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REVERSAL BY CITROVORUM FACTOR OF METHOTREXATE-INDUCED SUPPRESSION OF CELL-MEDIATED AND HUMORAL IMMUNE RESPONSE IN MOUSE MODEL SYSTEMS

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Abstract—C_3H/HeHa mice were immunized (day 0) with 5×10^8 sheep red blood cells (SRBC) or 3×10^7 EL-4 lymphoma cells (i.p.), and C57Bl/6J mice were immunized (day 0) with 3×10^7 P815 mastocytoma cells (i.p.). Methotrexate (MTX, 100 mg/kg) was given i.p. on day +2, with or without citrovorum factor (CF) at equimolar dose. In the absence of CF in C57Bl/6J mice, the complement-independent cellular cytotoxicity (CICC) response did not recover in 18 days from MTX suppression to levels seen in immunized controls, while in C_3H mice, with EL-4 as antigen, the response equalled that of controls by day 18 and was similar to or greater than that of controls through day 28; in both cases the serum antibody response returned to control levels by day 20. In both mouse strains, CF produced immediate recovery of the responses measured. In contrast, with SRBC as antigen, while the MTX suppression of complement-dependent cellular cytotoxicity (CDCC) in the absence of CF recovered by day 7, the CICC response recovered much more slowly. CF administration produced a rapid (day 4) return of CDCC activity to control levels, but only a partial restoration of CICC activity by day 6. In conclusion, the kinetics of recovery of the CICC and CDCC responses and of CF rescue of MTX-induced immunosuppression were dependent on mouse strain and on immunogens used, as well as on the type of response measured. Thus, selectivity of MTX action is indicated.

Methotrexate (MTX) has been shown to have immunosuppressive effects in systems stimulated by either soluble or cellular antigens [1]. It inhibits both B cell dependent functions, such as antibody production [2, 3], and T cell dependent functions, such as delayed hypersensitivity [4] and skin allograft rejection [5]. Since the discovery that folinic acid (CF) is capable of rescuing MTX-treated cells from toxicity [6], much interest has centered on the use of CF to selectively rescue immune functions following MTX-induced suppression. Berenbaum and Brown [7] observed that CF had to be administered either before or simultaneously with MTX to prevent the inhibitory effects of the antifolate on antibody titers to TAB (typhoid–paratyphoid A and B) vaccine, while whole body toxicity of methotrexate could be prevented by CF even when it was administered several hours after MTX. Medzihradsky *et al.* [8] reported a similar narrow time restriction for optimum reversal by CF of MTX-induced suppression of anti-SRBC humoral responses. CF reversal of the MTX-suppressed anti-P815 allogenic response *in vitro* has been characterized by this laboratory [9] and appears to be less time restricted.

Using methodologies developed in this laboratory [10, 11], the effects of MTX have been investigated in a xenogeneic system utilizing the well-characterized SRBC antigen and have been compared to the effects induced in two allogeneic systems utilizing P815 mastocytoma and EL-4 lymphoma cells as antigens. Thus, the immunosuppression induced by MTX, and its subsequent reversal by CF, have been evaluated using functions that are primarily B cell or T cell dependent and involve interactions among several cell types. The understanding of the manner in which MTX and CF modify these interactions and alter the kinetics of immune functions may be important for devising chemotherapy regimens that would minimize suppression of host defenses against tumor and may thereby improve the overall therapeutic effect of the treatment [12].

MATERIALS AND METHODS

Mice and antigens. Female C3H/He Ha mice, 6–8 weeks of age, were used for immunization with SRBC and EL-4 lymphoma. Female C57Bl/6J mice of the same age were used for immunization with P815 mastocytoma. The lymphoma was obtained from ascites tumor transferred in C57Bl/6J mice by the i.p. transplantation of 5×10^6 cells every 7 days. P815 mastocytoma was transferred every 6 days by the i.p. inoculation with 5×10^6 cells of female DBA/2Cr mice. Sheep erythrocytes in 50% Alsever's solution were purchased from the Grand Island Biological Co., Grand Island, NY.

