Production and Enhanced Anti-tumor Activity of Tumor Necrosis Factor in Mice Treated with Cyclophosphamide

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We demonstrated recently that the production of tumor necrosis factor (TNF) is induced in normal mice and in the immunosuppressed nude mouse model by the administration of muramyl dipeptide (MDP) derivatives followed by endotoxin (lipopolysaccharide). In the present study, the ability of this treatment to induce the production of TNF in mice receiving cyclophosphamide (CY) was examined. Two days following treatment with high-dose CY (250 mg/kg), mice exhibited leukocytopenia and drastically reduced splenic weight. However, these animals remained capable of producing TNF, albeit at lower levels, when treated with MDP derivatives and lipopolysaccharide (LPS), particularly when the lipophilic analogue MDP-dipalmitoyl glycerol (GDP) was utilized. TNF was also induced by the administration of MDP-GDP and LPS to Meth A sarcoma-bearing mice treated with this dose of CY. Furthermore, in all animals receiving this combination therapy, sarcoma necrosis and complete regression were obtained without any sign of tumor regrowth. A dose of 100 mg/kg CY was not effective for inhibiting tumor regrowth under the same experimental conditions. These results demonstrated that the anti-tumor activity of endogenously induced TNF is potentiated by combined therapy with a high dose of CY.

Key words: Anti-tumor activity — Tumor necrosis factor — Cyclophosphamide — Mouse

It has been well established by many investigators that sera containing tumor-necrotizing activity is obtained after administration of BCG followed by endotoxin.[1] Tumor necrosis factor (TNF),[2] a cytokine produced and elaborated by activated macrophages, has been shown to be responsible for such anti-tumor effects.[2-6] We have reported that pretreatment of mice with synthetic muramyl dipeptide (MDP) derivatives several hours before administration of lipopolysaccharide (LPS)-containing preparations resulted in enhanced production of circulating TNF.[7, 8] Several studies have reported the inhibition of normal immune responsiveness in advanced cancer patients and patients treated simultaneously with several chemotherapies.[9] The possibility exists that endogenously induced TNF production in such patients may contribute to the inhibition of tumor growth and the establishment of regression. Potentiation of the cytotoxic effects of TNF *in vitro* and *in vivo* in combination with established chemotherapeutic agents has been reported.[10] In this type of therapy, however, there are limitations to the extensive usage of exogenously administered TNF due to its toxicity. Therefore, inducing the endogenous production of this anti-tumor cytokine seems preferable to the administration of exogenous TNF.

In this study, we present evidence that mice treated with MDP derivatives in combination with CY therapy remain capable of producing TNF following the administration of endotoxin. In addition, these studies demonstrate a synergistic anti-tumor effect of endogenously produced TNF and cyclophosphamide.

MATERIALS AND METHODS

Mice Male Swiss mice (3 to 6 months old) and male BALB/c *nu/+* mice (6 to 8 weeks old) were obtained from Harlan Laboratories (Madison, WI). They were maintained on tap water and commercial animal food *ad libitum*. Male BALB/c *nu/nu* nude mice (6 to 8 weeks old) were obtained from Life Sciences (St. Petersburg, FL) and were maintained on sterilized water and animal food *ad libitum*. All animals were specific pathogen-free.

Materials Cyclophosphamide (CY) was purchased from Sigma Chemical Co. (St. Louis, MO). CY was dissolved in and diluted with sterile 0.9% saline solution. MDP (acetyl-muramyl-L-Ala-D-isoGln) was kindly provided by Institute Choay, Paris. MDP-GDP (MDP-1,2-dipalmitoyl-*sn*-glycerol) was prepared by P. Lefrancier and M. Level (Institute Choay). LPS from *Salmonella enteritidis* was obtained from Difco (Detroit, MI).

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[2] The abbreviations used are: TNF, tumor necrosis factor; MDP, muramyl dipeptide; MDP-GDP, MDP-dipalmitoyl glycerol; LPS, lipopolysaccharide; CY, cyclophosphamide; BCG, *Mycobacterium bovis*, strain bacillus Calmette-Guerin.

