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The Immunomodulatory and Antitumour Activities of Trichosanthin-An Abortifacient Protein Isolated from Tian-hua-fen *(Trichosanthes kirilowii)*

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SUMMARY **Trichosanthin, a basic protein purified from the root tuber of *Trichosanthes kirilowii,* has been used effectively in China to induce midterm abortion in humans. In this paper, we show that trichosanthin at non-cytotoxic concentrations markedly inhibited the mitogen-induced lymphoproliferative response and the generation of a primary alloreactive CTL response *in vitro.* Similarly, the production of IL-2 by Con A activated splenocytes and the *in vitro* effector functions of macrophages were also significantly suppressed. In contrast, the cytolytic activity of CTL and NK cells was unimpaired. Moreover, the *in vivo* activation of NK cells was not significantly altered by a single injection of a non-toxic microgram amount of trichosanthin into mice. However, other immune reactivities such as the induction of a DTH response and the humoral antibody formation to SRBC were markedly depressed. Our data suggest that trichosanthin is a potent immunosuppressive protein that could affect humoral immunity and a variety of cell-mediated processes. In addition, our preliminary results show that this abortifacient protein could also inhibit the growth of a murine malignant tumour (MBL-2), both *in vivo* and *in vitro.***

Tian-hua-fen, the root tuber of the Chinese medicinal herb *Trichosanthes kirilowii* Maxim of the Cucurbitaceae family, has been used as an abortifacient drug for centuries in China.[1] An active component, named trichosanthin, was isolated and purified from the crude extract of Tian-hua-fen.[2] It was shown to be a basic protein of molecular weight 24,000 daltons and its chemical structure has recently been elucidated.[3,4] Trichosanthin was found to be effective in terminating early pregnancy and inducing mid-term abortion in laboratory animals[5-7] and humans.[8,9] The abortifacient activity of trichosanthin has been attributed to its selective destruction of the placental trophoblasts.[10] In addition to its abortifacient effect, trichosanthin was also shown to be active against the abnormal growth of trophoblastic cells, both *in vivo*[11] and *in vitro*.[12] In view of the importance of trichosanthin as a naturally-occurring abortifacient plant protein, as a drug to induce mid-term abortion and as a treatment for ectopic pregnancies, hydatidiform moles and choriocarcinomas,[9,11] it was of interest to investigate whether trichosanthin possessed any immunomodulatory activity in its own right. Previous work in this laboratory had shown that trichosanthin could block the ^{3}H-thymidine (^{3}H-TdR) incorporation in mitogen-stimulated mouse splenocytes *in vitro*.[13] However, the effect of trichosanthin on specific T and B cell functions has not been examined. In this paper, we present evidence that trichosanthin is a potent immunosuppressive protein that could affect a variety of cell mediated and humoral immune responses. In addition, our preliminary results show that this abortifacient plant protein could also cause the growth inhibition of a murine syngeneic, transplantable tumour (MBL-2), both *in vitro* and *in vivo*.

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ABBREVIATIONS

Con A: concanavalin A; *CPM:* counts per minute; *CS:* culture supernatant; *CTL:* cytotoxic T lymphocytes; *DTH:* delayed type hypersensitivity; *FCS:* foetal calf serum; *^{3}H-TdR:* ^{3}H-thymidine; *IL-2:* interleukin-2; *IP:* intraperitoneal; *LPS:* lipopolysaccharide; *PBS:* phosphate-buffered-saline; *PEC:* peritoneal exudate cells; *PFC:* plaque-forming cells; NK: natural killer; *PHA:* phytohaemagglutinin; *SRBC:* sheep red blood cells.

MATERIALS AND METHODS

Mice

Inbred BALB/c (H-2^d) and C57BL/6J (H-2^b) mice were bred at the University Animal House, The Chinese University of Hong Kong. Mice of the same age (6-10 weeks old) and same sex were used in each experiment.

Abortifacient protein

Trichosanthin, a basic protein of molecular weight 24,000 daltons, was prepared from fresh root tuber of *Trichosanthes kirilowii* by techniques including acetone fractionation, ammonium sulfate precipitation and ion-exchange chromatography on CM Sepharose CL-6B columns (Pharmacia, Sweden).[14] The final preparation was shown to be homogeneous by SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis.[14] The abortifacient activity of trichosanthin was assayed by injecting various amounts (2-4 mg/kg body weight) of the protein intraperitoneally (IP) into pregnant mice on day 12 of gestation and this consistently resulted in death of over 90% of the fetuses and an abortion in all the pregnant mice. In addition, gross observations have shown that treatment of normal mice with these dosages of protein alone induced no general toxicity and no significant cellular depletion in various lymphoid organs. Trichosanthin was dissolved in phosphate-buffered-saline (PBS, pH 7.3) and sterilized by millipore filtration before use. Protein concentration was determined by the Folin-Lowry procedure.[15]

