lymphoblastic stimulation | | | Hemagglutination titer | |
|---|---|---|---|---|---|---|---|
| | Control | Treated animals | Unstimulated cells | PHA | LPS | Control | Treated animals |
| Spleen | 110 ± 4 | 85 ± 6<br>$p < 0.05$ | +38% ± 4 | +42% ± 10 | +58% ± 7 | | |
| | | | | | | 128 ± 0 | 1 ± 0.5 |
| Thymus | 31 ± 2 | 36 ± 1<br>$p < 0.02$ | −73% ± 3 | −69% ± 8 | | | $p < 0.001$ |

[a] Mice were treated daily with 1/12 DL50 *Fusarium* extract for fifteen days. The results are then mean of three experiments. In each test of lymphoblastic stimulation, 5 treated mice and 4 controls were killed the day after the last injection of toxin. The tests were made in triplicate, and the results were expressed as percent of control values. For determination of antibody titer, 5 treated mice and 4 controls were bled the day after the last injection of toxin, 5 days after SRBC injection. Serum titer is the inverse of the lowest dilution giving an unequivocal positive hemagglutination.

macromolecular synthesis are possibly responsible for the impairment of the immune system in the intoxication syndrome. However, the inhibiting effects of these toxins on translation have been demonstrated *in vitro* on acellular systems using higher doses than we used, and than encountered with natural contamination (11, 12).

It is particularly important to point out that the immune system appears sufficiently sensitive to this toxin to be impaired at doses not inhibitory for other organs. A certain number of acute alimentary intoxication caused by *Fusarium* toxins are well established but in most other cases the level of food contamination is too low to provoke cellular lesions or perceptible signs of toxicity. However our results suggest that an immunosuppression may occur, thus decreasing resistance to infection.

*Summary.* The effects of *Fusarium* extract and its principal components ($T_2$-toxin, diacetoxyscirpenol and Butenolide) on the immune system were tested in mice.

Animals were treated with these mycotoxins, and the mitogen responsiveness of spleen

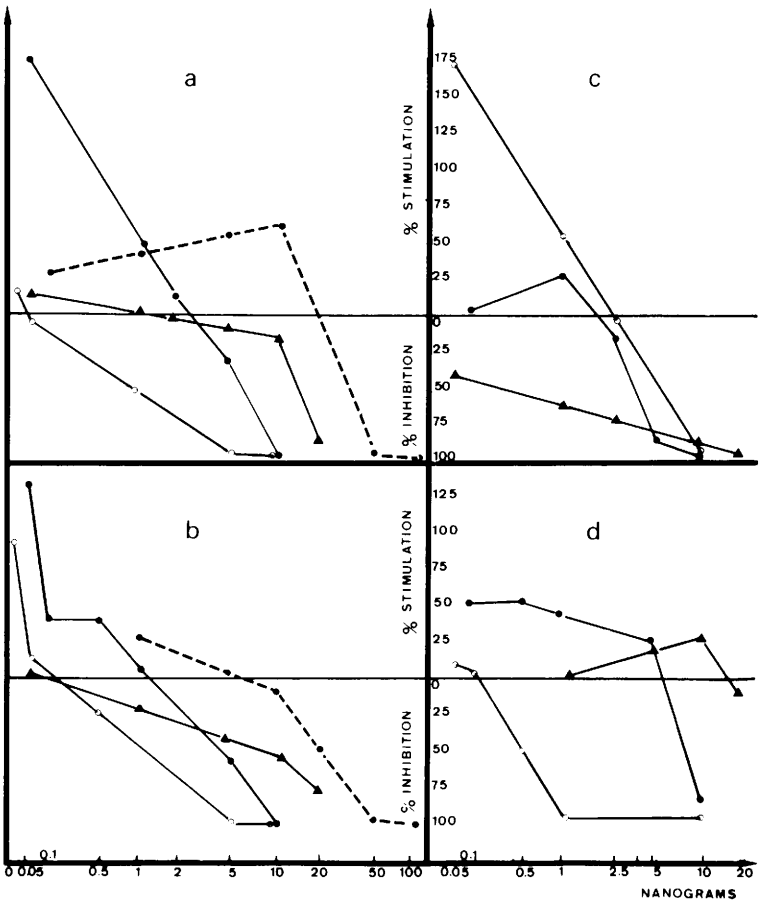


FIG. 4. Abscissa: ng of mycotoxins tested. Ordinate: inhibition or stimulation expressed as % of the control values. ●——● Fusarium extracts; ○——○ $T_2$-toxin; ▲——▲ Diacetoxyscirpenol; ●- - -● Butenolide: Activity of *Fusarium* extracts, $T_2$-toxin, diacetoxyscirpenol and Butenolide on spleen cells stimulated by PHA (a), or LPS (b) or, on thymic cells stimulated by PHA (c), and fibrosarcoma tumour cells (d). The tests were made in triplicate and the results are the mean values of 4 experiments. Mycotoxins were dissolved in DMSO or acetone then diluted 1: 2000 in PBS. Controls received the same quantity of solvent.

or thymic cells was examined. The stimulation of both T and B cells was found to be reversibly inhibited. Also, the ability to synthesize anti-SRBC antibodies was reversibly suppressed.

The direct effect of mycotoxins was tested *in vitro* in lymphocyte and fibrosarcoma cell cultures. These compounds exerted a direct cytostatic effect at high concentrations, and a stimulating effect at low concentration. Profound histological changes were observed in thymus and spleen after treatment, while under the experimental conditions employed, the histology of other organ systems was not affected.

This work was supported in part by "Le Ministére de la qualité de la vie". We thank Dr. Burtin for helpful discussion and advice and Mr. N. Lynch and Eric Kraus for assistance in preparation of the manuscript.

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Received October 12, 1978. P.S.E.B.M. 1979, Vol. 160.