Effect of Cyclophosphamide Administration on Immunoprophylaxis and Immunotherapy with Glutaraldehyde-treated MOPC-315 Syngeneic Tumour Cells*

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SUMMARY **The effect of cyclophophamide (CY) injection on immunoprophylaxis and immunotherapy with glutaraldehyde-treated MOPC-315 (GA-MOPC) plasmacytoma tumour cells was investigated.**

Immunoprophylaxis comprised two or three immunising injections of GA-MOPC cells. An injection of CY (100 mg/kg) before the last immunising injection of GA-MOPC resulted in a marked increase in the resistance to challenge of immunised mice which survived after the first inoculation of tumour cells. Chemotherapy with CY on days 4, 8, or 13 after inoculation cured approximately 60 per cent of the inoculated mice, but only mice injected with CY on the 13th day after inoculation were resistant to challenge. Combined chemotherapy with CY and immunotherapy with GA-MOPC cells was more effective than injection of CY alone on the 13th day after inoculation, as expressed by increased resistance to challege and an increase of antitumour cytotoxic response *in vitro*.

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We[1] have previously shown that immunisation with glutaraldehyde modified MOPC-315 syngeneic tumour cells (GA-MOPC) induced partial protection to subcutaneous inoculation with a tumourigenic dose, and, furthermore, that a combination of cyclophosphamide (CY) and inoculation with GA-MOPC resulted in induction of cytostatic activity *in vitro* of macrophage-enriched spleen cells against MOPC-315 tumour cells.[2]

The selective effect of CY on suppressor T cells involved in various cell-mediated immune type reactions is well documented.[3-6] With regard to a MOPC-315 experimental tumour, it was shown that therapy with a low dose of CY in mice bearing a large tumour cured the mice as a result of cooperation between the drug's tumoricidal effect and the host's antitumour immunity.[7]

The aim of the present work was to investigate the effect of CY administration on immunoprophylaxis or immunotherapy with glutaraldehyde-treated MOPC-315 tumour cells. We hereby show that, under certain conditions, the administration of CY increased the effectiveness of immunoprophylaxis and immunotherapy by modified tumour cells.

MATERIALS AND METHODS

Animals

Male BALB/c mice 8-12 weeks old, bred at the Weizmann Institute of Science, Rehovot, Israel, were used.

Tumour cells

BALB/c myeloma MOPC-315 cells were initially provided by Dr. D. Givol of the Weizmann Institute. These cells secrete IgA λ2 immunoglobulin endowed with activity as anti-TNP antibody.[8] The tumour cells were maintained *in vivo* by serial subcutaneous transfers. Preservation of the property to secrete antibodies against the TNP hapten was ascertained every few months by direct passive haemagglutination of the ascitic fluid with TNP-SRBC conjugate.[9]

Glutaraldehyde treatment[1]

Volumes of 0.2 ml of a suspension of 2×10^7 viable tumour cells/ml in PBS pH 7.2 were mixed with

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1.8 ml of a solution of GA (TAAB Lab., England) of desired concentration, and the mixture was left for 10 minutes at room temperature. The treated cells were washed by three subsequent centrifugations at 100 x g for seven minutes at 4°C. The packed washed cells were then resuspended in PBS for injection.

In vivo treatment

All injections (0.2 ml each) of tumour cells were given subcutaneously. Immunisation was performed with MOPC-315 cell suspensions treated with GA and adjusted to contain $5x10^5 - 1x10^6$ cells/0.2 ml. The concentrations of GA used, the time interval between injections and the number of viable cells used for inoculation are specified under "Results" CY (Taro, Haifa, Israel) was injected intraperitoneally at a volume of 0.5 ml (100-200 mg/kg).

