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TIME LIMITATIONS IN THE REVERSAL BY CITROVORUM FACTOR OF METHOTREXATE-INDUCED IMMUNOSUPPRESSION IN MICE*

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Abstract—Methotrexate was found to inhibit both the complement-dependent and the complement-independent cellular cytotoxicity of spleen cells from mice immunized with sheep erythrocytes. The inhibition was essentially complete when a single dose of 100 mg/kg (about one-half the LD_{50}) was given 2 days after antigen administration, and the response was measured on day 4. Recovery from this inhibition was incomplete on days 5–7 and complete on day 14. The inhibition measured on day 4 could be partially prevented by citrovorum factor (200 mg/kg), but only if this compound was administered 1–5 hr before methotrexate. In contrast, the residual inhibition measured during recovery, on day 7, was completely reversed if citrovorum factor was injected even as late as 6 hr after methotrexate on day 2. The data suggest that protection of the majority of the rapidly proliferating, antigen-stimulated, spleen lymphoid cells from the effects of methotrexate can be provided only by preloading these cells with citrovorum factor.

The immunosuppressive effects of methotrexate (MTX) have been studied extensively in a number of different experimental systems utilizing a variety of soluble and cellular antigens [1–4]. The reversal of the antiproliferative effects of MTX by citrovorum factor (CF) was demonstrated long ago in toxicity studies as well as in experimental tumor models [5, 6]. More recently, the reversal of the immunosuppressive effects of MTX by CF was shown in mice immunized with TAB (typhoid-paratyphoid A and B vaccine) vaccine [7] or grafted with allogeneic skin [8]. In these studies, it was found that the effect of the antifolate on the immune responses could not be reversed by CF unless this compound was given 1 hr before or within 1–2 hr after the administration of the inhibitor. This was in contrast to the greater latitude offered in the case of MTX effects on body weight.

Recently, the reversal of MTX toxicity by CF has been exploited clinically in antitumor treatments involving high doses of MTX followed by CF rescue [9–11]. In view of the possibility that host defenses play a role in the therapeutic effects of certain antitumor treatments, and considering the known potential for immunosuppression of most anticancer agents, it is important to design treatments with limited suppression of host defenses [12, 13]. In view of the clinical interest in MTX–CF rescue treatments, it was considered desirable to re-investigate the kinetics of reversal by CF of MTX-induced immunosuppression, utilizing methodology developed recently in this laboratory which allows measurement of immunosuppressive effects of drugs based on an evaluation of the function of effector cells [14, 15].

The results presented herein indicate that unexpected time-dependent restrictions exist in the ability of CF to reverse the inhibitory effects of MTX on complement-dependent and complement-independent cellular cytotoxicity responses of mouse spleen cells to sheep erythrocytes. These restrictions are much greater than those illustrated in previous studies utilizing other cell proliferation model systems.

MATERIALS AND METHODS

Mice. The C3Hf/HeHa and C3Hf/HeJ female mice used in this study were obtained from the Roswell Park mouse breeding colony and from Jackson Laboratories respectively. They were used when weighing 18–22 g, at approximately 6–8 weeks of age. No differences in results were noted in the two mouse sublines used.

Drugs. In order to ensure strict reproducibility of dosing, the MTX used (Nutritional Biochemicals Corp., Cleveland, Ohio) was purified by DEAE cellulose column chromatography using a linear gradient of 0.05 to 2.0 M ammonium acetate according to the procedures reported previously [16]. The purified MTX was suspended in saline and then dissolved by the addition of measured amounts of 1 N NH_4OH; care was taken to maintain the pH of the solution within 7.0 to 7.5. Aliquots of MTX solutions were kept frozen at −15° in the dark and individually thawed shortly before use. The single dose of 100 mg/kg of MTX given intraperitoneally was chosen for these studies based on preliminary toxicity tests in the mouse lines used and represented less than one-half the LD_{50} (250 mg/kg). The *d,l*-CF (Lederle Laboratories, Pearl River, N.Y.) was dissolved in saline shortly before injection. The dose of 200 mg/kg of CF was selected for intraperitoneal administration in this study.

