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Endogenous Tumor Necrosis Factor Induction with *Bordetella pertussis* Vaccine as a Triggering Agent and Its Therapeutic Effect on MM46 Carcinoma-bearing Mice

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Induction of endogenous tumor necrosis factor (TNF) by administration of *Bordetella pertussis* vaccine (BPV) as a triggering agent and its therapeutic effect against MM46 carcinoma were investigated in C3H/He mice. Test triggering agents were injected intravenously into mice after intravenous injection of 4-fold dilution of macrophage activating factor (MAF) or 10^4 units of murine interferon-γ (Mu-IFN-γ). Then sera were obtained from the mice, and their TNF activities were assayed on L-929 cells by the method of Ruff and Gifford. The triggering activity of BPV was the highest among those of conventional triggers, such as lipopolysaccharide (LPS) of *Escherichia coli*, and OK-432. The levels of serum TNF activity triggered by BPV (4×10^9 cells), LPS of *E. coli* (3 μg) and OK-432 (3 KE) were 5350, 85 and 102 units/ml, respectively. Growth of MM46, a spontaneous mammary carcinoma cell line of C3H/He was observed for 35 days after tumor inoculation and was suppressed significantly by intravenous injection of MAF and BPV (4×10^9 cells). On local injection of BPV (2×10^9 cells) into murine tumors, complete regression was observed in 67% of the mice tested with or without MAF priming on day 25 after tumor inoculation, and intratumoral TNF activity was observed even in the case of the single injection of BPV.

Key words: *Bordetella pertussis* vaccine — Tumor necrosis factor — Therapy

In 1975, Carswell *et al.* reported that the sera of endotoxin-treated animals infected with Bacillus Calmette-Guérin (BCG) caused hemorrhagic necrosis of various tumors in mice without apparent side-effects on the host.[1] This activity of the serum was named tumor necrosis factor (TNF). TNF can be induced by two-stage stimulation, priming and triggering. Since then, many studies have been made to apply TNF for cancer therapy, mainly focused on the production of a large amount of TNF by the recombinant DNA technique. A brief overview of studies on TNF has been published.[2] In previous studies we developed an experimental model for endogenous production of TNF which is clinically applicable, because various commercial preparations of biological response modifiers (BRM) could be used as primers or triggers.[3–5]

With a combination of PPD plus OK-432 or IFN-γ plus OK-432[8] we achieved partial regression of lung or liver tumors in patients.[8] These clinical trials are still in progress, but new triggers are urgently needed, since OK-432 sometimes shows no effect due to tolerance of the tumor patients.[8] So it is necessary to find other triggers for clinical purposes. LPS, a conventional trigger of TNF, is derived from *E. coli*, a Gram-negative bacterium, and its chemical conformation is similar to that of lipoteichoic acid derived from Gram-positive bacteria including *Streptococcus* (OK-432).[3–5,9] Thus, we tested vaccines of *Bordetella pertussis* as a trigger, expecting that less toxic LPS should be present in this bacterium. In this paper we report that *Bordetella pertussis* vaccine (BPV) induces high TNF activity in the sera of mice when MAF is used as a primer. As the priming effect of MAF (Con A sup.) in this study was neutralized by anti-Mu-IFN-γ, the priming activity can be induced by IFN-γ of MAF.

In local therapy, we showed that single injection of BPV induced intratumoral TNF activity.

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Materials and Methods

Animals Male C3H/He mice of 7 weeks old were purchased from Shizuoka Experimental Animal Farm (Shizuoka).

Cell Line A transformed cell line (L-929) originally derived from a C3H/He strain mouse was grown in Eagle's minimum essential medium (MEM; Nissui Seiyaku Co., Tokyo) supplemented with 5% fetal calf serum (FCS; Hyclone Laboratories, USA) and was passaged every 3 or 4 days.

Chemical Reagents BCG was obtained from Japan BCG Laboratory (Tokyo) and glycogen from E. Merck (West Germany). Lipopolysaccharide (LPS) from *E. coli* 0127:B8 was from Difco Lab. (Detroit, Mich.), OK-432 from Chugai Seiyaku Co. (Tokyo) and BPV was from Chiba Institute (Chiba). Anti-murine interferon-γ monoclonal antibody (anti-Mu-IFN-γ MoAb) was kindly provided by Dr. Yoshimi Kawade (Institute for Virus Research, Kyoto University, Kyoto). Recombinant murine interferon-γ (Mu-IFN-γ) was from Toray Industries, Inc. (Tokyo).

