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Effects of Two Metabolites of Ochratoxin A, (4*R*)-4-Hydroxyochratoxin A and Ochratoxin α, on Immune Response in Mice

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The metabolites of ochratoxin A, (4*R*)-4-hydroxyochratoxin A and ochratoxin α, were investigated for immunosuppressive properties in BALB/c mice. The standard plaque-counting technique for the estimation of antibody-producing spleen lymphocytes was used. (4*R*)-4-hydroxyochratoxin A was found to be an immunosuppressor almost as highly effective as ochratoxin A. Doses of 1 μg of (4*R*)-4-hydroxyochratoxin A per kg administered to mice caused an 80% reduction in the number of cells producing immunoglobulin M (90% with ochratoxin A) and a 93% reduction in cells synthesizing immunoglobulin G (92% with ochratoxin A). Ochratoxin α, however, was ineffective. A possible mode of action is discussed.

Ochratoxin A (OTA), a dihydroisocoumarin derivative linked through a 7-carboxy group to L-phenylalanine by an amide bond, is produced by strains of various species of the fungal genera *Aspergillus* and *Penicillium*. Among other toxic effects, it has been shown to cause nephropathy in swine (12). It is also presumed to be involved in a fatal human kidney disease encountered in certain districts of Bulgaria, Romania, and Yugoslavia (13).

It has been shown that in mice, OTA inhibits the primary immune response (mostly immunoglobulin M [IgM]) to sheep erythrocytes (SRBC) (7). We have recently found that the production of IgG response is also inhibited by OTA (E. E. Creppy, G. Lorkowski, R. Röschenthaler, and G. Dirheimer, Abst. IUPAC Symp. Mycotoxins and Phycotoxins, Vienna, Austria, 1982, p. 289).

When OTA is incubated together with pig liver microsomes and NADPH, two hydroxylated metabolites are formed in approximately equal amounts. They have been identified as (4*R*)- and (4*S*)-4-hydroxyochratoxin A (4*R*-OH-OTA and 4*S*-OH-OTA) (Fig. 1). These metabolites are also formed by microsomes from humans and from rat liver, although in different ratios (15).

When hepatocytes from rats are incubated with OTA, only 4*R*-OH-OTA is formed in significant amounts (6). Only traces of 4*S*-OH-OTA are detected. Rats given the toxin (OTA) intraperitoneally or by mouth excrete this compound together with ochratoxin α (OTα) and the 4*R*-epimer in the urine. The 4*S*-epimer is not found (O. Støren, H. Holm, and F. C. Størmer, Abstr. IUPAC Symp. Mycotoxin and Phycotoxin, Vienna, Austria, 1982, p. 221).

In this paper, we report on the effect of 4*R*-OH-OTA and OT on the IgM and IgG immune response to SRBC in BALB/c mice.

MATERIALS AND METHODS

OTA, 4*R*-OH-OTA, and OT. OTA was prepared from wheat kernels infected with *Aspergillus ochraceus* NRRL 3174, generously provided by A. Ciegler, Northern Regional Center for Agricultural Research, Peoria, Ill. Isolation and purification were carried out as previously described (2) by chromatography on Sephadex LH20 (Pharmacia, Uppsala, Sweden) and on silica gel columns (E. Merck, Darmstadt, Federal Republic of Germany).

4*R*-OH-OTA was isolated from incubation mixtures of pig liver microsomal fractions in the presence of OTA and reduced NADPH (15), and OT was isolated from the urine of rats given the toxin intraperitoneally or by mouth (Støren et al., IUPAC abstract). Both metabolites were purified by extraction, thin-layer chromatography, and high-performance liquid chromatography.

Calculation of the concentrations of OTA and its metabolites was based on their molecular extinction coefficients (mol^{-1} cm^{-1}): 5,500 at 333 nm for OTA (14), 6,400 at 334 nm for 4*R*-OH-OTA, and 5,600 at 338 nm for OTα (8).

Animals, antigen, and immunization method. The animals were 8- to 12-week-old BALB/c mice obtained from IFFA-CREDO (L'Abresle, France). All experiments were done with groups of two animals instead of

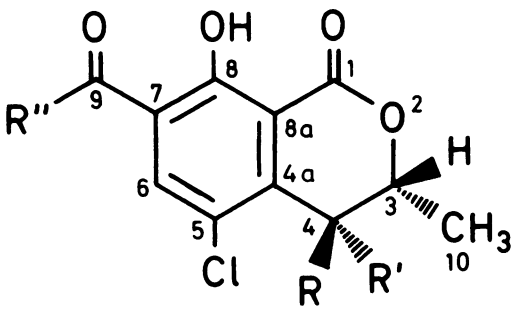

FIG. 1. OTA and its metabolites. OTA: R = R' = H, R'' = L-phenylalanine. OTα: R = R' = H, R'' = OH. Epimers of 4*R*-OH-OTA: R = H, R' = OH, R'' = L-phenylalanine and R = OH, R' = H, R'' = L-phenylalanine.

the four to five animals used for the first kinetic studies.