Compounds. Methotrexate was purchased from Nutritional Biochemicals, Cleveland, OH, and purified by the column chromatography method of Zakrzewski and Sansome [13]. The *d,l*-CF was purchased from the Grand Island Biological Co. and was used at twice the desired concentration to correct

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for the inactive steroisomer. Methotrexate and CF solutions were made immediately before use in sterile saline; a small amount of ammonium acetate was added to solubilize MTX. Radioactive [^{3}H]methotrexate (250 mCi/mmole) was purchased from Amersham-Searle, Arlington Heights, IL, and kept in the dark at −50° until it was reconstituted with sterile water and used.

Immunization. P815 mastocytoma in ascites form was removed from the host animal 6 days after implantation and washed three times in saline with centrifugation at 800 *g* for 5 min (4°). The final pellet was resuspended in saline and the cell density was adjusted to 6×10^7/ml. C57Bl/6J mice were immunized by i.p. injection of 0.5 ml of this suspension. EL-4 lymphoma in ascites form was removed from the host 7 days after implantation and washed three times in RPMI 1640 medium as described above. The final cell pellet was adjusted to a density of 6×10^7 cells/ml, and 0.5 ml was injected i.p. into C3H/He Ha mice for immunization. SRBC were washed three times in saline as above, the cells were resuspended at a density of 10^9 cells/ml and 0.5 ml was injected i.p. into C3H/He Ha mice for immunization.

Effector and cytotoxic cells. The immune responses to the three antigens used were quantitated by measuring the release of ^{51}Cr from labeled target cells that were identical to the cells used for immunization. The assays for measuring complement-independent cellular cytotoxicity (CICC), complement-dependent cellular cytotoxicity (CDCC), and serum antibody were carried out as previously described [14, 15] and are briefly outlined below. Effector cells producing cytotoxicity to ^{51}Cr targets were isolated from mouse spleens removed by established procedures from animals at various times after immunization [16].

Target cells. Target cells (5×10^7) were incubated in a volume of 0.1 ml with 50 μCi of sodium chromate-51 (0.1 ml) in a round bottom centrifuge tube for 20 min at 37° in an incubator containing a 5% CO_2 and 95% humid air atmosphere. The labeled cells were washed twice with cold RPMI 1640 medium containing 1 unit/ml penicillin and 1 μg/ml streptomycin, and were resuspended in 20 ml of the medium containing 5% fetal calf serum, 1 unit/ml penicillin and 1 μg/ml streptomycin. This suspension was returned to the incubator for 45 min and allowed to leak ^{51}Cr from any damaged cells. The target cells were pelleted by centrifugation in an International-PR-200 centrifuge at 500 *g* for 5 min (5°), resuspended in RPMI 1640 medium containing 5% fetal calf serum, and the density of viable cells was adjusted to 2×10^5 cells/ml. A volume of 0.1 ml (2×10^4 labeled cells) was added together with the desired number of effector or cytotoxic cells into duplicate 12 × 75 mm plastic tubes (Falcon No. 2052, Oxnard, CA), shaken, and incubated in a 5% CO_2–air mixture at 37° for the appropriate time.

CDCC, CICC and serum antibody assays. The CICC assay involved the incubation of spleen effector cells (0.1 ml) with labeled target cells (2×10^4 cells in 0.1 ml) for 4 hr. Effector-to-target ratios of 100:1 and lower were used. The CDCC assay involved the same initial mixing of effector and target cells. After 30 min at 37°, 0.4 ml of rabbit complement (Grand Island Biological Co.), diluted 1:20 with medium containing 5% fetal calf serum, was added with mixing. The incubation was continued for another 30 min. Serum antibody activity was determined by mixing 0.1 ml of the appropriately diluted serum with 0.1 ml of target cells (2×10^4 cells/ml), incubating them for 30 min at 37°, and then adding 0.4 ml of the diluted rabbit complement and continuing the incubation for another 30 min. All reactions were stopped by the addition of 2 ml of cold RPMI 1640 medium, centrifugation at 800 *g* for 5 min, and separation of the pellet and supernatant fraction. The percent release of radioactive chromium is given by:

$$\%\ ^{51}\text{Cr release} = \frac{\text{cpm in supernatant fraction}}{\text{cpm in supernatant fraction} + \text{cpm in pellet}} \times 100$$

Controls involved using either non-immune spleen cells or serum from non-immunized mice. The percent "specific release" was determined by subtracting the percent release in controls from the percent release in experimental samples.