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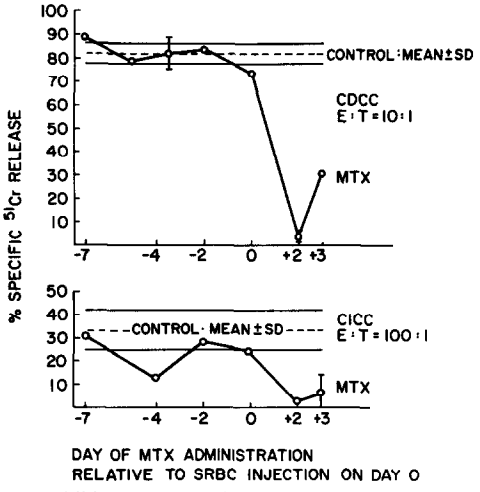

Fig. 1. Inhibition of complement-dependent (CDCC) and complement-independent (CICC) cellular cytotoxicity by methotrexate (MTX) as a function of the time of MTX administration when evaluated on day 4 after antigen administration. The data shown are the results of ten CDCC and twelve CICC experiments. The points represent an average of three to five experiments where standard deviations (S.D.) are indicated by vertical bars, or single experiments where no S.D. is indicated. At each experimental point, effector cells were obtained from pooled spleens (three to fifteen mice). E:T = ratio of the number of effector cells to target cells.

Antigen and immunization. Sheep erythrocytes (SRBC) in 50% Alsevers solution were purchased from Grand Island Biological Co. (GIBCO) and were washed three times using saline and centrifugation for 5 min at 100 *g* before counting in a haemocytometer. Immunization was by a single intraperitoneal injection of 5×10^8 SRBC in 0.5 ml saline.

Effector cells and cytotoxicity assays. Spleen cells were obtained at various times after immunization, as indicated in the text. The procedures used for the preparation of single-cell suspensions were identical to those reported previously [17]. The complement-dependent (CDCC) and complement-independent (CICC) cellular cytotoxicity assays are based on measurements of ^{51}Cr release from SRBC prelabeled with sodium chromate. The assay procedures were identical to those recently reported in detail from this laboratory [14]. In short, the CDCC response was measured after incubation of a certain number of spleen effector cells with ^{51}Cr-labeled target SRBC in RPMI 1640 medium with 5% fetal calf serum (total volume 0.2 ml) for 45 min at 37° in 10% CO_2, followed by a further 45-min incubation under the same conditions in the presence of 0.4 ml of guinea pig complement (GIBCO) diluted 1:40 with RPMI 1640. The reaction was stopped by adding 2 ml of cold RPMI 1640 medium. The CICC assay was carried out in the same way except that complement was omitted and the incubation period was 20 hr. After centrifugation for 5 min at 800 *g* in the cold, the label present in both the supernatant and the pellet was measured in a gamma-counter (Packard model 3375) and the per cent release was computed as follows:

$$\% \text{ release} = \frac{\text{cpm in supernatant}}{\text{cpm in supernatant} + \text{cpm in pellet}} \times 100$$

Specific release is equal to the per cent release obtained with sensitized spleen cells minus the per cent release with non-sensitized spleen cells.

RESULTS

The effects of MTX on CDCC and CICC were initially evaluated on days 4 and 7 after antigen administration in order to identify the optimal time of MTX administration. As shown in Fig. 1, when the responses were evaluated on day 4 after antigen administration, it was found that MTX was most inhibitory when given on day 2 after antigen. Drug administration before antigen was not significantly effective. When the responses were measured on day 7 at high ratios of the number of effector cells to target cells (E:T = 30:1–100:1), MTX was again most effective when given on day 2 (data not shown).

The kinetics of the inhibition of CDCC and CICC caused by a single injection of MTX given on day

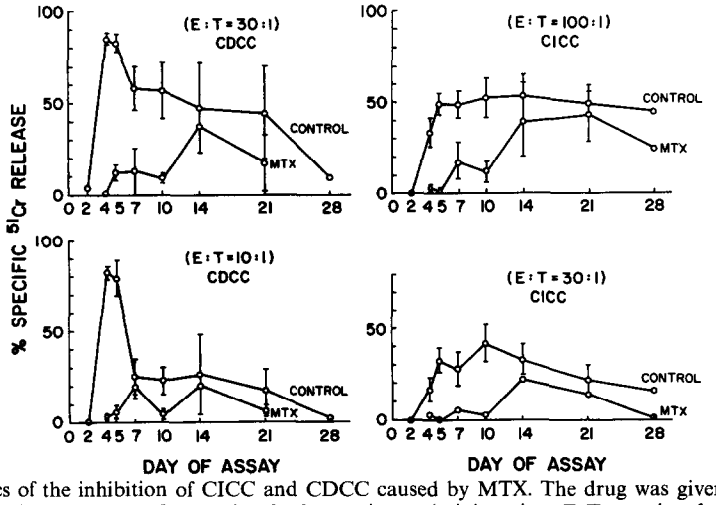

Fig. 2. Kinetics of the inhibition of CICC and CDCC caused by MTX. The drug was given intraperitoneally at the dose of 100 mg/kg on day 2 after antigen administration. E:T = ratio of the number of effector cells to target cells. The data points represent an average of five to fifteen experiments (fifteen to forty-five mice/point). The day of assay was counted from the day of antigen administration.