In vitro generation of cytotoxic spleen cells

The method for *in vitro* sensitisation of spleen cells in the presence of tumour cells has been described.[10] MOPC-315 stimulator cells were treated with mitomycin C (Sigma, U.S.A.) 50 $\mu g/10^7$ tumour cells for 30 minutes at 37°C and washed three times. A quantity of $75x10^6$ responder cells originating from pooled spleens of specified groups of mice was mixed with $2x10^6$ stimulator cells in a 250-ml tissue culture flask (Nunc, Denmark) in a final volume of 50 ml RPMI-1640 medium, supplemented with 1% non-essential amino acids, 5% foetal calf serum (Gibco, U.S.A.), 100 U/ml penicillin, 100 μg/ml streptomycin and 25 U/ml mycostatin. On the day of experiment, 2-mercaptoethanol was added to freshly prepared medium in a final concentration of $5x10^{-5}$ M. Cultures were incubated for six days at 37°C in a humidified atmosphere of 5% CO_2 in air. A control of spleen cells without stimulator MOPC-315 cells was set for each experimental group.

Antitumour cytotoxicity assays

In vitro sensitised spleen cells were tested for antitumour cytotoxicity in a ^{51}Cr release assay at an effectors: target ratio of 100:1 as described.[10] Briefly, $5x10^6$ MOPC-315 tumour cells were labelled with 100 μCI $Na^{51}CrO_4$ (Nuclear Research Center, Negev, Israel) at 37°C for 45 minutes, and washed three times in RPMI-medium. Mixtures of 0.4 ml of labelled target cells ($5x10^4$) and 0.5 ml of cultured spleen cells ($5x10^6$) were incubated in 15x75 mm plastic tubes (Falcon, U.S.A.) for three and a half hours at 37°C. At the end of the incubation period, cells were centrifuged at 250 x g for 10 minutes and both pellets and supernatants were counted in a gamma-counter (Packard, U.S.A.). Triplicate cultures were used for each experiment. The percentage of specific cytotoxicity was calculated using the following formula:

$$\text{specific } ^{51}Cr \text{ release} = \frac{T - C}{M - C} \times 100$$

Where: T = per cent of lysis with test spleen cells

C = per cent of lysis with control spleen cells (cultured without stimulator cells)

M = per cent maximal ^{51}Cr release obtained from labelled target cells disrupted by freezing and thawing.

Evaluation of treatment effect

The mean survival time (MST) of treated mice was compared with MST of untreated, inoculated mice. The effectiveness of immunoprophylaxis and of immunotherapy (with or without the addition of CY) was expressed as a per cent increase in life span (%ILS) calculated as follows:

Table 1 Effect of cyclophosphamide administration on the extent of antitumour protection induced by immunisation with glutaraldehyde-treated syngeneic MOPC-315 tumour cells

Group No.	Schedule of immunisation and inoculation	1st inoc TF/total	Challenge TF/total	MST	%ILS	P
1	0.08%—21d—0.02%—7d—1st inoc—41d—chall	26/81 (32%)	4/14 (28%)	36.1 ± 4.4	74.4	< 0.001*
2	0.08%—20d—CY—1d—0.02%—7d—1st inoc—41d—chall	20/82 (24%)	10/15 (66%)	50.4 ± 3.7	143.5	< 0.001* < 0.02**
3	——————————————————inoc		0/23 (0%)	20.7 ± 0.8		

All injections were given sc; immunisation: 5 x 10^5 glutaraldehyde-treated MOPC-315 cells per injection; 0.08% and 0.02% final concentration of glutaraldehyde used for treatment of cells; 1st inoculation: 1 x 10^4 viable MOPC-315 tumour cells/mouse; challenge and inoculation of group 3 (control): 1 x 10^5 viable MOPC-315 tumour cells per mouse; Cyclophosphamide (CY): 0.5 ml of 100 mg/kg intraperitoneally; d = days.

TF/total = tumour-free/total number of BALB/c mice.

MST = mean survival time ± SE.

%ILS = increase in life span by comparison with group 3.

*comparison with group 3.

** comparison between groups 1 and 2.

$$ILS = \frac{MST_t - MST_c}{MST_c} \times 100$$

Where: t = treated group
c = control (untreated inoculated) group

Mortality was recorded until the 60th day after inoculation. For calculations, an arbitrary survival time of 60 days was considered for mice remaining alive beyond the 60th day after inoculation. Statistical analysis was performed by using the two-tailed Mann-Whitney U test. Differences among the compared groups were considered significant when P was less than 0.05.