Mitogen-induced lymphocyte transformation

Mouse spleen cells were cultured in flat-bottomed 96-well microtiter plates (Flow Lab. Ltd.,U.K.) at 5×10^5 cells/well in a final volume of 0.2 ml RPMI 1640 medium (GIBCO, U.S.A.) supplemented with 10% foetal calf serum (FCS) (GIBCO, U.S.A.) and containing 100 units/ml of penicillin G, 100 μg/ml of streptomycin sulfate and 3 μg/ml of fungizone (GIBCO, U.S.A.). Mitogen at predetermined optimal concentration was added to cultures containing various concentrations of trichosanthin and the cultures were kept in a humidified atmosphere containing 10% CO_2 in air. Mitogens (Sigma Chem. Co., U.S.A.) used included concanavalin A (Con A, 3 μg/ml), phytohaemagglutinin (PHA, 10 μg/ml) and lipopolysaccharide (LPS, 30 μg/ml). After 48 hours of incubation at 37°C, 0.5 μCi ^{3}H-thymidine (2 Ci/mmol, Amersham, U.K.) was added to each well and incubation was continued for 6 hours. Cells were harvested with a Titertek multiharvester (Flow Lab. Ltd., U.K.) and radioactivity was counted in a liquid scintillation counter (Beckman LS1801, U.S.A.). Incubations were usually done in quadruplicate and DNA synthesis, as measured by ^{3}H-TdR incorporation, was expressed as counts per minute (CPM).

In vitro generation of alloreactive cytotoxic T lymphocytes and assay of cell-mediated lympholysis

The procedure described by Engers *et al*[16] was essentially followed. In brief, 2×10^5 mitomycin C (Sigma Chem. Co., U.S.A.) treated (50 μg/ml for 30 minutes at 37°C) BALB/c splenocytes were co-cultured with 10^5 C57BL/6J splenocytes in each well of the round-bottomed 96-well microtiter plate (Costar, Cambridge, U.S.A.). Various concentrations of trichosanthin were added to a final volume of 0.2 ml per well and the cultures were incubated at 37°C under a gas phase of 10% CO_2 in air. Each well was replenished on day 2 and day 5 with 50 μl RPMI medium supplemented with 10% heat inactivated FCS. Cytotoxicity of the effector cells generated was measured on day 6, using the standard ^{51}Cr release assay. For the measurement of cell-mediated lympholysis, 5×10^6 P815 targets (H-2^d) were labelled with 200 μCi ^{51}Cr ($Na^{51}CrO_4$, 350-600 mCi/mg chromium, Amersham, U.K.) for 1 hour at 37°C. The cells were then washed twice with RPMI medium and cell concentration adjusted to 10^5/ml. On day 6, 100 μl supernatant was removed from each well of the primary culture and 10^4 ^{51}Cr-labelled P815 cells in 0.1 ml volume were added. After a further incubation period of 8 hours, 100 μl supernatant was carefully sucked up from each well and radioactivity counted in a gamma counter. For spontaneous lysis, 0.1 ml RPMI medium was added to 0.1 ml labelled targets. For maximum releasable ^{51}Cr, 0.1 ml 10% Triton-X100 was added to 0.1 ml labelled target cells. The percentage specific lysis was calculated as follows:

$$\%\text{Specific lysis} = \frac{\text{Test culture} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

Measurement of delayed-type hypersensitivity

C57BL/6J mice in groups of four were given an IP injection of trichosanthin (4 mg/kg body weight) either two days before, on the same day of, or two days after intravenous sensitisation with 10^6 sheep red blood cells (SRBC). Control mice were injected with an equal volume of PBS at the same

day of SRBC sensitisation. Four days after antigen sensitisation, each mouse was challenged with 10^8 SRBC in 50 μl, injected subcutaneously into the right hind footpad. The same volume of PBS was injected into the left hind footpad as a control. Footpad thickness was measured at 24, 48 and 72 hours after antigen challenge and results were expressed as the percentage mean increase in footpad thickness as described in detail previously.[17]

Production of and assay for interleukin-2

The method for the production of interleukin-2 (IL-2) was essentially the same as described previously.[18] Briefly, splenocytes from mice pretreated either with trichosanthin (4 mg/kg given IP 2 days before) or PBS were suspended at a density of 10^7/ml in culture medium (RPMI + 10% FCS) and stimulated with Con A (5 μg/ml) for 24 hours at 37°C in a humidified atmosphere containing 10% CO_2 in air. Cell-free supernatants were absorbed with Sephadex G-75 (2 mg/ml) at 4°C overnight to remove residual Con A. All IL-2 preparations were millipore-filtered and kept at −20°C until use.

