

Combination therapy using intratumoral bacillus Calmette-Guerin (BCG) and vincristine in dogs with transmissible venereal tumours: therapeutic efficacy and histological changes

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ABSTRACT

Therapeutic efficacy and histological changes after bacillus Calmette-Guerin (BCG), vincristine and BCG/vincristine combination therapy of canine transmissible venereal tumours (CTVT) were studied. Twenty dogs with naturally occurring CTVT in the progression stage were divided into 4 groups and treated with intratumoral BCG, vincristine, BCG/vincristine combination therapy or intratumoral buffered saline (control group). Tumour sizes were determined weekly and tumour response to therapy was assessed. Tumour biopsies were taken weekly to evaluate histological changes. Complete tumour regression was observed in all the dogs treated with BCG, vincristine and BCG/vincristine combination therapy. BCG/vincristine combination therapy had a statistically significantly shorter regression time than BCG or vincristine therapy. No tumour regression was observed in the control group. Intratumoral BCG treatment resulted in the appearance of macrophages and increased numbers of tumour infiltrating lymphocytes (TILs) followed by tumour cell apoptosis and necrosis. Treatment with vincristine resulted in increased tumour cell apoptosis, reduction in the mitotic index and a decrease in the number of TILs. Tumours from dogs on BCG/vincristine combination were characterised by reduction in the mitotic index, and appearance of numerous TILs and macrophages followed by marked tumour cell apoptosis and necrosis. This study indicates that combined BCG and vincristine therapy is more effective than vincristine in treating CTVT, suggesting that the clinical course of this disease may be altered by immunochemotherapy.

Keywords: bacillus Calmette-Guerin, canine transmissible venereal tumour, immunochemotherapy, vincristine.

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INTRODUCTION

Canine transmissible venereal tumour (CTVT) is a canine-specific naturally occurring contagious round cell neoplasm located mainly on the external genitalia of both male and female animals¹³. The tumour is unique in that it is the only proven example of a naturally occurring tumour that can be transplanted across MHC barriers^{14,24}. Experimentally induced CTVT and some natural spontaneous CTVT exhibit a predictable growth pattern with progressive growth (P phase), static growth (S phase) and regression (R phase)^{4,12}. Different types of tumour-infiltrating lymphocytes (TILs)

are present during the S and R phases. Increased numbers of TILs, particularly T-lymphocytes, are associated with tumour regression in CTVT suggesting that the host immune system is important in tumour regression^{8,24}. Owing to these characteristics, CTVT is a good model to study immunobiology of tumours. CTVT responds well to chemotherapeutic agents such as vincristine¹. However, it is not clear whether chemotherapeutic agents interfere with the host immune reaction to the tumour.

Immunotherapy aiming at the modulation of the immune system of the patient is a promising alternative to conventional methods of treating cancers. Immunostimulants, including bacillus Calmette-Guerin (BCG)¹⁹ and a mycobacterial cell wall extract from *Mycobacterium phlei*¹¹, have been evaluated in preclinical and clinical studies as therapy or adjuvant

therapy for several tumours.

Bacillus Calmette-Guerin was developed as a live-attenuated vaccine for immunisation against tuberculosis²¹. Local immunotherapy with BCG has been used to treat several tumours^{2,6,10} and can prevent the recurrence and progression of human superficial bladder cancer⁵. However, the anti-tumoral mechanism of BCG is still unclear. Several lines of evidence suggest that BCG attachment to tumour cells and presentation of BCG antigens to T-helper cells are required to trigger effective anti-tumoral activity². *In vitro* studies have provided evidence that cytokines and non-specific cytolytic mechanisms are involved in the anti-tumoral mechanism of BCG¹⁹. In addition to the above, there is some evidence that BCG also decreases the capacity of tumour cells to proliferate²⁰.

In spite of remarkable results associated with BCG use in the treatment of superficial bladder cancer, no studies have been done on the efficacy of combined BCG and vincristine on animal tumours. The objectives of this study were to compare the therapeutic efficacy of BCG/vincristine combination therapy, BCG immunotherapy and vincristine chemotherapy and use the results as a model to try to improve vincristine therapy of CTVT.