Preparation of Rabbit Tumor Necrosis Serum (TNS) TNS was prepared by a modification of the procedure of Carswell *et al.*[1] Viable BCG (1.4×10^9 cfu/head) were injected intravenously into rabbits and 2 weeks later, 80 μg of LPS was injected intravenously. Rabbits were exsanguinated 2 hr later and their sera were pooled and stored at -80° until use. This TNS was used as a standard preparation of TNF in all cytotoxicity assays *in vitro*.

Preparation of MAF MAF was prepared by a modification of the procedure of Fidler *et al.*[6] Spleen cells were obtained from C3H/He mice 3 weeks after intravenous injection of viable BCG (4×10^7 cfu/head) and were incubated with concanavalin A (Con A) agarose (E. Y. Lab., USA) (a final concentration of 100 or 200 μg per ml) for 48 hr at 37° in RPMI 1640 medium (Nissui Seiyaku Co.). The Con A-stimulated cell cultures were centrifuged at 3,000 rpm for 10 min to remove the cells and Con A agarose, and the cell-free supernatant was concentrated 10-fold, filtered through a sterile 0.45 μm Millipore membrane and stored at -80° until use. For use as a MAF, it was diluted 4-fold with phosphate-buffered saline (PBS; pH 7.2) and volumes of 0.2 ml per mouse were injected intravenously.

Preparation of Cytotoxic Serum Mice primed with MAF or Mu-IFN-γ (10^4 unit/head) 3 hr previously were treated intravenously with triggers in 0.2 ml of PBS and 2 hr later, the mice were exsanguinated, and their sera were separated. All sera were stored at -80° until use.

Neutralization Assay Mice were injected with 25 mg of glycogen, and 5 days later 3 ml of Hanks' solution was injected into the peritoneal cavity. Peritoneal exudate cells (PEC) were subsequently obtained from the mice. In a 96-well flat-bottomed microtiter plate, Mu-IFN-γ (final concentration 50 units/ml) or MAF (final dilution; 1:20, 200, 2,000) was mixed with PEC (5×10^4 cells) and/or anti-Mu-IFN-γ MoAb (final dilution; 1:10) in 0.2 ml of MEM supplemented with 5% FCS, and incubated in a humidified atmosphere of 5% CO_2 in air at 37° for 3 hr. After this time, each well was washed with warmed medium and 200 μg of LPS (final concentration 15 μg/ml) was added to adherent cells and incubated for 2 hr. The supernatant were drawn for TNF assay.

TNF Assay TNF activity of serum preparations was measured by using an *in vitro* cytotoxicity assay with L-929 cells as a target, by the method of Ruff and Gifford.[7]

Cells (8×10^4) in 0.1 ml of MEM supplemented with 5% FCS were dispensed in 96-well flat-bottomed microtiter plates (Corning Glass Works, USA) and incubated in a humidified atmosphere of 5% CO_2 in air at 37° for 3 hr. Test serum preparations or standard rabbit TNS in 0.05 ml of MEM were added to the wells followed by the same volume of a solution of actinomycin D (Sigma Chemical Co., USA) to give a final concentration of 1 μg/ml.

After incubation for 18 hr, the medium was discarded and the wells were washed with PBS. Residual cells were stained with crystal violet and solubilized with 0.1 ml of 0.5% sodium dodecyl sulfate solution.

The absorbance at 590 nm was measured photometrically in an Immuno Reader NJ-2000 (Japan Inter Med Co., Tokyo) and the survival ratio was calculated by means of the formula: Survival ratio = absorbance of test sample/spontaneous (cytotoxic serum-free culture) absorbance. The dilution of cytotoxic serum giving a half survival ratio (ED_{50}) was obtained from a dose-response curve. The relative TNF activity (unit) was calculated as the ratio of the ED_{50} of the cytotoxic serum to that of rabbit TNS giving a half survival ratio of 10^5 to $10^{5.5}$. The cytotoxic activity of this rabbit TNS was equivalent to 6×10^3 units of recombinant human TNF (Asahi Chemical Industry, Japan).

Inoculation of Tumor Cells MM46 cells were derived from a spontaneous mammary carcinoma in a C3H/He mouse and were passaged weekly in the peritoneal cavity. For the systemic combination therapy, washed MM46 cells (4×10^6 cells) were inoculated subcutaneously into the inguinal region of male C3H/He mice of 10 weeks old. For the local combination therapy, washed MM46 cells (1×10^6 cells) were inoculated intradermally into the abdomen of male C3H/He mice of 11 weeks old.

