ORIGINAL ARTICLES

Enhancement of Cytostatic Activity of Macrophages Against MOPC-315 Tumour Cells by Combined Cyclophosphamide Administration and Immunisation with Glutaraldehyde-treated Tumour Cells*

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SUMMARY Spleen cells of BALB/c mice injected with cyclophosphamide (CY:100 mg/kg i.p.), and one day later with glutaraldehyde-treated MOPC-315 tumour cells (GA-MOPC:25x10^{6} cells/mouse i.p.), exhibited cytostatic activity *in vitro* against MOPC-315 tumour cells, when tested on the sixth day but not when tested on the 28th day after CY injection. Spleen cells from mice injected with CY alone or with GA-MOPC cells only were not cytostatic. The cytostatic activity (decrease in thymidine incorporation by tumour cells) was detected in unfractionated spleen cell suspensions, in spleen cell suspensions depleted of T cells, in the glasswool-adherent macrophage-enriched population but not in the nylon wool nonadherent T-enriched population. The cytostatic activity of the macrophage-enriched population was not affected by the addition of T cells. The T-enriched population was devoid of cytostatic activity even when supplemented with various amounts of macrophages. Most mice injected with CY and GA-MOPC cells were resistant to inoculation with a tumourigenic dose of MOPC-315 tumour cells.

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It has been reported that cyclophosphamide (CY) protentiates cell-mediated immune responses,[1] such as delayed-type hypersensitivity,[2] contact sensitivity,[3] auto-immune response[4] and antitumour immune response.[5] Moreover, the eradication of established MOPC-315 tumours was assumed to be due to cooperation between the CY effect and the host's antitumour immunity.[6] It was also shown that the combination of CY with immunotherapy was more effective than of either one alone against tumours.[7-10] Potentiation of cell-mediated immunological response by CY was related to its selective effect on suppressor T cells[5,11-13] or to the selective depletion of B cells.[14] We have shown[15] that immunisation with syngeneic MOPC-315 tumour cells treated with glutaraldehyde (GA-MOPC cells) afforded partial protection against inoculation with a tumourigenic dose of MOPC-315 cells. The aim of the present work was to determine whether administration of CY would increase the immune response induced by the injection of GA-MOPC cells as expressed by the generation of cytostatic activity of spleen cells *in vitro* against MOPC-315 tumour cells.

MATERIALS AND METHODS

Mice and tumour

BALB/c male mice between 8 and 12 weeks of age, selected from a breeding colony of the Hebrew University at Jerusalem, were used in this study. The MOPC-315 myeloma cell line was derived from a plasma tumour originally induced in BALB/c mice by the intraperitoneal injection of mineral oil.[16] An *in vitro* line of MOPC-315 tumour cells, adapted to growth in culture,[17] was maintained by serial passages in RPMI 1640 medium

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(Grand Island Biological Company, Grand Island, NY, USA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mmole/ml L-glutamine and 10 per cent foetal calf serum (complete medium). The viability of tumour cells in cultures was approximately 90 per cent as shown by trypan blue dye (0.1 per cent) exclusion test. The *in vitro* grown line was used in all experiments.

GA treatment of tumour cells (GA-MOPC)

Tumour cells were washed three times in PBS, pH 7.2, and resuspended at a concentration of 2x 10^7 viable cells/ml. A volume of 0.2 ml of the cell suspension was mixed with 1.8 ml of 0.02 per cent GA (TAAB, England) solution in PBS and kept for 10 minutes at room temperature. The GA-treated cells were washed by three subsequent centrifugations at 100xg for 7 minutes at 4°C and resuspended in PBS to a concentration of 5x 10^7 cells/ml for injections.

***In vivo* treatments**

Groups of mice were injected with a freshly prepared solution of 100 mg/kg CY in distilled water (Taro, Haifa, Israel, lyophilised powder), i.p. on day 0, and one day later were immunised with 25x10^6 GA-MOPC cells/0.5 ml i.p. and sacrificed on the sixth day (if not stated otherwise) for harvesting of spleen cells. Groups of untreated mice, of mice injected with CY only or of mice receiving only GA-MOPC cells served as controls. Mice injected with CY and GA-MOPC tumour cells and with CY alone was also inoculated i.p. with a tumourigenic dose of 5x10^4 viable MOPC-315 tumour cells eight days after CY administration. The mortality of tumour-bearing mice was recorded and compared with that of inoculated, untreated mice.