[^{3}H]*MTX uptake.* Tumor cells were washed three times in RPMI 1640 medium and resuspended at a final concentration of 3×10^6 cells/ml in medium with 5% fetal calf serum. To determine drug uptake, [^{3}H]MTX was added to triplicate cultures at a final concentration of 0.8 μM (0.2 μCi) and incubated for 15, 30, or 45 min at 37° in 5% CO_2–air. After washing the tumor cells, intracellular methotrexate was extracted with 0.1 N acetic acid by the method of Kessel *et al.* [17]. Aliquots of the radioactive extract (0.2 ml) were counted in 5 ml of an Aquasol scintillation mix using a Beckman liquid scintillation counter.

RESULTS

Effect of MTX on the immune responses to SRBC. The CDCC response is a measure of the humoral response to antigen and peaks on day 4, at which time primarily IgM production is measured. MTX (100 mg/kg, i.p.), administered on day 2 following antigen, induced marked immunosuppression (Fig. 1) which recovered spontaneously by days 6–7. Equimolar CF given at the same time as MTX prevented the suppression observed on day 4 and, therefore, appeared to produce a rapid and complete recovery. The spontaneous recovery of the CDCC response seen on day 6 after MTX administration paralleled an increase in [^{3}H]thymidine incorporation observed in the same spleen cell population when pulse labeling was carried out on day 6 (data not shown).

The CICC response to SRBC antigen measures at least two components, one involving antibody formation (IgG) and its release, the other involving interaction of non-T, non-B, killer cells with the antibody-coated target cell [18]. This response showed a slower rate of spontaneous recovery than CDCC from MTX immunosuppression (Fig. 2). Equimolar CF given at the same time as MTX increased the rate of recovery of this response, but it did not produce a return to control levels.

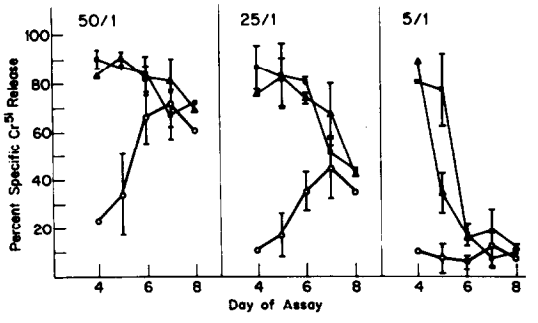

Fig. 1. Kinetics of the immune response of C3H/He Ha mice to 5×10^8 SRBC administered i.p. The complement-dependent cellular cytotoxic activity was measured at three effector-to-target cell ratios, using a fixed number (2×10^4) of ^{51}Cr-labeled SRBC target cells. MTX (100 mg/kg) was injected i.p. 2 days after antigen, as was CF (200 mg/kg) which was injected 10 min after MTX. The assay conditions are described in Materials and Methods. Bars indicate ± standard deviations. Points without bars represent the mean of four separate animals. Treatment groups are as follows: MTX (○); MTX + CF (▲); and controls (■).

Effect of MTX on the immune responses to P815 and EL-4. In contrast to the CICC response measured to SRBC antigen, the CICC response to the nucleated antigens, P815 and EL-4, measures the development of a T-killer cell response [11, 14]. Following administration of MTX (100 mg/kg, i.p.) on different days after EL-4 antigen stimulation (Fig. 3), it was found that MTX was most effective in inducing suppression of the CICC response when given 2 days after antigen. This confirmed the observation by Medzihradsky *et al.* [8], who found the same timing for maximum immunosuppression of the SRBC response, and indicated that nucleated and non-nucleated cellular antigen stimulated systems behave similarly in this type of immunosuppression. When MTX was given 2 days after antigen and CF was given at different times relative to MTX in the EL-4 antigen system (Fig. 4), no strict time restriction in producing reversal was seen. Similarly,