IL-2 activity was measured by the maintenance assay as described in details by Lafferty *et al.*[19] In essence, assays were carried out in flat bottomed 96-well microtiter plates. Serial two-fold dilutions of the culture supernatants (CS) containing IL-2 were prepared in 50 μl of culture medium and 50 μl of Con A-activated lymphocytes (4×10^5/ml) was added and the mixtures cultured for 20 hours at 37°C. The cells were then given a 5 hour pulse with 0.5 μCi ^{3}H-TdR and radioactivity incorporated was determined.

Primary humoral immune response to sheep red blood cells

C57BL/6J mice in groups of four were either pretreated with triochosanthin (4 mg/kg given IP) or PBS two days before IP immunisation with 4×10^8 SRBC. The number of direct (IgM) plaque-forming cells (PFC) in the spleens were enumerated four days later[20] whereas the levels of serum haemagglutinating antibody were determined at days 7, 14 and 21 after antigen injection.[21]

Natural killer cell assay

Natural killer (NK) cell activity was assessed by the ability of *Corynebacterium parvum* activated mouse spleen cells to lyse the NK-sensitive YAC-1 target cells in a 4 hour ^{51}Cr release assay as described in detail elsewhere.[22] Briefly, BALB/c mice were injected intravenously with 350 μg formalin-killed *C. parvum* (Wellcome Research Lab., U.K.) and splenic effector cells were assayed for NK activity 4 days later. The effect of trichosanthin was determined by either pretreatment of mice with the protein (4 mg/kg given IP 2 days before injection of *C. parvum*) or by adding the protein (100 μg/ml) directly to the mixture of effector and target cells in the assay.

Phagocytosis assay

Mice were injected IP with 1 ml 10% proteose peptone (Difco Lab., Detroit, MI). Three days later, the peritoneal exudate cells (PEC) were harvested, adjusted to 2×10^6/ml and then incubated with control medium or trichosanthin (final concentration was 100 μg/ml) for 6 hours at 37°C in a siliconised borosilicate culture tube. The phagocytic activity of the resultant PEC was assessed by the latex uptake method as described by Kohl *et al.*[23]

Assay of macrophage-mediated cytostasis

This was carried out as described by Ruffmann *et al.*[24] Briefly, 2.5 mg picolinic acid (Sigma Chem. Co., U.S.A.) dissolved in PBS was injected into C57BL/6J mice. Three days later, the PEC were harvested and resuspended in RPMI medium + 10% FCS at 2×10^6/ml. Next, 0.1 ml PEC suspension was added into each well of a flat-bottomed 96-well microtiter plate together with 0.1 ml trichosanthin (final concentration was 100 μg/ml) or control medium. After 6 hours of incubation at 37°C, nonadherent cells and trichosanthin were removed by three washes with warm culture medium. 10^4 MBL-2 cells (a Moloney virus-induced T cell lymphoma of C57BL/6J mice) supplemented with LPS (2 ng/ml) were added to each well in a final volume of 0.2 ml. After incubating for 48 hours at 37° in a humidified atmosphere containing 10% CO_2 in air, the cells in each well were pulsed with 0.5 μCi ^{3}H-TdR and then harvested 5 hours later using a Titertek multiharvester. The percentage growth inhibition of MBL-2 cells induced by macrophages was calculated as follows:

$$\%\text{Cytostasis} = \left[1 - \frac{^3\text{H} - \text{TdR incorp with M}\phi}{^3\text{H} - \text{TdR incorp without M}\phi}\right] \times 100$$

Evaluation of antitumour activity

C57BL/6J mice in groups of six were injected IP with a syngeneic, transplantable tumour MBL-2 (10^5 cells per mouse). Two days later, one group was given a single IP injection of trichosanthin (4 mg/kg) and the control group was injected with an equal volume of PBS. Antitumour activity was

evaluated by counting the number of MBL-2 cells recoverable from the peritoneal cavity of mice 10 days post tumour challenge.

The *in vitro* antitumour activity of trichosanthin was assessed in two ways: (a) by examining the direct cytotoxic effect of the protein on MBL-2 cells cultured *in vitro*, as assayed by the trypan blue dye exclusion method;[25] and (b) by examining the growth-inhibitory activity of the protein on MBL-2 cells cultured *in vitro*, as measured by the ^{3}H-TdR incorporation assay.

Statistical analysis

All results were expressed as the arithmetic mean ± standard error. Student's *t* test was used to determine the confidence limits in group comparisons.