Target tumor cell line Mycoplasma-free WEHI-164 murine fibrosarcoma cell line was obtained from Dr. J. Djeu (University of South Florida, College of Medicine, Tampa, FL). The cells were maintained in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% FBS (Hyclone, Logan, UT), 1.0 ml L-glutamine (Gibco Laboratories), 25 mM Hepes buffer (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid, Sigma) and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). **TNF assay** WEHI-164 cells (5×10^3) from a 3-day culture were seeded in a total volume of 0.1 ml/well into flat-bottomed microtiter plates (Falcon, Oxnard, CA) containing serial two-fold dilutions (between 200- and 2,560,000-fold) of the test sample. The plates were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 after which 1 μCi of ^{3}H-thymidine was added to each well. Plates were incubated for an additional 18 h and cells were harvested with a multiple cell harvester (Skatron, Flow Laboratories, Helsinki). The incorporation of ^{3}H-thymidine was determined by liquid scintillation spectrometry. The percentage of cytostasis was evaluated by using the formula:

$$\% \text{ of growth inhibition} = \frac{\text{cpm control} - \text{cpm experimental}}{\text{cpm control}} \times 100.$$

Cytostatic units were calculated by plotting the regression lines of the log reciprocal dilution of tested sample versus the percentage of growth inhibition. One unit is equal to the reciprocal of the dilution that caused a 50% inhibition of ^{3}H-thymidine incorporation. By using this assay, we obtained a specific activity of 0.8 to 1.0×10^7

U/mg with recombinant human TNF (Biogen and BASF, Geneva), comparable to that obtained with actinomycin D-treated L929 cells. Additional assays have shown that the cytostatic effect against WEHI-164 cells present in serum of mice treated either with BCG and LPS or with MDP and LPS was inhibited by rabbit antibodies (dilution of 1:5000) raised against murine recombinant TNF (generously provided by Dr. W. Fiers and Dr. J. Tavernier, University of Ghent, Belgium). Non-immune rabbit serum used at the same dilution did not affect the cytostatic effect of mouse serum TNF.

In vivo TNF production MDP or MDP-GDP (300 μg/mouse) was administered intravenously to mice. Five hours later, mice were challenged with LPS (6.0 μg/mouse) and were bled from the orbital plexus 2 h later. Sera were stored at $-20°C$ until assayed for the presence of TNF. For the induction of TNF in CY-treated mice, the animals were treated with 250 mg/kg of CY intraperitoneally 2 days prior to injection of MDP or MDP-GDP.

In vivo tumor assay Meth A (methylcholanthrene-induced) fibrosarcoma was maintained as ascites by serial passage in BALB/c mice. For these experiments, 10^6 tumor cells (in a volume of 0.05 ml) were injected intradermally into the depilated flank of BALB/c mice. After 2 weeks, tumor-bearing mice (diameter of tumor mass approximately 8–10 mm) were treated intravenously and tumor necrosis was evaluated after 48 h as described.[7] Tumor regression was evaluated after 20 days. For the determination of the anti-tumor activity in mice receiving CY therapy combined with treatment to produce endogenous TNF, tumor-bearing mice (diame-

Table I. Changes in Circulating Leukocytes and Spleen Weight

| Pre-treatment | TNF induction | | Circulating | Spleen |
| | Priming | Eliciting | leukocytes (mm³) | wt. (mg) |
−48 h	0 h	+5 h	+7 h	+7 h
CY (−)	Saline	Saline	8100±2100	78.8±4.7
CY (+, ip)	Saline	Saline	1400±700	40.1±5.4
CY (+, iv)	Saline	Saline	1700±450	38.8±7.9
CY (−)	MDP-GDP	LPS	2100±150	110.3±13.9
CY (−)	MDP	LPS	6400±530	118.9±34.2
CY (+, ip)	MDP-GDP	LPS	450±190	69.5±25.6
CY (+, ip)	MDP	LPS	660±340	67.2±11.5**
CY (+, iv)	MDP-GDP	LPS	600±360	61.2±18.0
CY (+, iv)	MDP	LPS	780±270	55.7±10.9*

Swiss mice received 300 μg of priming agent intravenously, and after 5 h the animals received 6 μg of LPS. In animals treated with cyclophosphamide, 250 mg/kg was administered intraperitoneally or intravenously 48 h prior to injection of the priming agent.
* $P < 0.05$ [CY (+, iv) and saline vs. CY (+, iv), MDP and LPS].
** $P < 0.01$ [CY (+, ip) and saline vs. CY (+, ip), MDP and LPS].