Cell preparations

Intact spleen cell suspensions, T-depleted spleen cell suspension, macrophage-enriched and T-enriched spleen cell fractions were used. Spleen cell suspensions were prepared in RPMI medium (without FCS) and finally resuspended in complete RPMI medium supplemented with 5x10^{-5} M 2-mercaptoethanol. T-cell depletion was achieved by incubating 1x10^7 viable spleen cells/ml with an equal volume of a 1:5,000 dilution of anti-Thy 1,2 monoclonal antibody (Booth, England) and an equal volume of 1:20 fresh guinea pig serum as the source of complement. Preliminary tests showed that approximately 98 per cent of thymocytes were killed by this procedure. A T-enriched fraction was obtained by passage through a glass-wool column[18] and subsequent passage through a nylon-wool column.[19] The nylon-wool nonadherent fraction contained more that 98 per cent anti-Thy 1,2 sensitive cells as shown by cytotoxic assay with monoclonal anti-Thy 1, 2 antibody. The macrophage-enriched population was obtained by elution of cells adherent to the glasswool column with warm PBS containing 0.02 per cent EDTA.[18]

***In vitro* Cytostatic assay**

The assay is based on the inhibition of DNA synthesis of MOPC-315 tumour cells by spleen cells *in vitro* and was performed as described.[20] A mixture of 0.05 ml of 1x10^5 spleen cells and 0.05 ml of 1x10^3 target tumour cells per well (E/T ratio of 100/1) in complete medium supplemented with 5x10^{-5} M 2-mercaptoethanol was incubated in 96-well flat-bottom tissue culture plates (Linbro, USA) at 37°C in a humidified atmosphere of 5 per cent CO_2 for 24 hours. A quantity of 0.5 μCi (^{3}H) thymidine [(^{3}H) dT, Nuclear Center, Negev, Israel] in 0.05 ml/well was then added and the incubation was continued for another 18 hours. The cultures were terminated by adsorption onto glass filter disc papers by the use of an automatic harvester and counted in a Packard beta counter. Each combination mixture was performed in 4-6 parallel samples. The incorporation of (^{3}H) dT within MOPC-315 cells was calculated by substracting the background value of spleen cells alone from the value of total incorporation in mixed cultures of spleen cells and tumour cells. Parallel cultures containing mitomycin-treated tumour cells were also included in order to evaluate the possible stimulation of DNA synthesis in effector cells by tumour antigens. The rate of (^{3}H) dT incorporation did not differ appreciably between effector cells alone and effector cells mixed with mitomycin-treated tumour cells. For comparison, the rate of (^{3}H) dT incorporation was also determined in tumour cells incubated without the addition of Spleen cells.

Statistical analysis

The significance of cytostatic effect was evaluated by the Student's t test. Differences were considered significant when P was less than 0.05.

RESULTS

Antitumour cytostatic activity in intact spleen-cell populations

The cytostatic activity was evaluated in spleen cells of untreated mice, mice injected with CY and GA-MOPC cells and mice injected either with CY or with GA-MOPC cells. As shown in Figure 1, the intact spleen cell population taken on the sixth day from mice injected with CY 100 mg/kg (day 0) and 25x10^6 GA-MOPC cells (day 1) exhibited cytostatic activity *in vitro* towards MOPC-315 tumour cells whereas spleen cells of normal mice, of mice injected either with CY or with GA-MOPC cells, were not cytostatic. Usually no significant difference was found in the rate of (^{3}H) dT incorporation between tumour cells incubated with spleen cells from normal mice and tumour cells incubated without the addition of spleen cells (Figs. 1,2,3).