RESULTS

Inhibition of the mitogen-induced lymphocyte transformation

C57BL/6J mouse splenocytes stimulated with the T cell mitogen (Con A or PHA) and with the B cell mitogen (LPS) were incubated with different concentrations of trichosanthin at the beginning of the culture period. As shown in Table 1, the mitogen-induced lymphoproliferative response was greatly suppressed (~ 90% suppression) at protein concentrations of 50-100 μg/ml. The magnitude of suppression was clearly dose-dependent and it was found that trichosanthin was more inhibitory to the PHA response than to the Con A and LPS responses (Table 1). In order to demonstrate that the inhibition of the mitogen-induced lymphocyte transformation was not due to the direct cytotoxic effect of trichosanthin on mouse lymphocytes *in vitro*, normal mouse splenocytes were incubated with a high concentration (100 μg/ml) of trichosanthin, and cell viability was assessed at different times (12-48 hours) after incubation using the trypan blue dye exclusion method.[25] It was found that trichosanthin exhibits no appreciable direct cellular lymphotoxicity under the prescribed experimental conditions *in vitro* (data not shown).

Effect of trichosanthin on the generation of a specific T cell response *in vitro* and *in vivo*

Results in Table 2 showed that trichosanthin at concentrations as low as 1 μg/ml exhibited a marked inhibitory effect on the generation of a primary alloreactive cytotoxic T lymphocyte (CTL) response *in vitro*. The inhibition occurred in a dose-dependent manner and almost complete suppression was seen at a protein concentration of 50 μg/ml (Table 2). In contrast, the cytolytic activity of the alloreactive effector CTL was not significantly diminished by preincubation of the effector cells with trichosanthin (50 μg/ml) for 6 hours before addition of target cells (specific lysis: no trichosanthin, 75.3 ± 2.6; with trichosanthin, 68.3 ± 1.4).

The effect of trichosanthin on the generation of a specific T cell response *in vivo* was also examined. As shown in Fig. 1, trichosanthin administered 2 days prior to, on the same day of, or 2 days after SRBC sensitisation of mice, all demonstrated a significant

Table 1 Effect of *in vitro* trichosanthin exposure on the response of murine splenocytes to mitogens*

Protein added to culture	Protein concentration (μg/ml)	^{3}H-TdR incorporation (CPM ± S.E. × 10^{-3}) Con A (3 μg/ml)	% suppression*	LPS (30 μg/ml)	% suppression*	PHA (10 μg/ml)	% suppression*
Nil		105.4±6.5		40.9±2.5		4.1±0.1	
Trichosanthin	0.1	106.4±8.3	-1	32.8±0.9+	20	2.0±0.2+	51
	1	95.5±3.1	9	28.5±1.5+	30	1.3±0.1+	68
	5	N.D.§		N.D.		0.9±0.05+	78
	10	71.2 ±2.2+	32	21.0±0.08+	49	0.6±0.03+	85
	50	N.D.		N.D.		0.3±0.2+	93
	100	11.3±3.5+	89	4.2±0.2+	90	N.D.	

* Mouse spleen cells (5 × 10^5/well) were stimulated with a predetermined optimal concentration of mitogen in the presence or absence of trichosanthin. Cells cultured for 48 hours were given a 6-hour pulse with ^{3}H-TdR (0.5 μCi/well) and incorporated radioactivity was measured.

* % suppression was obtained by comparison with the corresponding control culture in which no trichosanthin was added.

+ Significantly different from the control $P<0.05$

§ N.D. = not determined

inhibitory effect on the subsequent generation of a delayed type hypersensitivity (DTH) response *in vivo*, when treated mice were challenged with antigen 4 days after sensitisation. The observed suppression is unlikely to be due to a shift in the kinetic parameters of the immune response since the kinetics of the DTH response in both the control mice and protein-treated mice were quite similar and since significant suppression of the DTH response was seen at both 24 and 48 hours after footpad challenge (Fig. 1).

Table 2 Effect of trichosanthin on the generation of a primary alloreactive cytotoxic T lymphocyte response *in vitro**

Protein added to culture*	Protein concentration (μg/ml)	% specific ^{51}Cr release (mean + S.E.)	% suppression
Nil		80.2±10.1	
Trichosanthin	1	49.9±11.0+	38
	5	29.5± 6.3+	63
	10	19.0± 1.0+	76
	50	2.6± 0.6+	97

* Primary alloreactive CTL were generated by co-culturing 10^5 C57BL/6J (H-2^b) splenocytes with 2×10^5 BALB/c (H-2^b) splenocytes for 6 days at 37°C. Cytotoxicity was measured on P815 (H-2^d) targets using a 8-hour ^{51}Cr release assay.

* Various concentrations of trichosanthin were added at the beginning of the mixed lymphocyte culture.