MATERIALS AND METHODS

Animals

All experiments were approved by the University of Zimbabwe Laboratory Animal Research Committee. Twenty dogs of mixed breed (7 males and 13 females) with progressing natural CTVT restricted to the external genital membranes were obtained from cases admitted at University of Zimbabwe Teaching Veterinary Hospital. The mean age of the dogs was 4 years and the mean body weight was 15.6 kg. Tumour diagnosis was confirmed by histology and the tumours were clinically classified as progressing based on increasing size over 2 weeks. The base of the tumour was measured in 2 perpendicular directions and the surface area of the tumours was

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Table 1: Weekly changes in average size of canine transmissible venereal tumours after intratumoral Calmette-Guerin (BCG), vincristine or BCG/vincristine combination therapy.

Treatment	Tumour size (cm ²)					
	Before treatment	Week 1	Week 2	Week 3	Week 4	Week 5
Control	14.0 ± 2.0	14.1 ± 2.3	15.8 ± 2.2	15.7 ± 2.1	15.7 ± 2.0	15.9 ± 2.6
BCG	15.0 ± 2.1	15.2 ± 0.9	13.1 ± 0.7	9.2 ± 1.1	7.8 ± 0.7	6.3 ± 0.5
Vincristine	14.3 ± 1.8	13.1 ± 1.8	9.2 ± 2.0	5.8 ± 0.7	3.7 ± 0.9	3.3 ± 0.7
BCG/vincristine	14.7 ± 1.1	13.3 ± 0.2	7.5 ± 0.8	5.1 ± 1.1	2.9 ± 0.8	NA

NA = average could not be determined because some tumours had regressed
BCG = bacillus Calmette-Guerin.

calculated. The mean surface area of the tumours was 14.5 cm² (range 12.9–15.3 cm²). The dogs were kept under standard laboratory conditions and fed a standard diet and used after an acclimatisation period of at least 15 days.

Treatments

The dogs were randomly divided into 4 equal groups and the average size of tumours in each group was statistically similar (Table 1). In the 1st group (control), the tumours were injected with 5 ml normal buffered saline daily for 5 consecutive days. In the 2nd group, the tumours were injected intratumorally daily for 5 consecutive days with 5 ml *Mycobacterium bovis* BCG (Danish 1331 strain, Statens Serum Institut, Copenhagen, Denmark, with approximately 2.5 × 10⁸ CFU) dissolved in buffered saline. In the third group, animals were treated with vincristine IV (Abic, Netanya, Israel) at 0.025 mg/kg once a week for 5 consecutive weeks. In the 4th group, the tumours were treated intratumorally daily for 5 consecutive days with BCG, and the dogs were also subjected to treatment with vincristine at 0.025 mg/kg IV once a week for 5 consecutive weeks. Temperature, heart rate and respiration rates were taken daily during intratumoral injections and every week thereafter. Dogs were monitored daily for occurrence of side effects and followed-up for 84 days. Statistical analysis was performed by means of SPSS software (SPSS for Windows, Chicago, Illinois, USA). The average decrease in tumour size per week was obtained relative to the original size of the tumour. One-way analysis of variance was used to compare the decreases in tumour sizes for the different treatment protocols. *P*-value was set at 0.05.

Biopsies and histology

Before treatment the surface area of the tumours was measured and an incisional biopsy was taken. Subsequent biopsies were taken weekly and the surface area of the tumours was measured weekly until complete tumour regression. Complete regression was defined as the absence of

CTVT cells on histological examination. The biopsies were fixed in 10 % buffered formalin and routinely processed and sections were stained with haematoxylin and eosin (H&E). Lymphocytes are recognised as small round cells in H&E-stained sections. The number of TILs was obtained by counting the number of cells in at least 10 random ×400 high power field (HPF) using an optical light microscope and averaged. The number of mitotic cells was obtained in the same way as the number of TILs.