RESULTS

Effect of cyclophosphamide on immunoprophylaxis by modified tumour cells

Injections of glutaraldehyde-treated syngeneic MOPC-315 cells (GA-MOPC) induced resistance to inoculation in 32 per cent of the treated animals. Approximately a third of BALB/c mice immunised with GA-MOPC cells were also resistant to challenge. Administration of CY between the immunising injections of GA-MOPC had no effect on the degree of resistance to the first inoculation but resistance to challenge by the surviving mice was significantly higher than that of the immunised mice not treated with CY. The results are presented in Table 1. Figure 1 shows the kinetics of survival after inoculation and challenge.

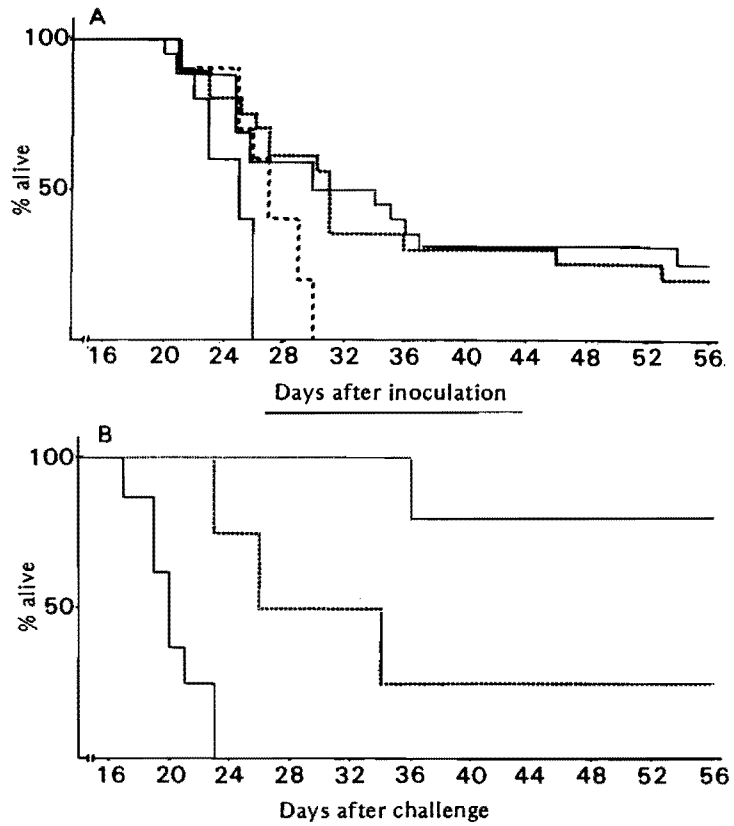

Fig. 1 Effect of cyclophosphamide on immunoprophylaxis by glutaraldehyde-treated MOPC-315 tumour cells.
5 x 10^5 GA-MOPC cells per immunising sc injection; cyclophosphamide (100 mg/kg) in 0.5 ml ip; sc inoculation of 1 x 10^4 viable tumour cells; challenge of 1 x 10^5 tumour cells 55 days after the first inoculation.

A. Immunisation schedule (injection of MOPC-315 tumour cells treated with 0.08%, 0.04% or 0.02% GA and inoculation)
- •••• 0.08% 14d 0.04% 7d 0.02% 7d inoculation
- ••••••• 0.08% 14d 0.04% 6d CY 1d 0.02% 7d inoculation
- - - untreated 20d CY 8d inoculation
- ——— untreated ——— inoculation

B. Challenge (1 x 10^5 viable tumour cells sc)
- •••• surviving mice of immunised group
- ••••••• surviving mice of immunised and CY-treated group
- ——— untreated mice

Effect of combined chemotherapy and immunotherapy

Mice inoculated with MOPC-315 tumour cells were given chemotherapy with CY and immunotherapy with GA-MOPC cells (Table 2). Treatment with CY was performed by a single injection on days 4, 8 or 13 after inoculation. Injection of CY on day 8 and especially on day 4 after inoculation cured approximately 60 per cent of the mice but the majority of cured mice succumbed after challenge. The addition of immunotherapy to CY-chemotherapy (day 4 or day 8) did not increase the extent of resistance to challenge of the surviving mice. The resistance to challenge of treated mice surviving after the first inoculation was higher in groups subjected to combined chemotherapy with CY on day 13 after inoculation and immunotherapy in comparison with mice treated with CY alone. As shown in Table 3, The *in vitro* antitumour cytotoxic response to MOPC-315 tumour cells of spleen cells harvested after the first challenge and subjected to combined chemotherapy and immunotherapy was much higher than that of spleens from mice treated with CY alone. A similar result was obtained when spleen cells were harvested from mice after the second challenge (Table 4): *In vitro* antitumour cytotoxic response was much higher in spleens of mice