\+ Significantly different from the control (no trichosanthin was added). $p<0.05$

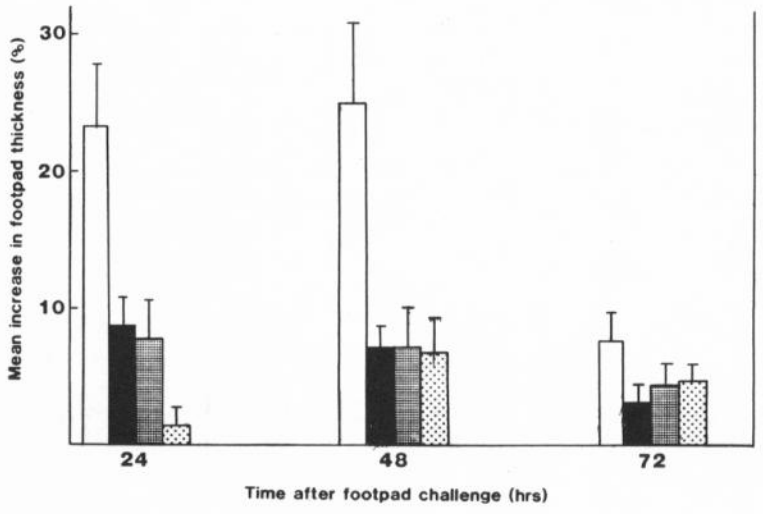

Fig. 1 Suppression of a DTH response by trichosanthin. Mice in groups of four were injected with trichosanthin (4 mg/kg given IP) either 2 days before (■), on the same day of (▦), or 2 days after (▨) intravenous sensitisation with 10^6 SRBC. Control mice (□) were injected with an equal volume of PBS at the same day of SRBC sensitisation. Four days after antigen sensitisation, each mouse was challenged with 10^8 SRBC into the right hind footpad. The DTH response, as determined by the % specific increase in footpad thickness, was measured 24-72 hours after antigen challenge. Vertical bars represent one standard error.

Effect of trichosanthin on the production of interleukin-2 from Con A-activated mouse splenocytes

Interleukin-2 is a T cell growth factor which is secreted by a subset of helper T cells, and it is an important biological regulator of T cell proliferation.[26] Therefore, we have examined the effect of trichosanthin on the synthesis of this lymphokine. Figure 2 showed that splenocytes obtained from trichosanthin-treated mice (4 mg/kg given IP 2 days before) had a reduced capacity to produce IL-2 when stimulated with Con A, as compared to control untreated mice. Similarly, the ability of Con A-activated normal mouse splenocytes to liberate IL-2 was also significantly impaired in the presence of trichosanthin (100 μg/ml) *in vitro* (Fig. 3).

Primary humonral immune response to SRBC in trichosanthin-treated mice

C57BL/6J mice in groups of four were either treated with trichosanthin (4 mg/kg injected IP) or PBS 2 days before immunization with SRBC (4×10^8 given IP). The splenic plaque-forming cell count was enumerated 4 days after SRBC sensitisation whereas the circulating haemagglutinating antibody titre was determined at days 7, 14 and 21 post-immunisation. Data in Table 3 show that pretreatment of mice with trichosanthin resulted in a marked reduction (~ 99%) in the number of antibody producing cells in the spleens of immunised mice. In addition, the levels of circulating antibody to SRBC, measured up to 3 weeks after antigen injection, were significantly depressed at all

times as compared to those of the control mice (Table 3).

Effect of trichosanthin on the *in vivo* generation and the *in vitro* activity of natural killer cells

BALB/c mice were injected IP with trichosanthin (4 mg/kg) two days before intravenous injection of *C. parvum* (350 μg/mouse) and the generation of splenic NK activity was compared to control mice receiving *C. parvum* only. It was found that the cytolytic activity of splenocytes from trichosanthin-treated mice towards NK-sensitive YAC-1 target cells was comparable to that of the control mice (Table 4). In addition, the NK activity of *C. parvum* activated splenocytes was not significantly altered by incorporation of trichosanthin (100 μg/ml) into the 4 hour ^{51}Cr release assay (Table 4). A similar result was obtained by preincubation of the splenic effector cells with trichosanthin for 6 hours before addition of the target cells (data not shown), thus suggesting that trichosanthin had no effect on the lytic function of activated NK cells, even when present at high concentrations.

Inhibitory effect of trichosanthin on macrophage functions *in vitro*

The effect of trichosanthin on the *in vitro* effector functions of macrophages was examined. As seen in Table 5, preincubation of the proteose peptone elicited PEC with trichosanthin (100 μg/ml) for 6 hours at 37°C significantly suppressed their phagocytic activity. Similar treatment of picolinic acid activated macrophages with trichosanthin also caused a marked reduction in the cytostatic activity of the activated macrophages (Table 5).

