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Augmentation of immune responses after methotrexate administration

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Summary. A single intraperitoneal injection of 0·5 mg methotrexate (MTX) has been found to increase the immune reactivity of spleen cells from (C57B1/6 × DBA/2)F_1 mice. Five days after injection, spleen cells from MTX-treated mice exhibited greater PHA responsiveness and GvH reactivity, and mice given SRBC at this time developed greater than normal direct PFC responses. This pattern of effects of MTX was particularly evident in mice that had been given high doses of BCG intravenously 14 days before testing, a treatment that generally depressed the measured activities. MTX enhancement of GvH was also observed in mice that had been depleted of short-lived T lymphocytes by adult thymectomy. We suggest that MTX-sensitive cells possibly exert, particularly in BCG-treated mice, a suppressive action on the responding cells.

INTRODUCTION

Antitumour chemotherapeutic drugs, by killing normal lymphoid cells as well as tumour cells, often induce an immunodepression. However, there are several reports in the literature demonstrating that immune responses may in some cases be enhanced by injection of cyclophosphamide (Cy) before immunization (Kerckhaert, Van Der Berg, Willers, 1974; Guttmann, 1974; Willers & Sluis, 1975). In the studies in which it was found that delayed type hypersensitivity (DHT) responses were augmented while antibody responses were concomitantly diminished, it was concluded that the Cy-induced decrease in antibody level was responsible for the increase in DTH (Katz, Parker & Turk, 1974; Lagrange, Mackaness & Miller, 1974; Neta & Salvin, 1974). However, it has also been demonstrated that Cy-mediated augmentation of DTH may be observed at doses which do not influence antibody responses, and it was suggested that cyclophosphamide-sensitive suppressor T cells might exist (Askenase, Hayden & Gershon, 1975).

We report here results showing that the administration of another frequently used chemotherapeutic drug, methotrexate (MTX), may also enhance some immune responses, particularly when these are modified by the prior use of high doses of BCG.

In previous work while attempting to explain the efficiency of BCG therapy in the control of some human and animal cancers, we noted that the intravenous injection of high doses of BCG led to

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decreased PHA and GvH reactivity of spleen cells. This was not due to a loss of responding cells, but to the development of suppressor cells (Orbach-Arbouys & Poupon, 1978).

Since recently developed therapeutic protocols often combine alternating cycles of chemo- and immunotherapy, we were interested in evaluating the effect of MTX on immune responses modified by prolonged or intensive BCG immunotherapy.

MATERIALS AND METHODS

Animals

Specific-pathogen-free C57B1/6 and (C57B1/6 × DBA/2)F_1 mice, 6–8 weeks old, were obtained from the breeding centre of the Centre National de la Recherche Scientifique in Orléans, France.

Enumeration of antibody-forming cells

Antibody-forming cells were assayed by the Cunningham technique (Cunningham & Szenberg, 1968) on day 6 after the intraperitoneal injection of 0·2 ml of a 10% SRBC suspension. Results are expressed as the number of direct plaque forming cells (PFC) per spleen, or, when total numbers of spleen cells were enumerated for each individual mouse, as PFC per 10^6 spleen cells. Each group consisted of six mice and the figures reported in the tables are the mean value ± standard deviation.

In vitro lymphocyte culture

Spleens from groups of three mice were aseptically removed, pooled and gently squeezed between two sterile glass slides. Cell suspensions thus obtained were then filtered through gauze and washed in cold sterile tissue culture medium 199.

For subsequent tests the suspensions were prepared in RPMI 1640 (Eurobio, France), supplemented with 2 mM glutamine (Gibco, Grand Island, New York, U.S.A.) and containing 5% fresh human AB serum (decomplemented by heating at 56° for 30 min), 100 iu/ml penicillin, and 100 μg/ml streptomycin.

To test the responsiveness of spleen cell populations to PHA, 5×10^5 cells were placed in each well of Falcon 3040 microplates in a volume of 250 μl. To some of the wells was added, at a dose which had been found to be optimal for stimulation of normal spleen cells (4 μg per well), phytohaemagglutinin (PHA, Wellcome Laboratories, England).

All cultures were performed in triplicate. The microplates were covered and incubated at 37° in an atmosphere of 5% CO_2 plus 95% air for 48 h, after which 1 μCi of tritiated thymidine (^{3}H-Tdr; TMM 48, Commissariat à l'Energie Atomique, Saclay, France; specific activity 27 Ci/mmole) was added to each well during the last 5 h of incubation. Cultures were harvested with a multiple automated sample harvester ('MASH', Microbiological Associates, Bethesda, Maryland, U.S.A.) on glass fibre filters (Reeve Angels, Clifton, New Jersey, U.S.A.). The discs containing the radioactive material were placed in toluene plus omnifluor (NEN, Dreieichnhaim, West Germany) and radioactivity measured in a Packard counter. Results are expressed as mean counts per minute (c.p.m.) of triplicate samples ± standard deviation.