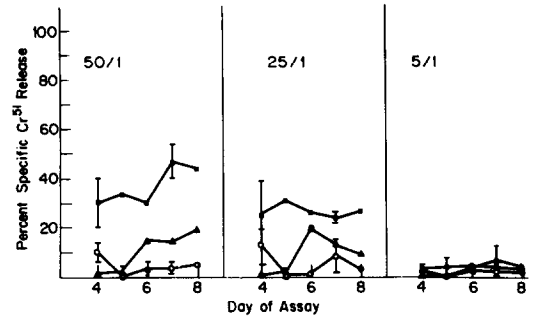

Fig. 2. Kinetics of the complement-independent cellular cytotoxic activity produced by immunization of C3H/He Ha mice with 5×10^8 SRBC (i.p.). MTX (100 mg/kg) and CF (200 mg/kg) were injected i.p. 2 days after antigen. Three effector-to-target cell ratios were used to determine activity. Bars indicate ± standard deviation. Points without bars represent the mean of four separate animals. Treatment groups are as follows: MTX (○); MTX + CF (▲); and controls (■).

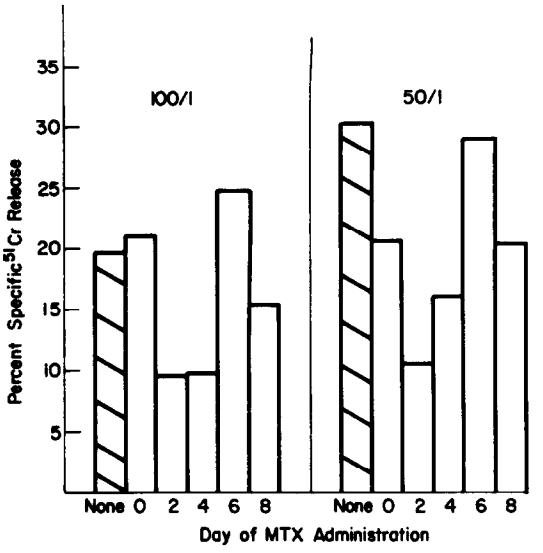

Fig. 3. Inhibition of the CICC response induced by immunization of C3H/He Ha mice with 3×10^7 EL-4 lymphoma cells (i.p.). Mice were killed and their spleens were removed for assay 14 days after immunization. MTX (100 mg/kg) was administered i.p. to immunized animals on the same day as antigen or on subsequent days. Two effector-to-target cell ratios were used to determine activity. The results shown are from one representative experiment out of three experiments carried out.

in the system using P815 as antigen (Fig. 5), CF was effective in reversing MTX immunosuppression when given either before or after the anti-folate. CF was not as effective in reversing MTX suppression of the T-killer response in the P815 system as it was in the EL-4 system when its addition was delayed by 24 hr.

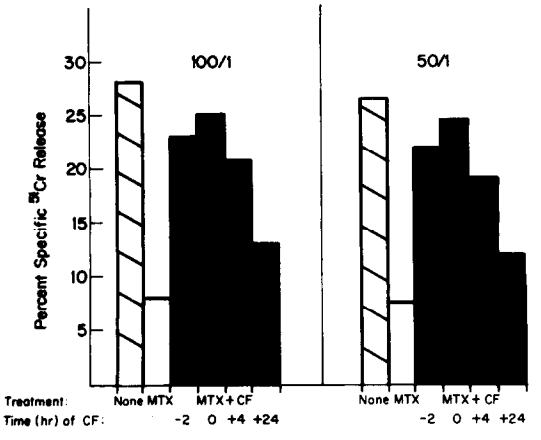

Fig. 4. Reversal of the MTX-induced inhibition of the CICC by CF in C3H/He Ha mice immunized with 3×10^7 EL-4 lymphoma cells (i.p.). Mice were killed and their spleens were removed for assay 14 days after immunization. MTX (100 mg/kg) was injected i.p. 2 days after antigen. CF (200 mg/kg) was administered i.p. before (−2 hr), at the same time (0 hr), or after (+4 or +24 hr) MTX administration at time 0. Two effector-to-target cell ratios were used to measure the CICC response. The data shown are from one representative experiment out of four experiments carried out.