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ter of tumor mass approximately 12–15 mm) were injected with 250 mg/kg of CY intraperitoneally followed by 300 μg of MDP-GDP and 6.0 μg of LPS administered intravenously. Tumor size was estimated twice weekly from caliper measurements of width and length of tumors in millimeters; data were expressed as relative tumor weight (mean tumor weight at given time/initial mean tumor weight).

Table II. TNF Production in Mice Treated with Cyclophosphamide

Pre-treatment	TNF induction		TNF level
−48 h	Priming 0 h	Eliciting +5 h	
CY (−)	MDP-GDP	LPS	20,011
CY (−)	MDP	LPS	9,403
CY (+, ip)	MDP-GDP	LPS	8,429
CY (+, ip)	MDP	LPS	5,129
CY (+, iv)	MDP-GDP	LPS	7,414
CY (+, iv)	MDP	LPS	2,045

Swiss mice received 300 μg of priming agent iv, and after 5 h the animals received 6 μg of LPS. Animals were bled 2 h after administration of LPS. TNF titer was expressed as serum dilution (fold) giving 50% cytotoxicity. Control animals which received saline alone or 300 μg of priming agent had a value of less than 200 for TNF activity. In animals treated with cyclophosphamide, 250 mg/kg was administered iv or ip 48 h prior to injection of the priming agent.

RESULTS

Alterations in circulating leukocytes and splenic weight induced by CY When mice were injected with CY ip or iv, significant decreases in the numbers of circulating leukocytes and splenic weights were observed (Table I). However, there was no difference in leukocyte counts or splenic weights between intraperitoneal versus intravenous injection. Injection of CY in mice pretreated with MDP or MDP-GDP and LPS to induce endogenous TNF production resulted in further decreases in the

Table III. TNF production in Meth A Tumor-bearing Mice

Pre-treatment	TNF induction		TNF level
−48 h	Priming 0 h	Eliciting +5 h	
CY (−)	Saline	Saline	<200
CY (+, ip)	Saline	Saline	<200
CY (−)	MDP-GDP	LPS	2,392
CY (+, ip)	MDP-GDP	LPS	1,420

BALB/c mice having intradermal Meth A implants (tumor mass diameter 12–15 mm) received 250 mg/kg of cyclophosphamide ip and 300 μg of priming agent iv. After 5 h, the animals received 6 μg of LPS. Animals were bled 2 h after administration of LPS. TNF titer was expressed as serum dilution (fold) giving 50% cytotoxicity.

Table IV. Tumor Necrosis and Anti-tumor Effect of Combination Therapy with Cyclophosphamide in BALB/c Mice

Treatment	Sarcoma necrosis score				Complete tumor regression (No./total) (%)
	+++	++	+	−	
CY (100 mg/kg)	0	0	0	7	1/7 (14.3)
CY (250 mg/kg)	0	0	0	8	1/8 (12.5)
MDP-GDP+LPS	6	1	0	0	2/7 (28.6)
CY+MDP-GDP+LPS (100 mg/kg)	7	0	1	0	5/8 (62.5)*
CY+MDP-GDP+LPS (250 mg/kg)	6	0	0	0	6/6 (100)**