Therapeutic Test On day 9 or on days 9, 16 and 23 after tumor inoculation, MAF and/or BPV was injected into tumor-bearing mice. For systemic combination therapy, mice were injected intravenously with MAF, and 3 hr later intravenously with 4×10^9 BPV cells in 0.2 ml of PBS. For local combination therapy, mice were treated intravenously with MAF, and 3 hr later 2×10^9 BPV cells in 0.1 ml of PBS were injected intratumorally. The largest and smallest diameters of each tumor were measured at intervals with a slide caliper and the average diameter (mm) was recorded.

Intratumoral TNF Activity in Local Therapy On day 9 after tumor inoculation, mice were treated intravenously with MAF and 3 hr later intratumorally with BPV. At 2 hr after BPV injection, tumors were removed and homogenized. A 5% homogenate of tumor in saline was centrifuged at 3,000 rpm for 10 min, and the supernatant was taken for TNF assay.

Results

Comparison of Various Triggering Agents The TNF activities triggered by LPS from *E. coli* (3 μg), OK-432 (3 KE) and BPV (4×10^9 cells) were compared in C3H/He mice primed with 4-fold dilution of MAF (Fig. 1). The levels of serum TNF activity triggered by LPS from *E. coli*, OK-432 and BPV were 85, 102 and 5,350 units/ml respectively. BPV (4×10^9 cells) induced the highest TNF activity among the triggering agents tested.

Time Course of Change in TNF Activity after BPV Administration The change of TNF activity with time after BPV (4×10^9 cells) injection in mice primed with 4-fold dilution of MAF is shown in Fig. 2. The serum TNF activity reached a maximum (16,700 units/ml) 2 hr after BPV injection, and then decreased, becoming negligible after 6 hr.

Effect of Dose of BPV on Serum TNF Production The serum TNF activities induced by various doses of BPV in mice primed with MAF or Mu-IFN-γ were measured (Table I). In tests with doses of 4×10^5 to 4×10^9 BPV

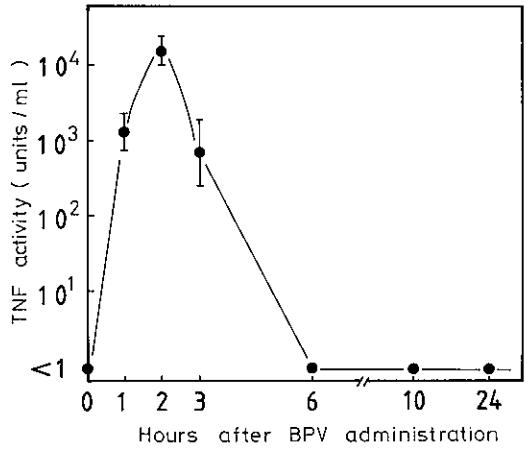

Fig. 2. Time course of change in TNF activity after BPV administration. Mice were treated intravenously with MAF, and 3 hr later with 4×10^9 cells of BPV per mouse. Their sera were obtained at the indicated times after BPV administration for measurement of TNF activities. Points and bars represent means and standard deviations for four individual samples.

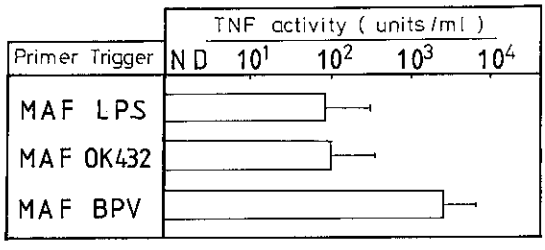

Fig. 1. TNF activities induced by various kinds of triggering agents. Mice were treated intravenously with MAF, and 3 hr later with 3 μg of LPS, 3 KE of OK-432 or 4×10^9 cells of BPV per mouse. A further 2 hr later their sera were obtained for measurement of TNF activities. Columns and bars represent means and standard deviations for four individual samples.

Table I. Effect of Dose of BPV on Serum TNF Production

Trigger	Primer		
BPV	Mu-IFN-γ	MAF	
		Exp. 1	Exp. 2
4×10^5			ND[a]
4×10^6	ND	ND	
4×10^7	175±0.20	127±0.16	239±0.30
4×10^8	1,090±0.14	549±0.18	
4×10^9	3,950±0.07		6,000±0.26

a) Not detected.

Mice were treated intravenously with 4-fold dilution of MAF or 10^4 units of Mu-IFN-γ, and 3 hr later with the indicated dose of BPV. A further 2 hr later their sera were obtained for measurement of TNF activities.