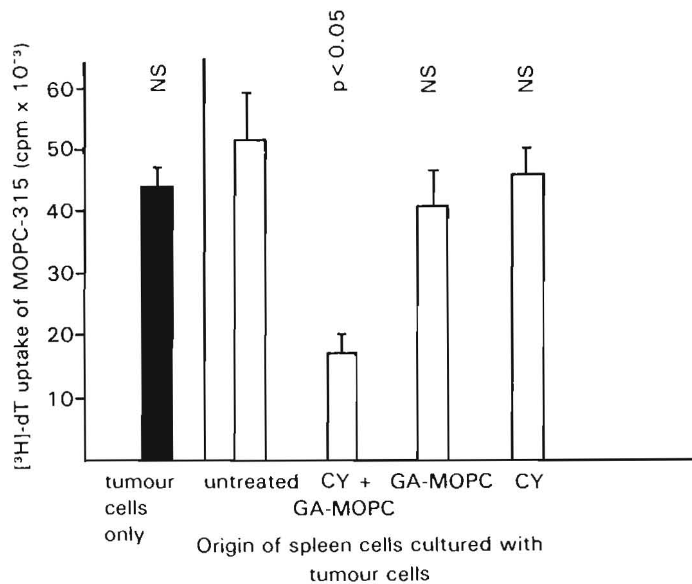

Fig. 1 Cytostatic activity of intact spleen cell populations towards MOPC-315 tumour cells following injection of cyclophosphamide (CY) and immunisation with glutaraldehyde treated MOPC-315 tumour cells (GA-MOPC), as expressed by the rate of [^{3}H] dT uptake. Tumour cells alone (■); tumour cells mixed with spleen-cell suspensions (□); CY (100 mg/kg) was given i.p. on day O; GA-MOPC (25×10^6/mouse) were injected i.p. on day 1; spleen cells were harvested on day 6 for evaluation of cytostatic activity; E/T - effector/target cell ratio of 100/1; P values were related to the mixture of tumour cells with spleen cells of the untreated mice; bars indicate mean ± SE of 4-6 paralled samples.

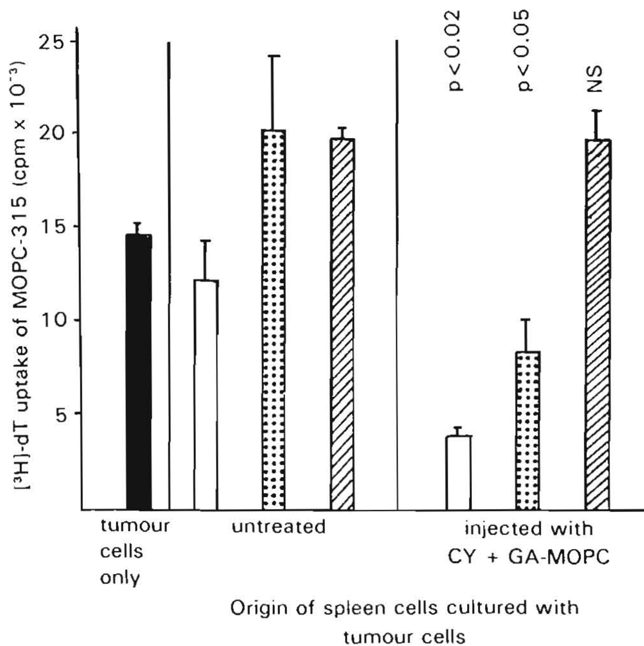

Fig. 2 Cytostatic activity of spleen cell populations towards MOPC-315 tumour cells following injection of CY and immunisation with GA-MOPC. Tumour cells alone (■); tumour cells were cultured with: intact spleen cell suspension (□); spleen cell suspension depleted of T cells (▦); and T-enriched nylon-wool nonadherent fraction of spleen cells (▨). See footnotes to Figure 1 for details.