BALB/c mice having intradermal Meth A implants (tumor mass diameter 12–15 mm) were injected intraperitoneally with cyclophosphamide (100 or 250 mg/kg). On the same day, in animals receiving MDP-GDP, 300 μg was injected iv 5 h before administration of 6 μg of LPS. Sarcoma necrosis was evaluated 48 h after injection of LPS. Grading of the necrotic changes of Meth A tumor is as followed; (−), no necrotic change; (+), necrotic change is observed in less than 50% of the tumor; (++), necrotic change is observed more than 50% but less than 75% of the tumor; (+++), necrotic change is observed more than 75% of the tumor. Complete tumor regression was evaluated 20 days after these treatments.
* $P<0.05$ (CY 100 mg/kg vs. CY 100 mg/kg+MDP-GDP+LPS).
** $P<0.01$ (CY 250 mg/kg vs. CY 250 mg/kg+MDP-GDP+LPS).

numbers of circulating leukocytes, relative to that of animals receiving either CY alone or MDP derivatives and LPS.

TNF production in mice treated with cyclophosphamide Table II demonstrates that mice which received CY retained their capacity to produce TNF, although the levels induced were less than that of normal mice. The lipophilic derivative, MDP-GDP, was a more effective priming agent for TNF induction in both normal and treated animals. There was little dependence of TNF production on CY administration routes.

TNF production in Meth A tumor-bearing mice Administration of MDP-GDP and LPS to tumor-bearing mice resulted in the production of TNF (Table III) although the levels of the cytokine induced in these mice were significantly lower than that present in non tumor-bearing animals under the same conditions (Table II). The decrease in TNF production in tumor-bearing BALB/c mice was not related to differences in the strain

of mouse, since injection of MDP-GDP and LPS induces approximately the same level of TNF in BALB/c and Swiss mice (data not shown). This treatment also induced TNF production in Meth A sarcoma-bearing mice which were treated concomitantly with CY. Two days following administration of MDP-GDP and LPS, remarkable tumor necrosis was observed (Table IV). It is well established that Meth A fibrosarcoma is highly sensitive to TNF.[11] Moreover, regression of Meth A tumor is easily observed at a tumor size of 8 to 10 mm. In this study, however, therapy was initiated when the tumor diameter reached 12–15 mm. As shown in Table IV, CY treatment alone at either dose did not induce sarcoma necrosis or regression following administration of this treatment in the absence of CY therapy. In the animals
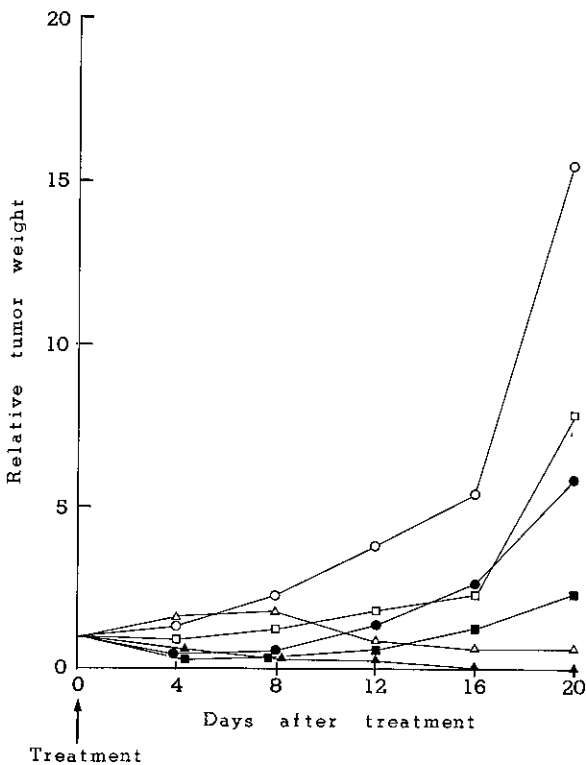