SRBC (Bio-Mérieux, Lyon, France) were employed as antigen. Before use, they were washed four times in phosphate-buffered saline (0.14 M NaCl and 0.003 M KCl in 0.008 M sodium phosphate buffer [pH 7.2]).

The mice were immunized by a single intraperitoneal injection of 2×10^8 SRBC in 0.5 ml of phosphate-buffered saline. In addition, the respective mice received single doses of OTA, 4*R*-OH-OTA or OTα. The doses were 1 μg/kg dissolved in 0.9% NaCl solution, injected intraperitoneally in 0.2-ml portions. The control mice received 0.2 ml of 0.9% NaCl solution.

Immunosuppression assay. The anti-SRBC response of mice was assayed according to the standard thin-layer technique of Jerne et al. (9) by counting the hemolytic plaque-forming cells (PFC) per spleen of each animal and per 10^6 spleen cells. The animals were sacrificed on days 3 to 15 after immunization. The immune response due to IgM production was determined by the direct plaque-counting technique from day 3 on, and from day 8 on the production of IgG was assayed by the indirect technique.

The complement used was from Bio-Mérieux, the anti-IgG was from Nordic Immunology (France), and the agarose was from Behring Institut (Federal Republic of Germany).

RESULTS AND DISCUSSION

The IgM response (Fig. 2) yielded the highest counts of PFC on day 5. OTA caused a 90% reduction and 4*R*-OH-OTA an 80% reduction in PFC with respect to the controls. With OTα, no immunosuppression occurred, but instead a slight stimulation, which was probably not significant. From day 5 on, the numbers of PFC decreased. The numbers remained consistently lower in animals which had been treated with OTA or 4*R*-OH-OTA, in comparison with those that received OTα or nothing at all.

The IgG response (Fig. 3) reached a maximum on day 10. Again OTA caused an immunosuppression of 92%, and 4*R*-OH-OTA caused an immunosuppression of 93%, whereas OTα had no effect on the number of antibody-producing cells.

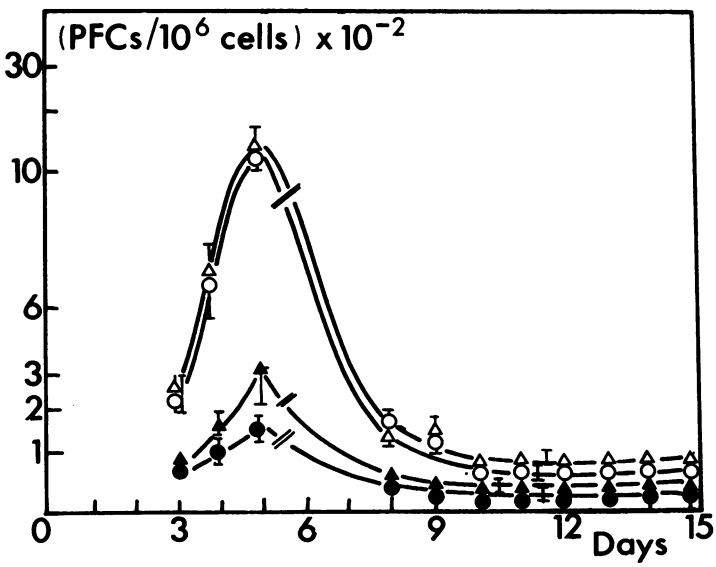

FIG. 2. Studies of the IgM immune response to SRBC in animals treated with OTA, 4*R*-OH-OTA, and OTα. SRBC were incubated for 45 min at 37°C in agar gel with spleen cells of each mouse, followed by the addition of the complement for 60 to 90 min. The complement was removed before the counting. Symbols: ○, control; ●, IgM immune response of mice treated with OTA; ▲, IgM immune response of mice treated with 4*R*-OH-OTA; △, IgM immune response of mice treated with OTα.