Table 2 Curative effect of combined cyclophosphamide chemotherapy and immunotherapy with glutaraldehyde-treated tumour cells in BALB/c mice inoculated with MOPC-315 tumour cells

Exp. No.	Group No.	Schedule of inoculation, chemotherapy and immunotherapy	TF/total After therapy	TF/total After challenge*
I	1	1st inoc—13d—CY—7d—0.08%—7d—0.04%—7d—0.02%—7d—0.01%—21d—chall	26/50 (52%)	14/18 (78%)
	2	1st inoc—13d—CY—49d—chall	37/48 (77%)	15/26 (58%)
	3	inoc		0/20 (0%)
II	1	1st inoc—8d—CY—9d—0.04%—7d—0.02%—7d—0.01%—7d—chall	12/20 (60%)	3/12 (25%)
	2	1st inoc—8d—CY—30d—chall	11/20 (55%)	4/11 (36%)
	3	inoc		1/10 (10%)
III	1	1st inoc—4d—CY—13d—0.04%—7d—0.02%—7d—0.01%—7d—chall	20/20 (100%)	0/20 (0%)
	2	1st inoc—4d—CY—chall	13/20 (65%)	1/13 (8%)
	3	inoc		1/10 (10%)

See footnotes to Table 1 for details and abbreviations

1st inoculation 1 x 10^6 viable MOPC-315 tumour cells sc, in all experiments; challenge and inoculation of control group: 1 x 10^6 viable MOPC-315 tumour cells sc in experiment I and 2 x 10^4 viable MOPC-315 tumour cells in experiments II and III; immunotherapy by injections of 1 x 10^6 GA-MOPC cells sc; CY: 200 mg/kg.

*Difference in TF/total after challenge between groups 1 and 2 in experiment I was found to be significant ($P < 0.002$); between groups 1 and 2 in experiments II and III were not significant.

Table 3 *In vitro* antitumour cytotoxic response to MOPC-315 tumour cells of spleen cells harvested after challenge from mice subjected to chemotherapy and immunotherapy

Origin of spleen cells	Schedule of chemotherapy and immunotherapy*	^{51}Cr specific release at effector/target ratio of 100/1**
Exp. I, Gr. 1	CY-GAMOPC	35.1 ± 6.2
Exp. I, Gr. 2	CY	16.6 ± 6.5
Exp. I, Gr. 3	None; inoculated	2.3 ± 0.8
Normal***	None	1.3 ± 1.3
Exp. II, Gr. 1	CY-GAMOPC	39.0 ± 0.8
Exp. II, Gr. 2	CY	21.9 ± 1.3
Exp. II, Gr. 3	None; inoculated	3.0 ± 1.3
Normal	None	4.1 ± 0.4

Spleen cells were harvested from BALB/c mice resistant to challenge, from mice inoculated, non-treated and bearing large tumours (app. 20 mm in diameter) and from normal, noninoculated mice (on day 26 after challenge or after first inoculation (group 3) from groups of mice mentioned in Table 2).

*See Table 2 for details on inoculations and therapies with cyclophosphamide and with glutaraldehyde-treated MOPC-315 tumour cells (GAMOPC).

** 5 x 10^6 effector spleen cells/5 x 10^4 target ^{51}Cr-labelled MOPC-315 cells.

***Noninoculated, untreated mice.

treated by combined chemotherapy and immunotherapy after the first inoculation than in spleens of mice treated with CY alone.

DISCUSSION

The main finding of the present work is that, under certain conditions, the administration of cyclophospamide in addition to the injection of modified tumour cells improved their immunoprophylactic and immunotherapeutic effectiveness.