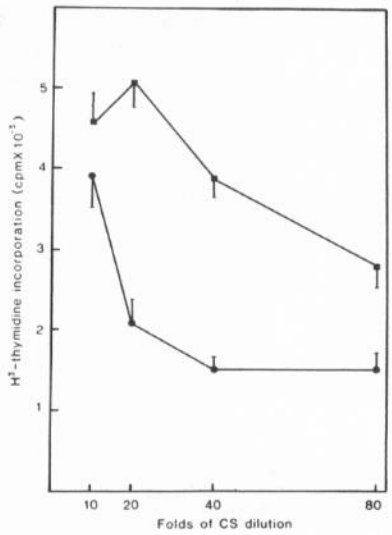

Fig. 2 Inhibition of IL-2 production by *in vivo* administration of trichosanthin
Spleen cells obtained from mice 2 days after IP injection with trichosanthin (4 mg/kg) (●) or PBS (■) were stimulated with Con A (5 μg/ml) for 24 hours. Each culture supernatant (CS) was serially diluted and assayed for IL-2 activity as described in detail elsewhere.[19] Vertical bars represent one standard error.

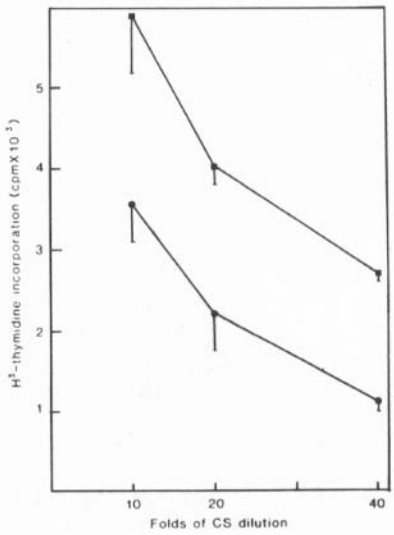

Fig. 3 Effect of trichosanthin on the *in vitro* production of IL-2 by Con A activated mouse splenocytes. Normal mouse spleen cells (10^7/ml) were cultured with Con A (5 μg/ml) in the absence (■) or presence (●) of trichosanthin (100 μg/ml). Culture supernatants (CS) were serially diluted and assayed for IL-2 activity as described in detail elsewhere.[19] Vertical bars represent one standard error.

Table 3 Primary humoral antibody response to SRBC in mice treated with trichosantin

Pretreatment of mice with*	Number of PFC/10^6 splenocytes*	Serum haemagglutinating antibody titre+ Day 7	Day 14	Day 21
PBS	644±25	320±0	300±20	200±23
Trichosanthin	9±7§	140±20§	65±15§	45±13§

* C57BL/6J mice in groups of four were pretreated with PBS or trichosanthin (4mg/kg given IP) 2 days before IP injection with 8×10^8 SRBC.

* The number of plaque-forming cells in spleens of mice were enumerated on the 4th day post SRBC administration using the Cunningham's modified hemolytic plaque assay.[20]

+ The haemagglutinating antibody titre was expressed as the reciprocal of the highest dilution of the serum which gave a positive result of haemagglutination.

§ Significantly different from control untreated mice, $p<0.05$.

Table 4 Effect of trichosanthin on the induction and effector function of mouse natural killer activity

Experiment	Pretreatment of mice with*	Co-culture of *C. parvum* activated spleen cells with*	% specific ^{51}Cr release at E/T 50:1	100:1
1	PBS	–	27.9±1.2	45.7±1.1
	Trichosanthin	–	26.2±1.2	42.2±2.1
2	–	Nil	50.6±3.1	64.6±1.3
	–	Trichosanthin	45.2±2.3	61.2±3.3

* Mice in groups of four were pretreated with PBS or trichosanthin (4 mg/kg given IP) 2 days before intravenous injection of *C. parvum* (350 μg/mouse). Splenocytes were assayed for NK activity 4 days later, using YAC-1 cells as the target.

* Day 4 splenocytes from *C. parvum* injected mice were co-cultured with trichosanthin (100 μg/ml) and assayed for NK activity on YAC-1 target cells. Trichosanthin alone was found to have no effect on the spontaneous release of the target cells over the 4-hour assay period.

Table 5 Effect of trichosanthin on macrophage effector functions *in vitro*

Preincubation of PEC with*	% Phagocytosis#	% Cytostatic activity+
Control medium	52.8±1.9	65.8±6.4
Trichosanthin	24.5±2.0§	15.6±4.8§

* PEC were preincubated with control medium (RPMI) or trichosanthin (100 μg/ml) for 6 hours before assay for the macrophage effector functions.

* Phagocytic activity of proteose-peptone elicited PEC was assayed by the latex uptake method.

+ Cytostatic activity of picolinic acid-activated PEC was measured by their ability to inhibit the growth of MBL-2 cells, as determined from the ^3H-TdR incorporation of the MBL-2 cells grown in the presence or absence of peritoneal macrophages.