Table II. Anti-MuIFN-γ MoAb Neutralization of Mu-IFN-γ Activity of MAF

Primer	Anti-MuIFN-γ MoAb	TNF activity
Mu-IFN-γ	−	23.5±0.0
	+	0.6±0.1
MAF (1/20)	−	13.6±0.1
	+	ND[a]
MAF (1/200)	−	7.6±0.1
	+	ND
MAF (1/2,000)	−	1.5±0.0
	+	ND

a) Not detected.
Mu-IFN-γ (final concentration 50 units/ml) or MAF (indicated final dilution) was mixed with glycogen-induced PEC (5×10^4 cells) and/or anti-Mu-IFN-γ MoAb (final dilution 1/10) at 37° for 3 hr. Each well was washed with warmed medium and 200 μl of LPS (15 μg/ml) was added. After 2 hr, the supernatant was drawn and TNF activity was determined.

cells, serum TNF activity was induced at doses of 4×10^7 cells or more. MAF showed the same priming effect as Mu-IFN-γ.

Neutralization of Priming Activity of MAF and Mu-IFN-γ by Anti-Mu-IFN-γ MoAb Neutralization of priming activity of Mu-INF-γ and MAF by anti-Mu-IFN-γ MoAb is shown in Table II. Adherent cells in PEC was primed with MAF or Mu-IFN-γ, and subsequently TNF activity was induced by successive LPS triggering. As in the case of Mu-IFN-γ, the priming activity of MAF was completely neutralized with anti-Mu-IFN-γ MoAb.

Systemic Therapy with MAF and BPV against MM46 Carcinoma The therapeutic effect of systemic induction of TNF by injection of 4-fold dilution of MAF and BPV (4×10^9 cells) against MM46 carcinoma inoculated subcutaneously into mice was examined. The tumor diameters were measured at intervals after tumor inoculation and the results are shown in Fig. 3. On day 35 after tumor inoculation, tumor diameters in the

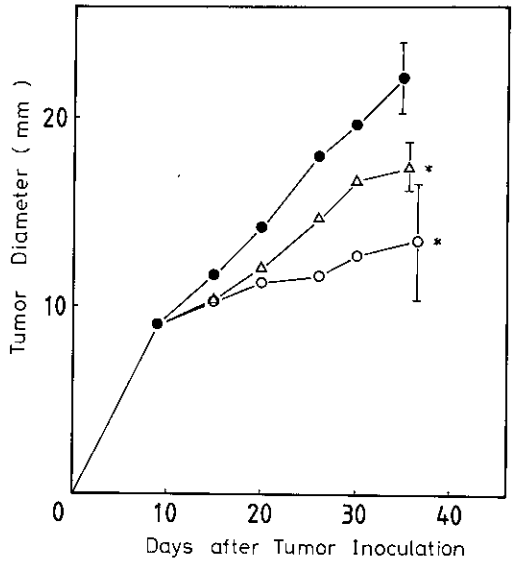

Fig. 3. Systemic therapy with MAF and BPV against MM46 carcinoma. Mice received subcutaneously inocula of 4×10^6 MM46 carcinoma cells on day 0. On day 9 or on days 9, 16 and 23, they were treated intravenously with MAF and 4×10^9 BPV cells. ●, Control; △, day 9 treatment; ○, days 9, 16 and 23 treatment. * Significant difference ($P<0.01$) from the value for untreated controls by Student's *t*-test.

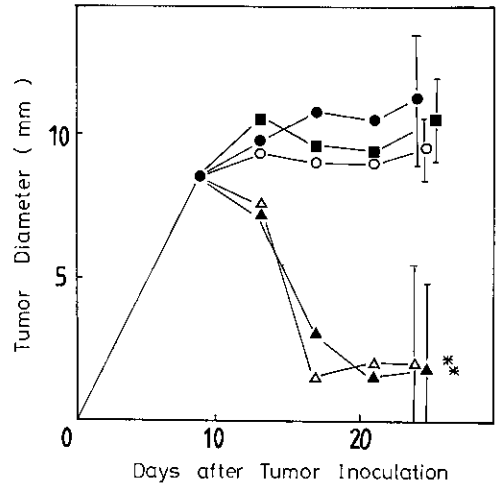

Fig. 4. Local therapy with MAF and BPV against MM46 carcinoma. Mice received intradermally inocula of 10^6 MM46 carcinoma cells on day. 0. On day 9, they were treated intravenously with MAF and/or intratumorally with 2×10^9 BPV cells. ●, Control; ○, PBS (intratumorally); ■, MAF; △, BPV; ▲, MAF and BPV. Symbols and bars represent means and standard deviations for six individual mice. * Significant difference ($P<0.01$) from the value for untreated controls by Student's *t*-test.