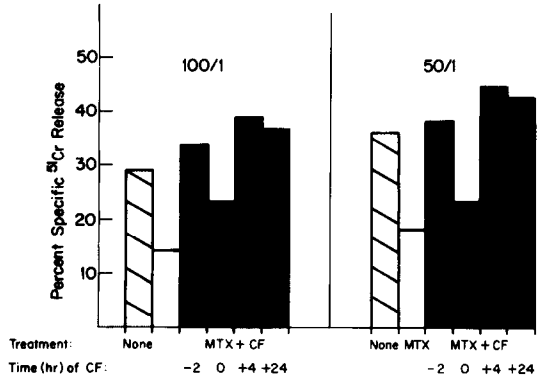

Fig. 5. Reversal of MTX-induced inhibition of the CICC response by CF in C57Bl/6J mice immunized with 3×10^7 P815 mastocytoma cells (i.p.). Mice were killed and their spleens were removed for assay 10 days after immunization. MTX (100 mg/kg) was injected i.p. 2 days after antigen. CF (200 mg/kg) was administered i.p. before (−2 hr), at the same time (0 hr), or after (+4 or +24 hr) MTX. Two effector-to-target cell ratios were used to measure CICC activity. The data shown are from one representative experiment out of four experiments carried out.

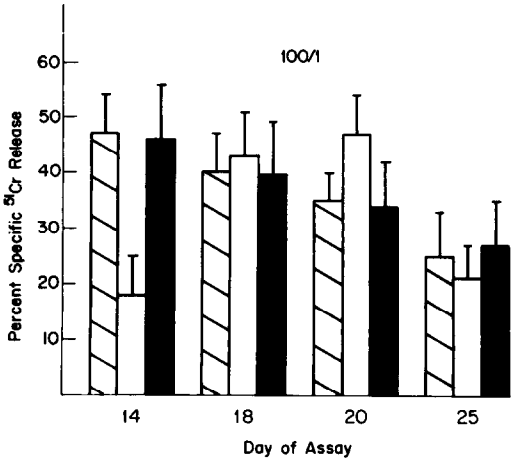

Fig. 7. Kinetics of recovery of MTX-inhibited CICC response in C3H/He Ha mice immunized with 3×10^7 EL-4 lymphoma cells (i.p.). MTX (100 mg/kg) was injected 2 days after antigen. CF (200 mg/kg) was administered 15 min after MTX. One effector-to-target cell ratio (100:1) is shown. Bars represent ± standard deviation. Treatment groups are as follows: (▨) controls; (□) MTX; and (■) MTX + CF.

Kinetics of recovery after MTX-induced suppression of the CICC response in the P815 and EL-4 systems. The kinetics of recovery of the CICC response to P815 mastocytoma following 100 mg/kg MTX given on day 2 was studied (Fig. 6), both with and without the simultaneous addition of equimolar CF. MTX-induced immunosuppression was maximum on day 7, but it showed some recovery already by day 10; day 10 is the peak day for this response [14]. Equimolar CF accelerated the rate of this recovery. The slight residual MTX-induced inhibition seen at late time points (day 14 or 18) may indicate an incomplete spontaneous recovery from the dose of antifolate used. The recovery of the CICC response to EL-4 antigen following MTX-induced inhibition is shown in Fig. 7. Immunosuppression was seen on day 14, but spontaneous recovery occurred by day 18. Equimolar CF prevented the effects of MTX seen on day 14. Therefore, on the days when peak CICC response to P815 (day 10) and EL-4 (day 14) antigens was measured, equimolar CF produced significant recovery from the MTX-suppressed activities. Experiments in which CF was given 4 hr after MTX showed the same recovery patterns as those seen with simultaneous MTX and CF, thus arguing against the possibility that the effect of CF is related to an interference with MTX uptake.

Effects on serum antibody responses. The serum antibody response to P815 antigen (Fig. 8) was inhibited by 100 mg/kg MTX and did not recover until day 18. Administration of equimolar CF with MTX induced recovery of serum antibody activity by day 8. This rapid effect was comparable in its

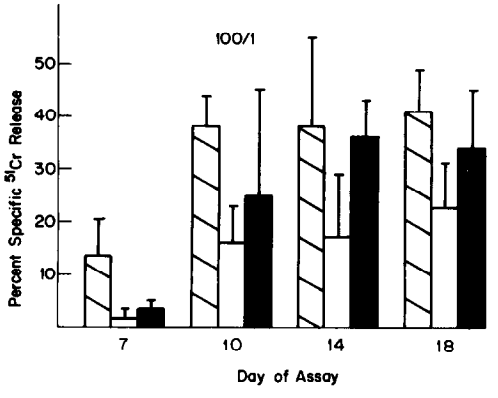

Fig. 6. Kinetics of recovery of MTX-induced inhibition of the CICC response in C57Bl/6J mice immunized with 3×10^7 P815 mastocytoma cells (i.p.). MTX (100 mg/kg) was injected 2 days after antigen. CF (200 mg/kg) was administered 15 min after MTX. One effector-to-target cell ratio (100:1) is shown. Bars represent ± standard deviation. Treatment groups are as follows: (▨) controls; (□) MTX; and (■) MTX + CF.