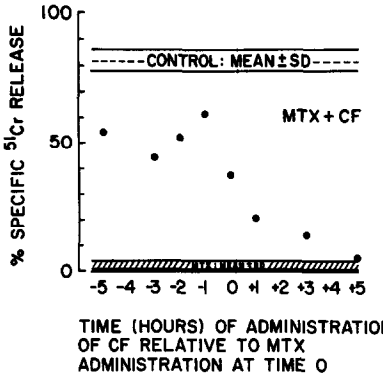

Fig. 3. Reversal by citrovorum factor (CF) of the inhibition of CDCC caused by MTX. Sheep erythrocytes (SRBC) (5×10^8) were given intraperitoneally on day 0; MTX and CF were given on day 2. The assay was carried out on day 4. E:T = ratio of the number of effector cells to target cells. Each experimental point represents an average of three individual mice. Control and MTX treatment groups represent an average of twenty-four mice.

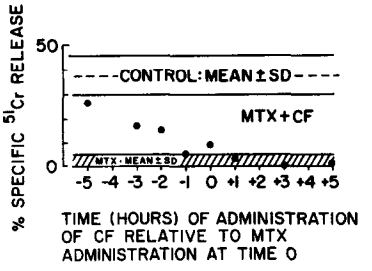

Fig. 4. Reversal by CF of the inhibition of CICC caused by MTX. Sheep erythrocytes (SRBC) (5×10^8) were given intraperitoneally on day 0; MTX and CF were given on day 2. The assay was carried out on day 4. E:T = ratio of the number of effector cells to target cells. Each experimental point represents an average of three individual mice. Control and MTX treatment groups represent an average of twenty-four mice.

2, at the time of maximum effectiveness, are shown in Fig. 2. The peak of the responses could be best identified when low E:T ratios (10:1–30:1) were used in the assays: that of the CICC was around day 10, that of the CDCC on days 4–5. For both responses, the MTX-induced inhibition was complete on day 4, regardless of the E:T ratio used in the assays. Afterward, both responses showed comparable tendencies toward recovery. In view of the complete inhibition observed on day 4, this time of assay was initially chosen for studies on CF reversal.

As shown in Fig. 3, at the doses used, CF was unable to completely reverse the effects of MTX on CDCC regardless of the time it was given, from 5 hr before MTX to 3 hr after it. In order to achieve approximately a 50 per cent reversal, it was necessary to give CF at the same time as MTX or before it. In the case of the CICC (Fig. 4), the restriction in time of CF administration was even stricter than in the case of the CDCC. In fact a 50 per cent reversal of MTX-induced inhibition could be obtained only when CF was given 2 or 3 hr before MTX. With this response, however, essentially a complete reversal was seen when CF was given 5 hr before MTX.

In view of the limitations in CF reversal of MTX effects seen when the responses were measured on day 4, and the fact that a tendency toward recovery from MTX effects was noted on day 7 (see Fig. 2), it was considered desirable to see whether CF would be able to accelerate this recovery, as possibly a more sensitive measure of reversal. As shown in Fig. 5, when the responses were measured on day 7, the effects of MTX were completely reversed by CF given as late as 6 hr after the drug. When the responses were measured on day 5, results were intermediate between those found on day 4 and those found on day 7.