Assay of DNA synthesis of lymphoid cells in response to alloantigens

This technique has been described in detail elsewhere (Gershon & Hencin, 1971; Gershon & Liebhaber, 1972). Groups of 5 to 6 (C57B1/6 × DBA/2)F_1 mice were irradiated with 950 rad and inoculated intravenously with C57B1/6 lymphoid cells on day 0.

On day 4, at the peak of the reaction, mice were injected intraperitoneally with 2 μCi of 5-^{125}iodo-2'-deoxyuridine (IUDR, Radiochemical Centre, Amersham, England), which is incorporated into DNA in place of thymidine. Twenty-four hours later, the spleens of these animals were removed and their radioactivity measured for 2 min in a Packard Autogamma Spectrometer. Also counted at this time were standard 0·2 ml aliquots of IUDR from the same solution used for injecting test animals. Results are expressed as mean uptake of IUDR (as per cent of injected dose) per spleen ± standard deviation.

BCG

When necessary, mice were inoculated intravenously with 3 mg BCG, kindly provided by the Pasteur Institute (Paris, France) in the form called 'Immuno BCG', which retains 95% viability after 3 months storage at 4°.

Preparation of T cells from the spleen

We employed the technique described by Julius, Simpson & Herzenberg (1973) using nylon wool columns.

RESULTS

Enhancement of the PHA responsiveness of spleen cells after MTX injection

The dose of MTX used throughout this paper was 0·5 mg per mouse, which in previous studies was found to be non-toxic and exerted maximal cytotoxicity against L1210 leukaemia cells *in vivo* (Pouillart, Hoang Huong, Brugerie & Lheritier, 1974).

In preliminary experiments we observed that the greatest increase of the PHA response occurred when MTX was injected 5 days prior to cell harvest. These conditions were thus used for all the experiments presented in this paper.

In Table 1 is presented the spleen cell responsiveness to PHA. Spleen cells from MTX-injected animals responded much better to PHA than cells from non-MTX treated animals whether from normal ($P<0·001$) or BCG-injected animals ($P<0·01$). In this experiment, as has been previously reported (Orbach-Arbouys & Poupon, 1978), a decrease ($P<0·02$) of the PHA reactivity of the spleen cells from BCG-treated animals can be seen. It can also be seen that the non-PHA stimulated thymidine incorporation of the MTX and BCG treated spleen cells was considerably elevated above normal.

Table 1. PHA responsiveness of spleen cells after MTX injection

Treatment	Alone	With PHA
Controls	549 ± 196	13,408 ± 710
MTX day −5	2,799 ± 531	27,246 ± 2559
BCG 3 mg day −14	5,588 ± 722	7,831 ± 987
BCG 3 mg day −14 + MTX day −5	3,855 ± 45	20,052 ± 1672

Enhancement of the GvH reactivity by administration of MTX to cell donor

GvH was induced by injecting spleen cells from C57Bl/6 mice into lethally irradiated (DBA/2 × C57Bl/6)F_1 hybrids. The [^{125}I]UDR incorporation in the spleen on day 4 of the GvH provides a measure of the intensity of the reaction. It can be seen in Table 2 that when cells were taken from MTX-injected animals, the [^{125}I]UDR incorporation in the recipient spleens was greater than when they were taken from normal animals ($P<0·001$). Similarly, the spleen cell reactivity of BCG-treated mice was augmented ($P<0·001$) by MTX injection.

Table 2. Enhancement of the GvH reactivity of spleen cells from normal and BCG treated mice 5 days after the injection of 0·5 mg methotrexate

Treatment of donor	% Spleen [^{125}I]UDR incorporation on day 4 of the GvH
Normal	0·099 ± 0·032
MTX day −5	0·214 ± 0·031
BCG 3 mg day −7	0·079 ± 0·024
BCG 3 mg day −7 + MTX day −5	0·159 ± 0·033

In an attempt to define whether the same effect could be observed if only slowly or non-dividing (long-lived) T cells were present, we repeated the experiment using as donors adult thymectomized animals, 6 weeks after surgery. It may be seen in Table 3 that in all cases, whole spleen or T-cells

Table 3. Enhancement of the GvH reactivity of spleen cells from normal and adult thymectomized mice 5 days after the injection of 0·05 mg methotrexate

Donor	Treatment	Population transferred	% Spleen [^{125}I]UDR incorporation on day 4 of the GvH	
Normal	Nil	Spleen cells	0·257 ± 0·075	p = 0·06
	MTX	Spleen cells	0·376 ± 0·081	
	Nil	Spleen T-cells	0·197 ± 0·060	p = 0·05
	MTX	Spleen T-cells	0·323 ± 0·086	
Thymectomized	Nil	Spleen cells	0·188 ± 0·037	p < 0·02
	MTX	Spleen cells	0·430 ± 0·160	
	Nil	Spleen T-cells	0·221 ± 0·043	p < 0·001
	MTX	Spleen T-cells	0·600 ± 0·061	

from MTX-injected donors were more reactive than cells from non-MTX-treated mice and that the greatest response was obtained with T cells from MTX-treated adult thymectomized mice.