§ Significantly different from the untreated PEC, $p<0.05$.

Effect of trichosanthin on the growth of MBL-2 tumour cells *in vitro* and *in vivo*

In view of the potent immunosuppressive effect of trichosanthin, it was of interest to know whether it could also suppress the growth of tumour cells both *in vitro* and *in vivo*. A Moloney virus-induced T cell lymphoma of C57BL/6J mice, MBL-2, was incubated with various concentrations of trichosanthin for 24 and 48 hours. Cell viability was monitored by the trypan blue dye exclusion method and cell growth of tumour cells was measured by the ^3H-TdR incorporation assay. It was found that incubation of MBL-2 cells with a high concentration (100 μg/ml) of trichosanthin for 24 hours did not result in any significant reduction in cell viability, but a 50% decrease in cell viability was seen at 48 hours of incubation. In contrast, trichosanthin exhibited a dose-dependent growth inhibitory effect on MBL-2 cells both at 24 and 48 hours of incubation, with a 50% inhibitory dose around 7 μg/ml and 1.5 μg/ml, respectively (Fig. 4A and 4B).

The *in vivo* antitumour activity of trichosanthin was also studied. It is clearly seen from Table 6 that a single injection of trichosanthin (4 mg/kg given IP) into C57BL/6J mice 2 days after MBL-2 implantation (10^5 cells per mouse) significantly suppressed the growth of the tumour *in vivo*, as demonstrated by a marked reduction (~ 14 fold) in the number of viable MBL-2 cells recoverable from the peritoneal cavity of mice 10 days post tumour challenge.

DISCUSSION

In an earlier report we have shown that trichosanthin, an abortifacient protein isolated from the

root tuber of the Chinese medicinal plant *Trichosanthes kirilowii*, could inhibit the mitogen-induced blastogenesis of mouse splenocytes *in vitro*.[13] In the present work, we show that this abortifacient plant protein exhibits a potent immunosuppressive effect for both cell-mediated and humoral immune responses. In addition, we have also demonstrated that trichosanthin could also cause the growth inhibition of a murine transplantable tumour (MBL-2), both *in vitro* and *in vivo*.

Although a number of plant proteins have recently been shown to be immunosuppressive,[27,28] trichosanthin is of particular interest to us as it acts specifically on the trophoblasts[10] and has been used clinically to terminate mid-term pregnancy (success rate was 99%) with only mild side effects.[9] Our results show that trichosanthin can markedly inhibit the lymphoproliferative response of mouse splenocytes to T cell mitogens such as Con A and PHA and to B cell mitogens such as LPS. Suppression occurred in a dose-dependent manner and complete abrogation of mitogen responsiveness was observed at a concentration of 50-100 μg/ml, a concentration that did not cause any significant loss of lymphocyte viability. Moreover, the *in vitro* generation of a primary alloreactive CTL response and the production of IL-2 by Con A-activated mouse splenocytes *in vitro* were significantly suppressed in the presence of trichosanthin. The mechanism whereby trichosanthin can lead to an impaired release of IL-2 from Con A-activated splenocytes is as yet unclear. Whether the protein exerts its action directly on IL-2 producer T cells or indirectly on IL-1 producing macrophages remains to be determined. Consistent with our *in vitro* observations, our *in vivo* studies showed that single injection of a non-toxic microgram amount of trichosanthin into mice could reduce the capacity of their splenocytes to liberate IL-2 in response to Con A stimulation *in vitro*. Similarly, trichosanthin administered *in vivo* not only resulted in a significant depression of the DTH response but also suppressed the primary humoral antibody response to SRBC. These taken together show that the abortifacient protein can not only inhibit nonspecific T and B cell proliferation *in vitro* but can also effectively suppress specific induction of T and B cell functions *in vivo*.