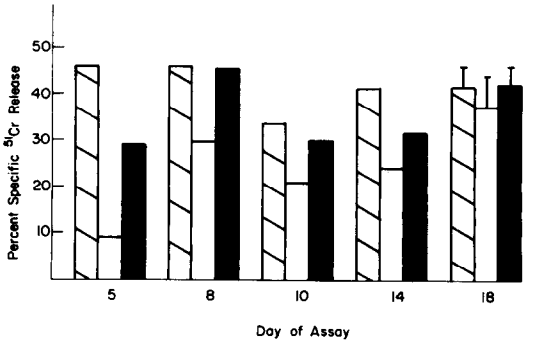

Fig. 8. Kinetics of recovery from MTX-induced inhibition of serum antibody response in C57Bl/6J mice immunized with 3×10^7 P815 cells (i.p.). MTX (100 mg/kg) was injected i.p. 2 days after antigen. CF (200 mg/kg) was administered 15 min after MTX. Serum was obtained from two to three mice, pooled, and diluted 1:200 for testing. The data shown are the mean of two experiments. Day 18 results represent the mean of three experiments. Bars indicate ± standard deviation. Treatment groups are as follows: (▨) controls; (□) MTX; and (■) MTX + CF.

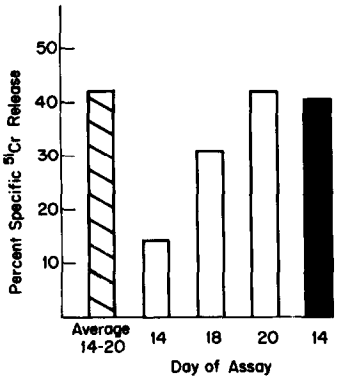

Fig. 9. Kinetics of recovery from MTX-induced inhibition of serum antibody response in C3H/He Ha mice immunized with 3×10^7 EL-4 cells (i.p.). The drug schedule was as indicated in the legend of Fig. 8. Serum was obtained from two to three mice, pooled, and diluted 1:500 for testing. The data shown are the mean of two experiments. Treatment groups are as follows: (▨) controls; (□) MTX; and (■) MTX + CF.

timing to the reversal of MTX-induced suppression of the CDCC against SRBC noted above.

The serum antibody response was also measured following immunization with EL-4 cells (Fig. 9). Spontaneous recovery from MTX-induced immunosuppression was seen by day 20. Equimolar CF induced a complete recovery of antibody response on day 14, the earliest time point investigated in this experiment.

[^{3}H]*MTX uptake in P815 and EL-4*. The immunosuppression of the CICC induced by 100 mg/kg MTX given i.p. appeared to be more severe in the system stimulated with P815 than in that stimulated with EL-4. Kessel *et al.* [17] have correlated the sensitivity of several tumor lines to MTX with the cellular uptake of the anti-folate. To investigate differential sensitivity of P815 and EL-4 to MTX, uptake studies with [^{3}H]MTX were carried out (Fig. 10). The results indicated that P815 indeed had a higher uptake of [^{3}H]MTX and may thus have been more sensitive to MTX. Further investigations are needed to clarify whether the different sensitivities of P815- and EL-4-induced CICC responses to MTX suppression are related to differential effects on antigen proliferation kinetics, or whether a stronger initial response to antigen is more susceptible to MTX inhibition.