Fig. 1. Tumor growth curves of Meth A sarcoma in BALB/c mice after cyclophosphamide endogenous TNF treatment. Curves represent the mean of the volumes of 6 to 8 tumors per treatment group. (○), Control; (△), CY 250 mg/kg; (□), CY 100 mg/kg; (●), MDP-GDP (300 μg/mouse) +LPS (6 μg/mouse); (▲), CY (250 mg/kg)+ MDP-GDP (300 μg/mouse) +LPS (6 μg/mouse); (■), CY (100 mg/kg)+MDP-GDP (300 μg/mouse) +LPS (6 μg/mouse).
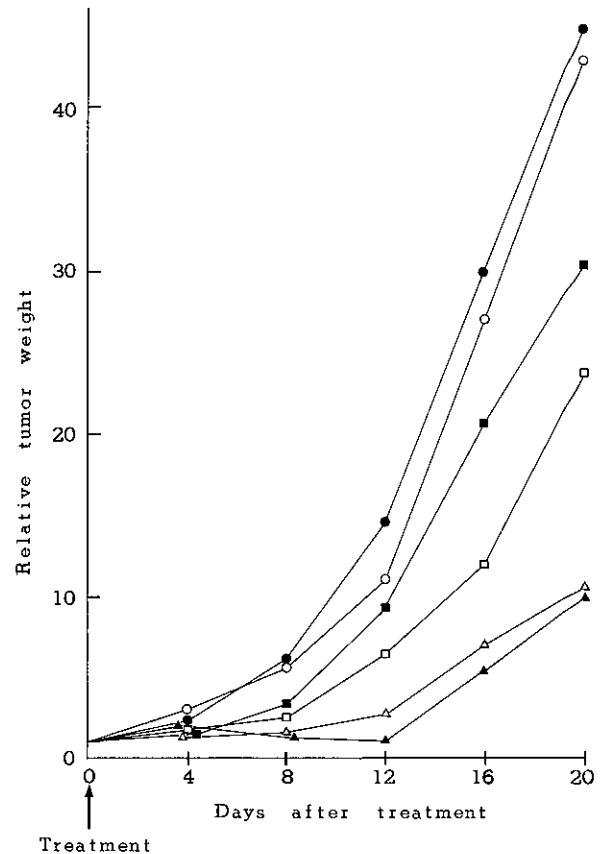

Fig. 2. Tumor growth curves of Meth A on BALB/c nu/nu nude mice after cyclophosphamide and endogenous TNF treatment. Curves represent the mean of the volumes of 5 to 12 tumors per treatment group. (○), Control; (△), CY 250 mg/kg; (□), CY 100 mg/kg; (●), MDP-GDP (300 μg/mouse)+ LPS (6 μg/mouse); (▲), CY (250 mg/kg) +MDP-GDP (300 μg/mouse)+LPS (6 μg/mouse); (■), CY (100 mg/kg)+ MDP-GDP (300 μg/mouse) +LPS (6 μg/mouse).

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Table V. Tumor Necrosis and Anti-tumor Effect of Combination Therapy with Cyclophosphamide in BALB/c *nu/nu* Nude Mice

Treatment	Sarcoma necrosis score				Complete tumor regression (No./total)
	+++	++	+	−	
CY (100 mg/kg)	0	0	0	6	0/6
CY (250 mg/kg)	0	0	0	5	0/5
CY + MDP-GDP (250 mg/kg)	0	0	0	6	0/6
MDP-GDP + LPS	1	2	4	5	0/12
CY + MDP-GDP + LPS (100 mg/kg)	0	2	2	1	0/6
CY + MDP-GDP + LPS (250 mg/kg)	0	0	0	6	0/6

BALB/c *nu/nu* nude mice having intradermal Meth A implants (tumor mass diameter 12–15 mm) were injected intraperitoneally with cyclophosphamide (100 or 250 mg/kg). On the same day, in animals receiving MDP-GDP, 300 μg was injected iv 5 h before administration of 6 μg of LPS. Sarcoma necrosis was evaluated 48 h after injection of LPS. Complete tumor regression was evaluated 20 days after these treatments. Grading of necrotic change is described in Table IV.

treated with 250 mg/kg CY, all tumors exhibited necrosis and showed complete regression after treatment with MDP-GDP and LPS, and no tumor regrowth occurred. Combination therapy with 100 mg/kg CY was less effective for producing complete regression.