TUNEL assay for apoptosis

For the quantitative analyses of apoptosis, sections from paraffin-embedded tumours were assayed using the terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labelling (TUNEL) method (Roche, Penzberg, Germany). The numbers of TUNEL-positive cells were counted in at least 10 random 400 HPF and averaged. Sections from canine thymus were used as positive controls.

RESULTS

Response to therapy

Complete tumour regression was observed in all the dogs treated with vincristine, BCG or BCG/vincristine combination therapy. BCG/vincristine combination therapy had a shorter regression time (mean regression time of 32 ± 5 days, range 28–35 days) than both vincristine therapy (mean regression time of 38 ± 4 days, range 37–41 days) and BCG therapy (mean regression time of 44 ± 6 days, range 40–49 days). No tumour regression was observed in the control group after observing the dogs for 84 days.

The therapeutic efficacy of BCG/vincristine combination therapy based on time to regression was significantly greater than that of the BCG or vincristine (*P* < 0.05). When the average decreases in tumour sizes per week were compared it was shown that BCG/vincristine combination therapy was the best treatment regimen, followed by vincristine

and BCG (Table 1). No side effects were observed in treated animals. Body temperature, heart and respiratory rate remained normal during the treatment period.

Histological findings

Before treatment all tumours had similar histological features (Fig. 1). The tumours were composed of dense cellular sheets with little fibrovascular stroma. The tumour cells had a moderate amount of cytoplasm, round central nuclei with granular chromatin and single nucleoli. Mitotic indices were high, averaging 8 per ×400 HPF. A few apoptotic cells (averaging 6 per ×400 HPF) and TILs were present (averaging 20 per ×400 HPF).

In the control group (animals treated with intratumoral buffered saline), tumours showed minimal oedema and hyperaemia with unchanged number of mitotic and apoptotic tumour cells and a minimal increase in the number of TILs at the end of the 1st week after treatment (Table 2). The tumour cell morphology remained unchanged. In the 2nd week, oedema was no longer evident while the number of mitotic and apoptotic tumour cells remained unchanged. These findings remained unchanged in the subsequent weeks (Table 2).

In tumours treated with BCG, oedema and hyperaemia were observed at the end of the 1st week after treatment. This was accompanied by the appearance of macrophages and an increase in the number of TILs. At the end of the 2nd week no oedema was present and there was a marked increase in the number of macrophages and TILs (Fig. 2). There was no change in number of mitotic tumour cells (Table 2). During the subsequent weeks there was a gradual increase in the number of TILs followed by tumour cell apoptosis and necrosis, and replacement of the tumour parenchyma by fibrovascular tissue.

In tumours treated with vincristine, no change was observed in tumour cell density at the end of the 1st week after treatment. At the end of the 2nd week there was increased tumour cell apoptosis, and reduction in the number of mitotic

Table 2: Average mitotic figures, tumour-infiltrating lymphocytes and apoptotic cell counts in canine transmissible venereal tumours after intratumoral Calmette-Guerin (BCG), vincristine, or BCG/vincristine combination therapy.