Antitumour cytostatic activity in spleen-cell fractions

In view of the results showing cytostatic activity of spleen cells from mice injected with CY and GA-MOPC cells, attempts were made to define the spleen cell population responsible for this activity. The cytostatic activity towards MOPC-315 tumour cells was detected in a spleen cell population depleted of T cells but not in a nylon-wool nonadherent T-enriched population (Fig. 2). As shown in Figure 3, cytostatic activity was detected in the macrophage-enriched glass-wool adherent fraction (mostly macrophages) from CY+ GA-MOPC-treated mice but not in the same fraction obtained from normal untreated animals. The addition of various amounts of the macrophage fraction to a T-enriched population did not render the T fraction cytostatic. Thus, slight cytostatic activity, although not significant, was observed with the mixture of macrophages + T cells only when the quantity of macrophages added was 4×10^4 cells out of a total of 1×10^5 effector cells (Fig. 3, 1/2.5 ratio columm). When the quantity of macrophages in the mixture of M+T cells was less than 4×10^4 no cytostatic effect was observed and the rate of thymidine incorporation was similar to that observed with the effector cell population containing only T cells (Fig. 3). The possibility that the T-enriched population contained cells suppressing the cytostatic activity of the macrophage-enriched fraction was examined by adding various quantities of cells from the T-enriched population to a cytostatic glasswool adherent fraction. As shown in Figure 4, a slight reduction in the cytostatic activity of macrophages against MOPC-315 tumour cells was observed in cultures supplemented with cells from a T-enriched population. However, the cytostatic activity in mixed cultures of T cells and macrophages was still significant. The persistence of cytostatic activity was examin-

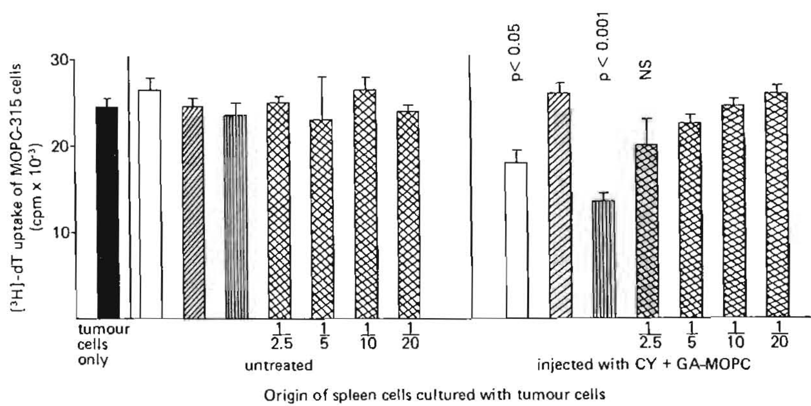

Fig. 3 Cytostatic activity towards MOPC-315 tumour cells in glass-wool adherent (macrophage-enriched) and T-enriched nylon-wool nonadherent spleen cell populations originated from mice injected with CY and GA-MOPC. Tumour cells alone (■); tumour cells were cultured with: intact spleen cell suspensions (□); T-enriched population (▨); macrophage-enriched population (▥); and T-enriched population completed to a total quantity of 1×10^5 cells/culture with the following amounts of macrophage-enriched population/culture (▩): 1/2.5, 1/5, 1/10 and 1/20. See footnotes to Figure 1 for details.