We[1] have previously reported that immunisation with glutaraldehyde-treated syngeneic MOPC-315 cells induced partial protection against a tumourigeneic dose inoculated subcutaneously. We now show that the administration of CY before the last immunising injection did not affect the degree of resistance to the first inoculation but increased significantly the resistance to challenge in the surviving mice. This "delayed" effect of CY may be related to the well-known selective effect of this drug on suppressor T-cell populations.[3-6] It might be that the selective removal of suppressor T cells by CY in our system increases the ability of GA-treated tumour cells and of the inoculum itself to stimulate specific effector cells involved in antitumour protection to challenge.

Chemotherapy by a single injection of CY performed on days 4, 8 or 13 after inoculation cured approximately 60 per cent of the treated mice. However, a marked difference was observed between the CY treated groups in their degree of resistance to challenge of cured mice. The resistance to challenge was much higher in mice treated with CY on the 13th day after inoculation as compared with mice treated with CY on day 8 and especially on day 4 after inocula-

Table 4 *In vitro* antitumour cytotoxicity and resistance to second challenge of BALB/c mice subjected to chemotherapy and immunotherapy

Group No.	Schedule of inoculation, chemotherapy and immunotherapy	TF/total after 1st inoc	TF/total after 1st chall	TF/total after 2nd chall	% specific ^{51}Cr release
1.	1st inoc—13d—CY—9d—0.04%—7d—1st chall—40d—2nd chall	16/21 (80%)	4/16 (25%)	3/4 (75%)	50.7 ± 2.9
2.	1st inoc—13d—CY—16d—1st chall—40d—2nd chall	13/19 (68%)	5/13 (38%)	3/5 (60%)	9.4 ± 3.7
3.	——————————inoc			1/5 (0%)	

See footnotes to Tables 1, 2 and 3 for details

1st inoculation: $1x10^6$ viable MOPC-315 tumour cell; 1st challenge: $2x10^4$ viable MOPC-315 tumour cells; 2nd challenge and inoculation in contol untreated group: $1x10^5$ viable tumour cells; spleen cells harvested on day 49 after 2nd challenge or inoculation in the control group. CY: 200 mg/kg. % specific ^{51}Cr release at an effector/target cell ratio of 100/1.

tion. This finding is in line with reports[11-13] showing that the injection of CY in mice bearing a large subcutaneous MOPC-315 tumour (day 12 after inoculation) is more effective and leads to the development of antitumour immune response compared with the injection of CY on day 4 after inoculation.

A certain discrepancy has to be noted between the data reported[11-13] and our data concerning the effect of therapy with a high dose of 200 mg/kg CY of mice bearing large MOPC-315 tumours: in our hands (unpublished observations), 60 per cent of mice cured by 200 mg/kg CY developed strong antitumour immunity (were resistant to challenge with a tumourigenic dose of MOPC-315), whereas in previously reported data,[11-13] mice cured by the high dose of CY had little antitumour immunity as shown by their susceptibility to challenge. Therefore, it is possible that a dose of CY which was shown to be highly immunosuppressive,[11-13] allowed still in our hands development of antitumour immunity and this may account for the beneficial effect of CY on immunoprophylaxis and immunotherapy in the present experiments. The difference in the response of mice bearing large MOPC-315 tumours to high dose of CY between previously reported data[11-13] and ours may be due to the fact that substrains of BALB/c mice vary in their susceptibility to CY and to MOPC-315 tumour and/or to variation in the properties of MOPC-315 tumour line used by us and that used by other workers.[11-13] This point is now under investigation.

The difference in the effect of CY in relation to the time of chemotherapy was also expressed in groups subjected to combined chemotherapy with CY and immunotherapy with GA-MOPC-treated cells. Thus, the combination of chemotherapy and immunotherapy was more effective (as expressed by resistance to challenge) when CY was given on day 13 after inoculation than when CY was given at an earlier time after inoculation. The advantage of combined chemotherapy and immunotherapy over chemotherapy alone was also shown by the finding that *in vitro* antitumour cytotoxic response to MOPC-tumour cells was higher in the spleens of mice subjected to CY and GA-MOPC treatment than in the spleens of mice subjected to CY treatment alone. The synergistic effect between chemotherapy and immunotherapy in the development of antitumour protection and the cure of treated mice was described in several systems.[14-17] Our results show that under certain conditions, a synergistic effect between CY treatment and specific immunisation can be detected in the MOPC-plasmacytoma system.

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