Group	Parameter	Average counts at $\times 400$ HPF					
		Before treatment	Week 1	Week 2	Week 3	Week 4	Week 5
Control	Mitotic figures	8.3 \pm 1.5	8.5 \pm 2.0	7.9 \pm 0.6	8.8 \pm 1.1	8.1 \pm 3.0	7.8 \pm .5
	TIL	19 \pm 4	22 \pm 5	23 \pm 3	19 \pm 3	17 \pm 2	22 \pm 5
	Apoptotic cells	6.8 \pm 0.9	7.8 \pm 2.0	6.0 \pm 0.9	7.1 \pm 2.0	5.0 \pm 0.8	7.7 \pm 0.6
BCG	Mitotic figures	8.6 \pm 2.0	6.2 \pm 1.1	7.9 \pm 1.3	6.6 \pm 0.8	7.5 \pm 0.6	8.0 \pm 1.1
	TIL	22 \pm 6	50 \pm 7	230 \pm 8	235 \pm 10	247 \pm 12	220 \pm 13
	Apoptotic cells	4.7 \pm 1.0	4.5 \pm 0.8	9.1 \pm 0.3	10.1 \pm 0.4	12 \pm 1.1	15.5 \pm 0.4
Vincristine	Mitotic figures	7.7 \pm 0.8	3.5 \pm 0.7	3.1 \pm 0.2	3.0 \pm 0.4	2.9 \pm 0.5	1.8 \pm 0.7
	TIL	21 \pm 3	14 \pm 2	10 \pm 2	7 \pm 2	5 \pm 1	5 \pm 1
	Apoptotic cells	6.1 \pm 1.1	17 \pm 5.1	22.0 \pm 5.1	28 \pm 3.0	29 \pm 7.1	32.0 \pm 6.0
BCG/vincristine	Mitotic figures	7.5 \pm 0.9	2.8 \pm 0.3	2.7 \pm 0.2	1.0 \pm 0.3	0.0	NA
	TIL	17 \pm 4	80 \pm 15	150 \pm 13	200 \pm 10	240 \pm 7	NA
	Apoptotic cells	6.0 \pm 1.3	18.6 \pm 1.1	28.0 \pm 3.0	33.2 \pm 2.8	32.1 \pm 2.5	NA

BCG = bacillus Calmette-Guerin.

TILs = tumour-infiltrating lymphocytes.

NA = average could not be determined because some tumours had regressed.

tumour cells and a decrease in the number of TILs (Fig. 3) (Table 2). During subsequent weeks, there was gradual increase in apoptosis of tumour cells and reduction in mitotic figures and increase in fibrovascular tissue (Table 2).

In tumours treated with BCG/vincristine combination therapy, tumours had oedema and hyperaemia accompanied by a marked increase in the number of macrophages and TILs and increased apoptosis at the end of the 1st week after treatment (Table 2). At the end of the 2nd week, tumours were characterised by marked increase in apoptosis, and necrosis of tumour cells and complete loss of mitotic tumour cells. At the end of the 4th week after treatment most of the tumours were completely replaced by fibrovascular tissue (Fig. 4).

DISCUSSION

Treatment is indicated in clinical cases of canine transmissible venereal tumour (CTVT) because spontaneous regression is not ordinarily expected in naturally occurring disease¹⁵. This was evident with the controls in this study which showed no tumour regression in the 84 days of this study.

The most effective chemotherapeutic agent used to treat CTVT is vincristine¹. The antitumour effect of BCG is well established, and is the choice of treatment in human recurrent bladder carcinomas⁵, and equine sarcoids¹⁰. This study shows that treatment of naturally occurring CTVT with intratumoral BCG is feasible. No side effects were associated with this treatment. Hess and colleagues showed that intralesional BCG therapy of CTVT was effective in causing tumour regression with a mean regression time of 63 days⁶. In this study the mean regression time was 44 days. The shorter regression time in this study could be attributed