Effect of CY dose on growth of Meth A sarcoma in normal versus BALB/c *nu/nu* nude mice We investigated the anti-tumor effect of combination therapy of MDP-GDP and LPS with either low- or high-dose CY treatment. In sarcoma-bearing BALB/c mice, administration of 250 mg/kg CY resulted in the stasis of tumor growth but these animals failed to achieve complete regression (Fig. 1). Mice receiving 100 mg/kg CY exhibited an inhibition of tumor growth initially after treatment but developed a four-fold increase in tumor size by day 20, whereas tumor regrowth was observed within 12 days after treatment of animals with only MDP-GDP and LPS. However, administration of high-dose CY combined with MDP-GDP and LPS resulted in complete regression by day 16 with no tumor regrowth. Mice which received combination therapy with low-dose CY exhibited increases in tumor size within 16 days following treatment.

Studies performed in nude mice demonstrated that, relative to normal animals, administration of MDP-GDP and LPS combined with either dose of CY was not effective for the long-term inhibition of tumor growth (Fig. 2), although limited sarcoma necrosis did occur in animals receiving MDP-GDP and LPS alone or together with 100 mg/kg CY (Table V). Even though initial sarcoma growth was suppressed during 8 to 12 days

following experimental treatments, significant regrowth had occurred by day 16.

DISCUSSION

We have previously demonstrated that following priming with MDP and particularly its lipophilic analogue, MDP-GDP, TNF production is elicited in normal or athymic mice by the administration of endotoxin-containing preparations.[7, 8] The present data demonstrate that mice exposed to CY remain capable of producing TNF in response to MDP-GDP and LPS, although the levels produced are lower than those in normal mice. Similarly, tumor-bearing mice exhibit lower levels of TNF, but these animals also retain their capacity for production of the cytokine following CY treatment. In Meth A-bearing normal mice, therapy with MDP-GDP and LPS combined with 250 mg/kg CY resulted in necrosis and complete regression with no regrowth. The same conditions of combination therapy with the lower dose of CY resulted in a high level of necrosis but was less effective for producing complete regression. Animals which received MDP-GDP and LPS also exhibited tumor necrosis with incomplete regression. Although administration of high-dose CY resulted in the stasis of tumor growth, this dose given alone did not induced complete regression.

In contrast to the results obtained in tumor-bearing mice, nude mice having Meth A sarcoma exhibited reduced necrosis and failed to achieve complete tumor regression following exposure to CY with MDP-GDP

and LPS. Although therapy with high-dose CY administered alone or together with inducers of TNF initially caused stasis of tumor growth, this effect was followed by rapid sarcoma regrowth. Our data demonstrated that the TNF level in circulating blood is closely related to tumor hemorrhagic change. However, hemorrhagic change did not lead to complete tumor regression. It seemed that another treatment is required to obtain complete tumor regression after partial hemorrhagic change.

In early studies utilizing a murine model of lymphoma growth, we showed that MDP and its stereoisomer MDP (DD) were as effective as CY for inhibiting ascitic growth of thymoma and plasmacytoma cell lines.[12] We also demonstrated that treatment with MDP significantly increased nonspecific resistance to *Klebsiella* infection in BALB/c mice immunosuppressed by exposure to CY.[13] Subsequent investigation revealed that in addition to its anti-tumor effects, TNF is a mediator of MDP-induced enhancement of resistance to microbial infection.[14, 15] Although TNF is produced by mononuclear phagocytes, the role of additional immune cells, in particular the involvement of T lymphocyte cooperation, is not clear. Early studies demonstrated that the administration of endotoxin resulted in necrosis in sarcoma-bearing adult mice which had been made T cell-deficient by thymectomy and irradiation.[16] Congenitally athymic (nude) mice were also shown to possess serum levels of anti-tumor cytotoxic factors after administration of BCG followed by LPS.[17] Watanabe *et al.*[18] demonstrated that TNF production was induced in nude mice infected with *Propionibacterium acnes,* although the levels were lower than those present in normal mice after injection of *P. acnes.* However, we have reported that a larger yield of TNF activity were produced even in nude mice than BALB/c *nu/+* mice treated with MDP-GDP and *Bordetella pertussis* vaccine.[8] More recently, Regenass and co-workers[10] have shown that CY treatment enhanced the anti-tumor activity of exogenously administered recombinant TNF in sarcoma-bearing BALB/c

mice but not in nude mice. In contrast, treatment with either agent alone had no significant effect on tumor necrosis or regression. Our studies confirm and extend these observations by demonstrating that combination therapy with CY potentiates the anti-tumor effect of endogenously induced TNF in tumor-bearing BALB/c mice.