DISCUSSION

As demonstrated in this study, MTX inhibits completely the CDCC and CICC responses to SRBC in mice when it is given on day 2 after antigen administration and the responses are measured on day 4 using spleen cells as effectors. Recovery from this inhibition occurs within 7–14 days. At this time it is not possible to say whether the recovery is the result of proliferation of cell population(s) which was/were not affected by MTX on day 2, or the result of cells which were stimulated by residual antigen after the MTX

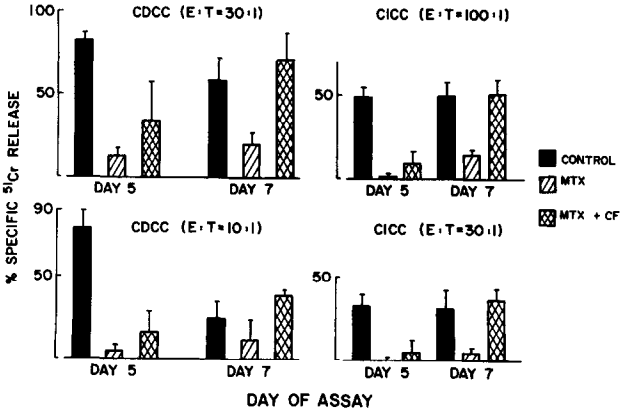

Fig. 5. Reversal by CF of the inhibition of CDCC and CICC caused by MTX. Sheep erythrocytes (SRBC) (5×10^8) were given intraperitoneally on day 0; MTX was given on day 2 followed 6 hr later by CF. The assay was carried out on the days indicated after antigen administration. E:T = ratio of the number of effector cells to target cells. The data represent an average of three experiments (nine mice/value).

concentration in blood had already decreased to ineffective levels. The possibility of a differential sensitivity to MTX of early and late responses to antigen cannot be ruled out in the case of CDCC, which measures concurrently both IgM and IgG production. Nevertheless, a greater inhibition of the IgG response by drugs is common [18, 19] and has been attributed in some cases to interference with the regulatory mechanism concerned with the switch from IgM to IgG production. Specifically, inhibition of both IgM and IgG responses by MTX with selective recovery of the IgM response was found to occur in humans [20]. In the case of the CICC, however, differential sensitivity of early and late responses seems unlikely due to the very nature of the response. In fact, this assay measures an antibody-dependent cellular cytotoxicity response where the IgG antibody is released by sensitized spleen cells during the assay incubation and is subsequently bound to the target cell, which then can be lysed by a killer cell. Thus, in the CICC response, only IgG appears to be involved [21]. It is possible that both a differential sensitivity of early and late responses and a late stimulation by residual antigen are involved in the recovery of CDCC but that only the latter phenomenon is involved in that of CICC.

The complete inhibition of both CDCC and CICC by MTX can be attributed to the antiproliferative effects of the drug. In fact on day 2 there is no measurable immune response, thus suggesting that among the rapidly proliferating sensitized cells the number of those which have already differentiated into antibody-producing cells is insufficient to give measurable responses. MTX had no significant effect when given on day 4, at the time when the responses are well developed as a result of differentiation of the antibody-producing cells. Therefore, in considering the reversal of MTX-induced effects by CF, one can assume that the majority of the cells proliferating on day 2 would have to be protected by CF in order for a complete reversal of MTX effects to be seen on day 4. The data shown in Figs. 3 and 4 indicate that only a partial protection could be achieved and that this could be done only if CF were given before MTX. These results suggest that the rapidly proliferating clones of lymphoid cells on day 2 have a great dependence upon newly formed reduced folate cofactors, possibly as a result of the presence of unusually small pools of these cofactors and/or preformed purines and thymidine in these cells, relative to proliferation requirements. Thus, it may be necessary to preload these cells with CF in order to allow enough of them to survive after exposure to MTX to subsequently develop a partial response on day 4. It cannot be said with certainty whether this restricted time requirement is also related to the possibility that pretreatment with CF may reduce the effects of MTX by competing with MTX uptake [22, 23], or whether it is related to restrictions in the rate of uptake of CF or in the rate of metabolic interconversion of CF into reduced folate cofactor pools. These alternatives require further investigation.

The recovery of responses on day 7, from the inhibition caused by MTX given on day 2, is greatly accelerated when CF is given as late as 6 hr after the drug. This finding is consistent with the observations by Berenbaum and Brown [7] in mice sensitized with TAB vaccine and with the known reversal of MTX toxicity by CF reported by others [5]. It is conceivable that the delayed administration of CF may have protected a quantity of cells which was not sufficient to give rise to a response on day 4 but was sufficient to provide a full response on day 7, perhaps in conjunction with those cells responsible for the spontaneous partial recovery seen on that day.

It is apparent that rapidly proliferating clones of antigen-sensitized lymphoid cells may provide a useful system to study *in vivo* the effects of agents on a population of cells whose functional expression is entirely dependent on the integrity of its total proliferating capability. Thus, the systems described herein may be developed for the study of the target cell determinants involved in the selective toxicity of antimetabolites.

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