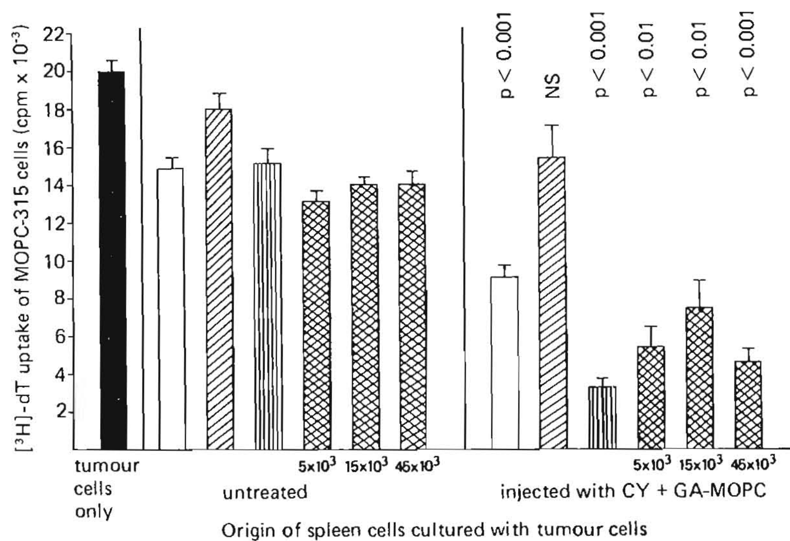

Fig. 4 Effect of addition of nylon-wool nonadherent (T-enriched) cells to macrophage enriched population on their cytostatic activity. Macrophage-enriched and T-enriched population originated form mice injected with CY and GA-MOPC. Tumour cells alone (■): tumour cells were cultured with: intact spleen cell suspension (□); T-enriched population (▨); macrophage-enriched population (▥); and macrophage-enriched population (1×10^5 cells/culture) supplemented with various amounts of T-enriched population (▩): 5×10^3 /culture, 1.5×10^4/culture and 4.5×10^4/culture. See footnotes to Figure 1 for details.

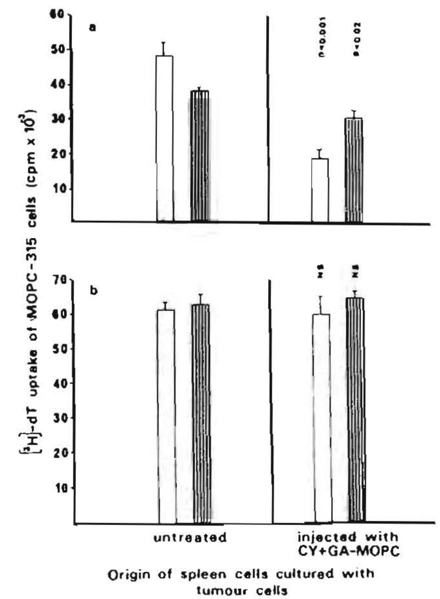

Fig. 5 Peristence of cytostatic activity in macrophage-enriched fraction of spleen cells from mice injected with CY and GA-MOPC. 5a - day 5 after CY injection; 5b - day 28 after CY injection. Tumour cells mixed with: intact spleen cell population (□); and macrophage-enriched population (▥). See footnotes to Figure 1 for details.

ed by evaluating this effect on the 28th day after the injection of CY (day 27 after immunisation with GA-MOPC cells), in comparison with day 7 after the CY injection (day 5 after immunisation with GA-MOPC cells). On the sixth day after the CY injection the cytostatic activity was again detected in the intact spleen cell population and in the glasswool adherent fraction but was not detected in spleen cells taken on the 28th day after the injection of CY (Fig. 5).

Tumour development in mice treated with CY and GA-MOPC cells

Mice injected with CY and GA-MOPC cells under conditions similar to those required for induction of cytostatic activity were inoculated with a tumourigenic dose of MOPC-315 tumour cells. As shown in Figure 6, most mice injected with CY and GA-MOPC cells, survived after the inoculation, whereas injection of CY alone had no effect on mortality. As shown previously,[15] a single injection of GA-MOPC did not induce a detectable level of protection against tumour inoculation.