The mechanism by which TNF exerts its anti-tumor activity has been attributed to several factors including direct binding to tumor cells via specific receptors[19-21] as well as producing alterations of vascular endothelium by inducing procoagulant activity[22-25] together with capillary toxicity,[26] leading to diminished tissue perfusion. In contrast, the antineoplastic effects of CY are associated with the selective down-regulation of suppressor mechanisms, resulting in enhanced immune responsiveness.[27] The ability of CY to yield complete tumor regression in BALB/c mice may therefore be related to the elimination of some suppressor mechanism(s) which negatively regulates TNF-induced effects. However, CY treatment in combination with endogenous TNF induction in nude mice did not result in enhanced anti-tumor activity. This effect is most likely due to the absence of functional T cells in these mice, since studies have shown that a late-stage influx of T cells may be a requirement for complete tumor regression to occur.[16]

The clinical usage of recombinant TNF has been limited by its high level of toxicity. We have shown that TNF is endogenously produced by the administration of nontoxic MDP derivatives together with either the conventional typhoid or pertussis vaccine preparations.[8] These results together with the present findings suggest that more acceptable clinical models may be feasible by combining endogenous TNF production with antineoplastic agents such as CY.

ACKNOWLEDGMENTS

REFERENCES

1) Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fione, N. and Williamson, B.   An endotoxin induced serum factor that causes necrosis of tumor. *Proc. Natl. Acad. Sci. USA,* **72,** 3666–3675 (1975).

2) Beutler, B., Greenworld, D., Humes, J. L., Change, M., Par, Y. C. E., Nathison, J., Ulevitch, R. and Cerami, A. Identity of tumor necrosis factor and the macrophage secreted factor cachectin. *Nature,* **316,** 552–554 (1985).

3) Matthews, N. Tumor necrosis factor from the rabbit. II. Production by monocytes. *Br. J. Cancer,* **38,** 310–315

(1978).

4) Mannel, D. N., Moore, R. N. and Mergenhagen, S. L. Macrophages as a source of tumoricidal activity (tumor necrotizing factor). *Infect. Immun.,* **30,** 523–530 (1980).

5) Beutler, B. and Cerami, A. Cachectin: more than a tumor necrosis factor. *N. Engl. J. Med.,* **316,** 376–385 (1987).

6) Beutler, B. and Cerami, A. Cachectin and tumor necrosis factor as two sides of the same biological coin. *Nature,* **320,** 584–588 (1987).

7) Parant, M., Parant, F., Vinit, J. C., Noso, Y. and Chedid,

L. Priming effect of muramyl peptides for induction by lipopolysaccharide of tumor necrosis factor production in mice. *Cancer Res.*, in press.

8) Noso, Y., Parant, M., Parant, F. and Chedid, L. Production of tumor necrosis factor in nude mice by muramyl peptides associated with bacterial vaccines. *Cancer Res.*, **48**, 5766–5769 (1988).

9) Hersh, E. M., Whitecar, J. P., McCredie, K. B., Bodey, G. P. and Freireich, E. J. Chemotherapy, immunocompetence, immunosuppression and prognosis in acute leukemia. *N. Eng. J. Med.*, **285**, 1211–1221 (1971).

10) Regenass, U., Muller, M., Curschellas, E. and Matter, A. Anti-tumor effects of tumor necrosis factor in combination with chemotherapeutic agents. *Int. J. Cancer*, **39**, 266–273 (1987).

11) Haranaka, K., Satomi, N. and Sakurai, A. Antitumor activity of murine tumor necrosis factor (TNF) against transplanted murine tumors and heterotransplanted human tumor in nude mice. *Int. J. Cancer*, **34**, 263–267 (1984).