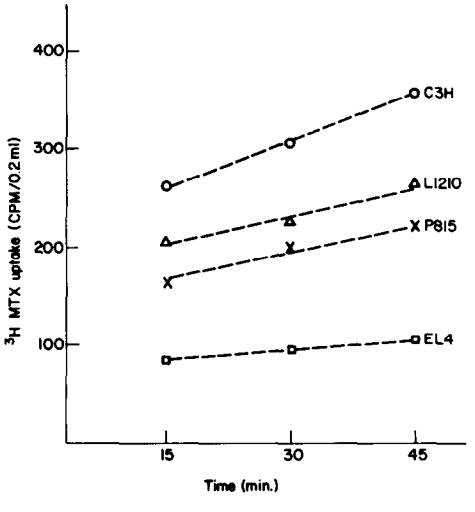

Fig. 10. Uptake of [^{3}H]MTX by 3×10^6 tumor cells *in vitro*. Tubes were run in triplicate with 0.8 μM (0.2 μCi) [^{3}H]MTX as described under Materials and Methods.

DISCUSSION

Suppression by MTX of immune responses to SRBC was optimal when MTX was administered 2 days after antigen, at which time the drug also exerts its maximum anti-proliferative effect [8]. Lin [19] has shown that MTX is an effective inhibitor of direct hemolytic plaque formation in response to SRBC if it is administered to animals after spleen cells have been stimulated to proliferate but that it is inactive if administered before such stimulation. CF accelerated the recovery or prevented the inhibition of both CDCC and CICC responses to SRBC which were inhibited by MTX, but the CICC response showed a less complete recovery. The CICC response to SRBC involves IgG [18, 20] and the CDCC response involves IgM [10, 20]. In patients, CF has caused a recovery of MTX-induced inhibition of IgM antibody titers but not of IgG titers [21]; other antiproliferative drugs have also been shown to effect a greater inhibition of the IgG than of the IgM response [22]. Thus, it is possible that the differential sensitivity of the anti-SRBC responses to MTX + CF treatment observed in this study is the result of a greater inhibition of the IgG response by MTX.

Since the CICC responses to P815 mastocytoma and EL-4 lymphoma cells are a measure of cell-mediated immunity [11, 14], the MTX-induced suppression of these responses indicated that the drug, on the schedule tested here, effectively inhibited T cell function. The effectiveness of CF in reversing this MTX-induced suppression was not restricted by narrow time limits, i.e. it was effective if given before, after, or simultaneously with MTX. This observation is different from the time restriction seen in the reversal of the inhibition of antibody responses to TAB vaccine [7], or in the CICC and CDCC responses to SRBC [8], and may reflect differences in the MTX effects on T cell responses as compared to effects on B cell responses. Differential effects of MTX and of other anti-proliferative agents have been seen on T cell versus B cell functions [22, 23], but these effects depend on the dose and timing of drug administration. The dose of MTX used in these studies, 100 mg/kg i.p., is less than one-half the LD_{50} in mice. This dose has been found to cause a three-log decrease in the number of direct plaque-forming cells (PFC) in response to SRBC on the peak day of response [19].

In both the P815 and EL-4 systems, CF accelerated the recovery of immune function after MTX suppression. CF has been shown to increase the rate of efflux of intracellular MTX and thus decrease the persistence of free MTX within the cell [24]. Different cell types can survive high levels of free MTX within the cell for different periods of time [25], and this has been proposed as a basis for the selectivity

of MTX plus CF regimens. Little is known about the requirements for persistence of free MTX in the different cell types involved in the immune response. CF also accelerated the rate of recovery of serum antibody against P815 and EL-4 which was suppressed by MTX. Friedman *et al.* [22] have reported that in the guinea pig MTX has less effect on delayed hypersensitivity than on antibody formation. This differential effect on cellular vs humoral responses was not seen in the studies reported here using nucleated tumor cells as antigen. It should be pointed out that these tumor cells proliferate for a number of days *in vivo* before the immune response leads to an allogeneic rejection effect. This is a complex effect, but the dose of MTX used in these studies has been shown not to increase the life span of syngeneic animals carrying these tumors (unpublished data). Thus, MTX and CF are not likely to affect the load of antigen present in these animals.

These studies indicate that CF is effective in protecting against MTX suppression of several immune functions in three different antigen-stimulated systems. Different time restrictions for CF addition have been shown, and different extents of MTX suppression and CF reversal in different functional assays have been observed. The characterization of the immune modulation induced by MTX plus CF is important for the ultimate design of selective chemotherapy regimens that may minimize toxicity to host immune functions.

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