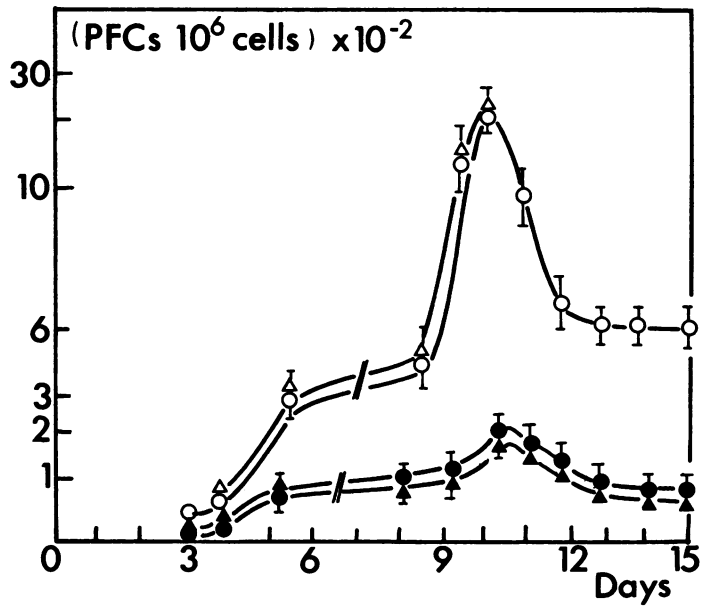

FIG. 3. Studies of the IgG immune response to SRBC in mice treated with OTA, 4*R*-OH-OTA, and OTα. Spleen cells are incubated first with SRBC in agar gel for 45 min, then for 60 min with anti-IgG, and finally for 60 to 90 min with the complement. Symbols: ○, control; ●, IgG immune response in OTA-treated animals; ▲, IgG immune response of mice treated with 4*R*-OH-OTA; △, IgG immune response of mice treated with OTα.

These results clearly show that OTα has no immunosuppressive action on BALB/c mice. Apparently, the phenylalanine moieties of the molecules are necessary for the inhibitory action to occur. The hydroxylation at the C-4 atom does not affect the toxic property of this OTA metabolite.

This conclusion leads to the question of the mechanism of action by which OTA and 4*R*-OH-OTA impair the immune response to SRBC in BALB/c mice.

We have previously shown that OTA inhibits protein synthesis of several organisms in vitro and in vivo (1, 5). The inhibition is caused by competition with phenylalanine in the phenylalanyl-tRNA synthetase-catalyzed reaction (1, 5, 11). Since the inhibition is competitive, it can be reversed by phenylalanine (4, 5). As phenylalanine can also prevent the immunosuppression by OTA of IgM in mice (7), it is possible that the inhibition of protein synthesis is the cause for the immunosuppression by OTA and probably by 4*R*-OH-OTA as well. Preliminary results show that 4*R*-OH-OTA strongly inhibits yeast phenylalanyl-tRNA synthetase in vitro (unpublished results).

On the other hand, it was found that lymphocytes in culture are extremely sensitive to OTA. Whereas hepatoma tissue culture cells exhibit a 50% reduction of protein synthesis at doses of 10 to 15 mg of OTA per liter, a dose of only 0.5 mg/liter is just as effective in a lymphocyte culture (3). This difference in sensitivity may not be due to degradation of OTA in liver cells, since at least the immediate metabolite, 4*R*-OH-OTA, is also toxic. One possible explanation for this difference is a selective uptake and concentration of the toxin by the lymphocytes.

If one assumes that 4*R*-OH-OTA acts like OTA, i.e., by inhibition of protein synthesis, the most sensitive step in the development of the immune response remains to be determined. This could occur at different levels: differentiation of lymphoblasts, activation and proliferation of lymphocytes after antigenic stimulation, or inhibition of immunoglobulin synthesis. As only a single dose of the immunosuppressor was employed, we believe that the activation of B- or T-cells might be impaired. If the activity occurred at a later stage, only very small plaques might be observed with PFC from treated animals.

However, we cannot exclude the possibility that OTA and 4*R*-OH-OTA may also act indirectly, e.g., at the macrophage level (10) or by direct inhibition of the immunoglobulin synthesis with deletion of affected cells.

In summary, the results of this study show that OTA and its immediate metabolite, 4*R*-OH-OTA, are highly effective immunosuppressors, whereas OTα is not. Thus, it must be concluded that the isocoumarin moiety as such is not

effective but must be bound to phenylalanine. Hydroxylation does not destroy the toxic property of OTA. Because of similar reactions of both compounds, a similarity in the mode of action can be expected.

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