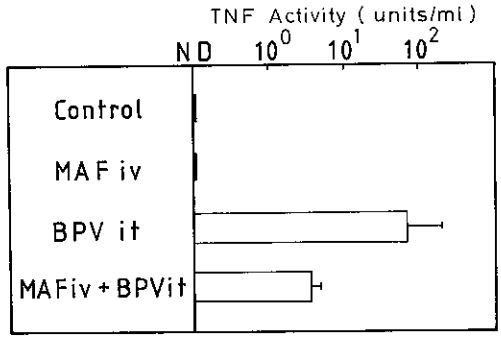

Fig. 5. Intratumoral TNF induction in MM46-bearing mice. On day 9 after tumor inoculation, mice were treated intravenously (iv) with MAF and 3 hr later intratumorally (it) with BPV. At 2 hr after the BPV injection, intratumoral TNF induction was measured.

cases of no treatment, day 9 treatment and days 9, 16 and 23 treatment were measured. The values were 22.2, 17.5 and 13.5 mm, respectively. Tumor growth was suppressed significantly ($P<0.01$) with the combination of MAF and BPV, and even with multiple combination therapy no mice were completely cured.

Local Therapy with MAF and BPV against MM46 Carcinoma The local therapeutic effect of 4-fold dilution of MAF and BPV (2×10^9 cells) against MM46 carcinoma was investigated. For this, mice were treated intravenously with MAF and then given BPV intratumorally, and tumor diameters were measured at various times after therapy. As shown in Fig. 4, tumor diameters in the cases of no treatment and treatment with PBS, MAF, BPV and MAF plus BPV on day 25 after tumor inoculation were measured. The values were 11.3, 9.5, 10.5, 2.0 and 1.8 mm, respectively. Intratumoral injection of BPV with or without MAF resulted in complete regression of tumors in 67% of the mice tested.

Intratumoral TNF Activity On day 9 after tumor inoculation, intratumoral TNF induction was measured (Fig. 5). Intravenous TNF activity was not observed when BPV (2×10^9 cells) was injected without MAF (data not shown). However, when BPV was injected intratumorally with or without MAF, intratumoral TNF activity was induced. Intratumoral TNF activity induced by a single BPV injection was 68.6 units/ml.

Discussion

In this study we used MAF and Mu-IFN-γ as a primer, since it is thought to be the actual primer molecule. We found that BPV was a potent trigger with low toxicity. Induction of TNF with BPV (4×10^7 cells/head) was almost the same as that with OK-432 (0.3 KE/head). The acute toxicity (LD_{50}) of BPV in C3H/He mice on intravenous injection was 6.8×10^{10} cells, while that of OK-432 was 5 KE per mouse (data not shown). The chemotherapeutic index of BPV was estimated to be about 100 times that of OK-432. Thus, of the triggers so far tested, BPV is the best and can clearly be used in place of OK-432, if necessary, since BPV is a commercial product that is widely available.

The real effector molecule may be the LPS present in this bacterium. However, no precise data on the LPS of BPV are available.

As the priming effect of MAF in this study was completely neutralized with anti-Mu-IFN-γ MoAb, the priming activity can be induced by IFN-γ of MAF. Consequently MAF can be replaced by IFN-γ and combination therapy with IFN-γ plus BPV against human cancer is expected to be effective.

The tumor regression observed in this study is caused not only by the induction of endogenous TNF, but also by the activation of cytotoxic cells such as macrophages and NK cells, as well as the induction of antitumor cytokines.

More interesting results were obtained on local therapy with BPV. Namely, complete regression of tumors was observed in 67% of MM46-bearing mice treated with BPV with or without MAF priming. Complete regression of tumors was observed in 75% of Meth A-bearing BALB/c mice by the same treatment, too (data not shown).

In this paper, we have shown for the first time that a single injection of BPV induced intratumoral TNF activity, and this might be one of the mechanisms of tumor regression in local therapy. This result indicates that the tumor itself acts as a primer in TNF induction. We will report on the priming effect of tumor cells in the next paper. It is suggested

that a single injection of BPV can be used for treatment of human tumors.

In clinical trials, partial regressions of lung and liver tumors were observed on systemic induction of TNF.[8] Intratumoral injection of BPV with or without IFN-γ should be effective after the systemic induction of endogenous TNF. We are now studying this possibility in experimental models.

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