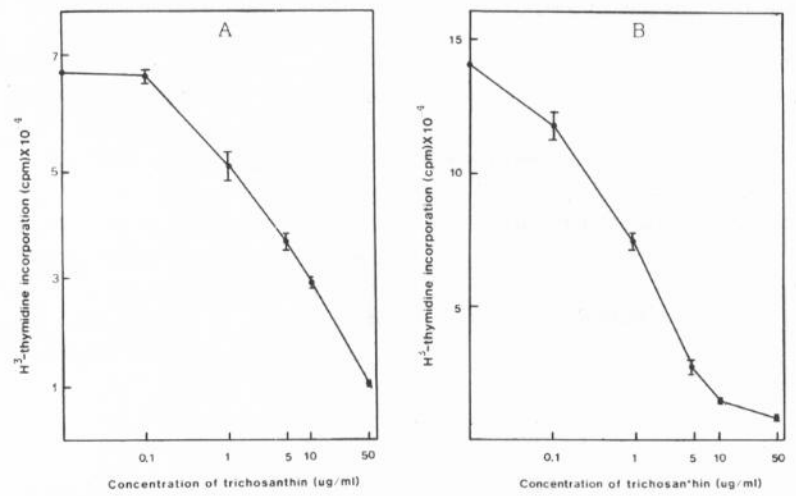

Fig. 4 Cytostatic activity of trichosanthin on the growth of MBL-2 tumour cells *in vitro*. 10^5 MBL-2 cells were seeded into each well of a flat-bottomed 96 well microtiter plated and incubated at 37°C with various concentrations of trichosanthin in a final volume of 0.2 ml culture medium (RPMI ± 10% FCS). After 24 (Fig. 4A) and 48 (Fig. 4B) hours of incubation, each well was pulsed with 0.5 μCi ^{3}H-TdR and the radioactivity incorporated was determined by liquid scintillation counting. Vertical bars represent ± one standard error.

Table 6 Effect of tricosanthin on the growth of MBL-2 tumour cells *in vivo**

Mice treated with	Tumour size* (× 10^{-6} cells)	P value+
PBS	150±25	
Trichosanthin	11± 2	<0.001

* C57BL/6J mice were injected IP with a syngeneic, transplantable tumour MBL-2 (10^5 per mouse). Two days later, one group was given a single IP injection of trichosanthin (4 mg/kg) and the control group was injected with an equal volume of PBS. Tumour size on day 10 post tumour challenge was measured.

* Data are expressed as mean ± S.E. for groups of 6 mice.

+ Significance of difference between values of control and trichosanthin treated group was determined by Student's *t* test.

The effect of trichosanthin on other immune cell types was also examined. Although we have found that single injection of trichosanthin *per se* did not appear to affect the number of resident peritoneal macrophages

(K.N.Leung, unpublished observation), the effector functions of macrophages, such as the cytostatic activity of picolinic acid activated macrophages and the phagocytic activity of proteose peptone elicited macrophages, were markedly depressed by *in vitro* exposure to trichosanthin. In contrast, similar treatment did not inhibit the effector phase of CTL and NK-mediated cytolysis. Likewise, the *in vivo* activation of NK cells was not appreciably affected by pretreatment of mice with trichosanthin. Thus, although trichosanthin appears to modulate a variety of immune reactivities, its suppressive effect is not totally non-selective. The available evidence so far suggests that NK cells may not be the primary target for the abortifacient protein as both the *in vivo* activation and the *in vitro* effector functions of this cell type were not significantly altered in the presence of the protein. Similar conditions were markedly depressive for other immune reactivities.

The mechanism(s) of immunodepression caused by trichosanthin has not been resolved. Immunosuppression cannot be attributed to the direct lymphocytotoxic effect of trichosanthin since exposure of mouse splenocytes to a high concentration (100 μg/ml) of the protein for at least 48 hours did not lead to any significant loss of cell viability. In addition, the kinetics of the DTH and humoral antibody responses to SRBC were not appreciably altered by pretreatment of mice with trichosanthin. This suggested that the observed suppression was not due to a time shift in the kinetic parameters of an immune response. Using a cell-free system, Xiong *et al*[29] have recently shown that trichosanthin is a very potent inhibitor of protein synthesis. Although protein synthesis inhibition could account for the immunomodulatory effect of trichosanthin, it has yet to be formally established that this is the only or the major mechanism involved in immunosuppression.

Besides its clinical use in terminating early pregancy[8] and inducing mid-term abortion,[9] trichosanthin has also been used therapeutically for the treatment of certain types of trophoblastic tumours.[9,11] A recent report has shown that trichosanthin can exert a selective cytotoxic effect on choriocarcinoma cells and melanoma cells whereas a number of cell types such as fibroblasts, hepatocytes and squamous carcinoma cells are relatively resistant.[12] In view of these findings and the fact that many immunosuppressive agents are generally cytotoxic to malignant cells, we evaluated the antitumour activity of trichosanthin, using a murine tumour model. Our preliminary results showed that although trichosanthin did not exert immediate cytotoxic effects on MBL-2 tumour cells *in vitro*, it could effectively inhibit growth of this malignant tumour both *in vitro* and *in vivo*. Since trichosanthin is shown to be immunosuppressive, it is unlikely that immune enhancement contributes to its antitumour effect. Rather, a direct interaction of the protein with the tumour cells was probably involved. Experiments to determine whether trichosanthin has any differential cytotosis. Although protein synthesis tumour cell lines are currently in progress. The mechanism(s) whereby trichosanthin may be transported and internalised in different types of lymphoid cells and malignant cells needs further investigation.

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