Enhancement of the splenic PFC responses to sheep erythrocytes after MTX injection

MTX was injected intraperitoneally 12, 5 or 2 days before intraperitoneal injection of 0·2 ml of a 10% SRBC suspension, and direct PFC per spleen were enumerated 6 days after antigen administration. As seen in Table 4, the maximum enhancement of the response was observed when MTX was given 5 days before antigen ($P=0·02$). It should be noted that the MTX effect on this test in normal animals is not strong and is somewhat variable. In Table 5 are reported the results of an experiment in which BCG given 14 days before SRBC led to an increased PFC response per spleen as compared to normal animals ($P=0·001$). Under these conditions, the injection of MTX increased the number of PFC per spleen ($P=0·01$) and per 10^6 cells ($P=0·001$).

Table 4. Enhancement of the splenic PFC response to SRBC by the injection of 0·5 mg methotrexate before SRBC

Protocol	Direct PFC/spleen
MTX day −12	57,600 ± 9172
MTX day −5	87,767 ± 29,914
MTX day −2	42,867 ± 18,642
Controls	45,767 ± 19,380

DISCUSSION

The data presented here indicate that PHA stimulation, GvH reactivity and anti-SRBC responses were enhanced 5 days after MTX injection. This stimulating effect was particularly evident in situations where these responses had previously been depressed by the i.v. injection of high doses of BCG. The dose of MTX (25mg/kg) producing these effects was that which is currently used in oncostatic trials by our Institute. This dose significantly prolongs the survival of mice injected with L1210 leukaemic cells (Pouillart *et al.*, 1974).

The two cytotoxic drugs that now appear to enhance immune responses under certain circumstances, cyclophosphamide and methotrexate, have different modes of pharmacological action. Cyclophosphamide is reported to act by crosslinking DNA strands, whereas MTX inhibits folic reductase.

At least two mechanisms may be envisaged to account for the fact that, on a spleen equivalent basis, the reactivity of the cells remaining after MTX injection was greater than that of the initial population.

Without implying that a given mechanism is responsible, it seems reasonable to suggest that a lymphoid population has been eliminated due to its MTX sensitivity, and that the remaining population thus responds better than in its presence. The cells which are killed by MTX possibly exert, particularly in BCG-treated mice and less so in normal mice, a suppressive action on the responding cells. Considering the GvH results, where the augmenting effect of MTX was still observed when the pool of short-lived T cells was depleted by adult thymectomy, it can be postulated that adult thymectomy partially depletes these suppressive cells, and that this is more complete when such animals are then treated with MTX. It may, therefore, be incorrect to assume that if the MTX-sensitive cells are T cells, they are eliminated on the basis of their mitotic activity.

The second possibility is that cell proliferation occurs after drug-induced cell destruction, which favours production of the reactive cell type. It is of interest that the non-PHA-stimulated thymidine

Table 5. Enhancement of the splenic PFC response to SRBC of normal or BCG treated mice by the injection of 0·5 mg methotrexate 5 days before SRBC

Treatment	PFC/spleen[a]	No. of cells/ spleen	PFC/10^6 cells
Nil	12,800 ± 1667	$4·32 \times 10^7$	336 ± 119
MTX day −5	15,800 ± 2688	$3·7 \times 10^7$	496 ± 169
BCG 3 mg day −14	143,833 ± 33,349	$34·6 \times 10^7$	371 ± 93
BCG 3 mg day −14 + MTX day 5	217,333 ± 35,529	$35·8 \times 10^7$	759 ± 188

incorporation of spleen cells from MTX-treated animals was considerably higher than the normal. This was also the case after BCG treatment, but in the latter situation suppression rather than enhancement was observed. It appears therefore that spleen cell proliferation alone does not necessarily lead to enhancement.

The fact that appropriate combinations of BCG and MTX led to a considerable increase of the anti-SRBC response on a whole spleen basis, suggests, if the first hypothesis is correct, that despite enhancement by BCG of total splenic PFC-forming potential, development of a high degree of immunosuppression occurs concomitantly. This is in agreement with our previous published findings (Orbach-Arbouys & Poupon, 1978). It may even prove possible to quantify the magnitude of a suppressor cell response by evaluating the augmentation induced by MTX treatment.

We have previously reported (Orbach-Arbouys, 1968) that both MTX and cyclophosphamide can break immune tolerance to SRBC in rats. This was, at that time, an unexplained observation, but may now be considered as also resulting from a similar mechanism. That is, since suppressor cells are held responsible for some forms of immune tolerance, their destruction by cytotoxic drugs might allow the animals to respond to the tolerated antigen.

Another point to underline is the ease with which we could enhance GvH reactivity of the donors. Most protocols using adjuvants or preimmunization do not always yield such results (Simonsen, 1970; Ford & Simonsen, 1971).

Consequently, in spite of the fact that the exact mechanism responsible for the MTX-induced increase in immune reactivity is not known, this empirical observation might nevertheless prove useful in diminishing the immunosuppressive activity of some adjuvants and allow the combination of optimal MTX administration with immunotherapy in the treatment of cancer.

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