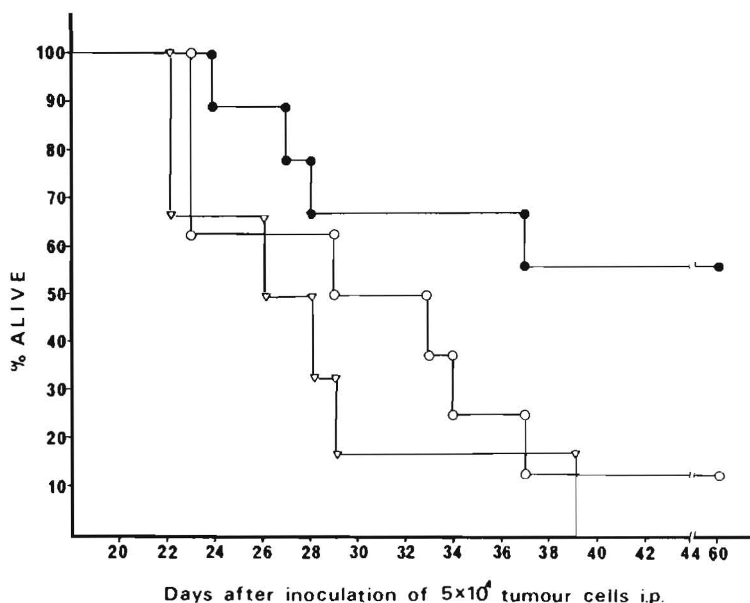

Fig. 6 Resistance of mice injected with CY and GA-MOPC to inoculation of MOPC-315 tumour cells. CY (100 mg/kg) injected in 0.5 ml i.p. on day 0. GA-MOPC cells (25 X 10^6/injection) injected on day 1. Viable tumour cells (5 X 10^4) were inoculated i.p. on day 8. (O—O) normal mice (8 mice per group); (▽—▽) CY injection mice (8 mice per group); (●—●) CY and GA-MOPC injected mice (9 mice per group).

DISCUSSION

The present experiments show that combined administration of cyclophosphamide and glutaraldehyde-treated syngeneic MOPC-315 tumour cells generated cytostatic activity against MOPC-315 tumour cells *in vitro*. On the other hand, injection of CY alone or of GA-MOPC cells only, did not induce cytostatic activity. It seems, therefore, that both CY and GA-MOPC injections are required for induction of cytostatic activity against tumour cells.

Selective effects of CY on suppressor T cells[5,11-13] and on B cells[14] were reported. It seems that under our conditions, the combined administration of CY and GA-MOPC cells resulted in increased macrophage activity. Thus, the cytostatic activity against MOPC-315 tumour cells was expressed by the macrophage-enriched fraction of spleen cells and was not detected in the T-enriched fraction. Moreover, the depletion of T cells from the intact spleen cell population did not affect its cytostatic activity against tumour cells. The lack of cytostatic activity in the T-enriched fraction is probably not due to dependence on macrophages for expression of cytostatic effect, because the addition of macrophages to the T-enriched fraction did not render this population cytostatic. The addition of T-cell fraction to macrophage-enriched fraction reduced slightly the cytostatic activity of macrophages against tumour cells. However, in view of the slight reduction in cytostatic activity, it does not seem possible to conclude whether the T-cell fraction contains a population suppressing the cytostatic effect. Although the full kinetics of the development and persistence of cytostatic activity has not been yet performed, it seems that this activity is transient as shown by its absence in spleen cells taken on the 28th day after CY administration.

Injection of CY may cause changes in the relative proportions of the different types of cells within the spleens, such as selective elimination of suppressor cells.[5,11-13] However, it seems unlikely that the induction of the cytostatic effect against MOPC-315 tumour cells by injection of CY and GA-MOPC cells is due exclusively to such changes because spleen cells taken from mice injected with CY alone were not cytostatic. Another possibility is that the thymidine incorporation assay in tumour cells is subjected to many errors when carried out in the presence of macrophages. This possibility seems also unlikely because the addition of unfractionated spleen cells or macrophage-enriched fraction of spleens from normal mice had no effect on the rate of thymidine incorporation in tumour cells.

It was reported[21] that cytotoxic activity against MOPC-315 target cells can be generated *in vitro* in cultures of spleen cells and that this activity is expressed by cytotoxic T cells. (For a review, see footnote[22]). It seems that the cytostatic activity reported here is different from the cytotoxic activity generated *in vitro* because in our hands, the T-enriched fraction was not cytostatic.

It was of interest to find out whether induction of cytostatic activity against tumour cells *in vitro* by CY and GA-MOPC administration has any bearing on the development of ascitic tumours of MOPC-315. We found in this respect, that most mice injected with CY and GA-MOPC cells survived after inoculation with an otherwise tumourigenic, lethal dose of MOPC-315 tumour cells, whereas injection of CY alone had no effect on the mortality rate after inoculation. We have also shown[15] that a single injection of MOPC-315 cells treated with 0.02 per cent GA did not confer protection against inoculation with a tumourigenic dose.

The mechanism of induction of cytostatic activity of macrophages against tumour cells by combined CY and GA-MOPC administration is not yet clear. A likely possibility[23] is that macrophages are activated

by soluble products released from T cells stimulated, in our case, by GA-MOPC antigen entity and that this stimulation is facilitated by CY-induced elimination of suppressor T cells.

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