12) Philips, N. C., Paraf, A., Bahr, G. M. and Chedid, L. Modulation of murine lymphoma growth by MDP, MDP (D-D) and cyclophosphamide. I. Inhibition of growth *in vivo. Int. J. Immunopharmacol.*, **5**, 219–227 (1983).

13) Parant, M. Biologic properties of a new synthetic adjuvant, muramyl dipeptide (MDP). *Springer Semin. Immunopathol.*, **2**, 101–118 (1979).

14) Chedid, L., Parant, M. Parant, F. and Riveau, G. Nonspecific effects of LPS or bacterial infections. *In* "The Immunobiology and Immunopharmacology of Bacterial Endotoxins," ed. A. Szentivanyi and H. Friedman, pp. 201–213 (1986). Plenum Press, New York.

15) Parant, M., Gatelli, A., Parant, F. and Chedid, L. Role of B lymphocytes in nonspecific resistance to *Klebsiella pneumoniae* infection of endotoxin treated mice. *J. Infect. Dis.*, **134**, 531–539 (1976).

16) Berendt, M. J., North, R. J. and Kirsten, D. P. The immunological basis of endotoxin induced tumor regression. Requirement for T-cell mediated immunity. *J. Exp. Med.*, **148**, 1550–1559 (1978).

17) Mannel, D. N., Metzler, M. S. and Mergenhagen, S. E. Generation and characterization of a lipopolysaccharide induced and serum derived cytotoxic factor for tumor cells. *Infect. Immun.*, **28**, 204–211 (1980).

18) Watanabe, N., Sone, H., Neda, H., Niitsu, Y. and Urushizaki, I. Mechanisms of production of tumor necrosis factor (TNF). Reconstitution experiment with nude mice. *Jpn. J. Cancer Chemother.*, **11**, 1284–1289 (1984).

19) Rull, F. C., Jr., Jacobs, S., Jr. and Cuatrecasa, O. Cellular receptor for ^{125}I-labeled tumor necrosis factor specific binding, affinity labeling and relationship to sensitivity. *Proc. Natl. Acad. Sci. USA*, **82**, 5756–5760 (1985).

20) Tsujimoto, M., Yip, Y. K. and Vilcek, J. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. *Proc. Natl. Acad. Sci. USA*, **82**, 7626–7630 (1985).

21) Tsujimoto, M., Feinman, R. and Vilcek, J. Differential effects of type I INF and INF gamma on the binding of tumor necrosis factor to receptor in two human cell lines. *J. Immunol.*, **137**, 2272–2276 (1986).

22) Gamble, J. R., Harlan, J. M., Klebanoff, S. J. and Vadas, M. A. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA*, **82**, 8667–8671 (1985).

23) Taracey, K. J., Beutler, B., Tavry, S. F., Merryweather, J., Wolpe, S., Milsark, I. W., Hariri, R. J., Fahey, T. J., III, Zentella, A., Albert, J. D., Shires, G. T. and Cerami, A. Shock and tissue injury induced by recombinant human cachectin. *Science*, **234**, 470–474 (1986).

24) Nowroth, P. P. and Stern, D. M. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J. Exp. Med.*, **164**, 740–745 (1986).

25) Bevliacqua, M. P., Pober, J. S., Majeau, G. R., Fiers, W., Cotran, R. S. and Gimbrone, M. A. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison to activity of interleukin I. *Proc. Natl. Acad. Sci. USA*, **83**, 4533–4537 (1986).

26) Sato, N. Goto, T., Haranaka, K., Satomi, N., Nariuchi, H., Mano-Hirano, T. and Sawasaki, Y. Action of tumor necrosis factor on cultured vascular endothelial cells: morphologic modulation, growth inhibition and toxicity. *J. Natl. Cancer Inst.*, **76**, 1113–1121 (1986).

27) Ehrke, M. J. and Mihich, E. Effects of anti-cancer agents on immune responses. *In* "Trends in Pharmacological Sciences," pp. 412–417 (1985). Elsevier Science Publishers B.V., Amsterdam.