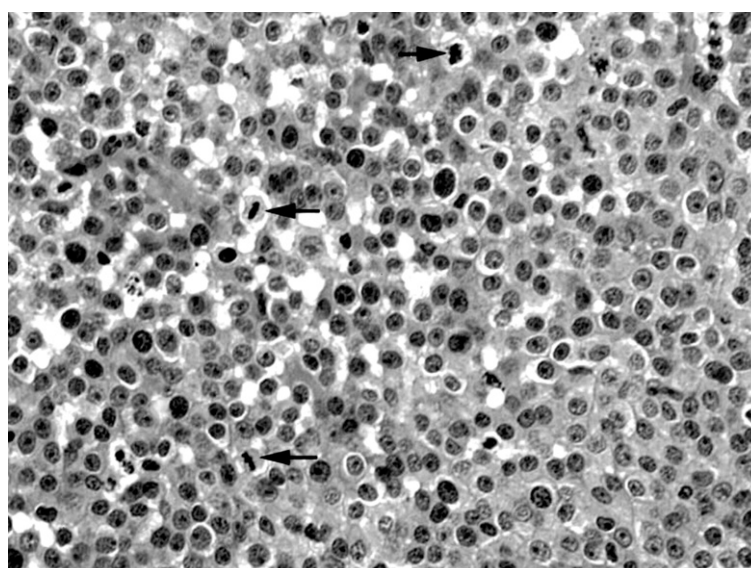


Fig. 1: Histology of CTVT during the progressive phase before treatment. The tumour is highly cellular and has many mitoses (arrows). Representative photomicrograph ($\times 400$ magnification) of H&E-stained sections.

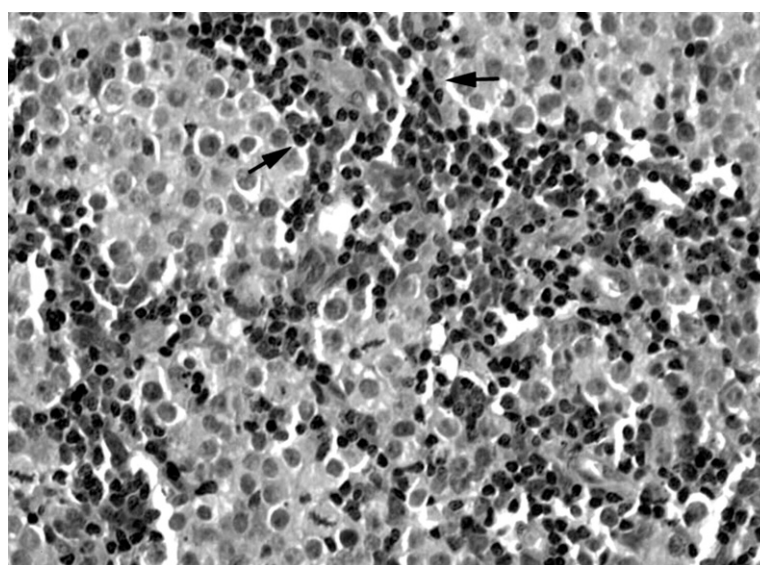


Fig. 2: Histology of CTVT 2 weeks after treatment with BCG. Increase in the number of tumour-infiltrating lymphocytes (arrows). Representative photomicrograph ($\times 400$ magnification) of H&E-stained sections.

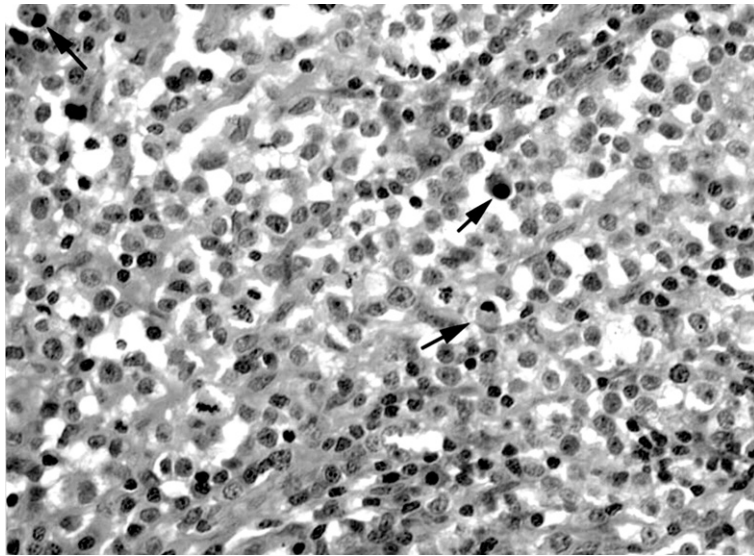


Fig. 3: Histology of CTVT 2 weeks after treatment with vincristine. Apoptosis of tumour cells (arrows) and reduction in the number of cells undergoing mitosis. Representative photomicrograph ($\times 400$ magnification) of H&E-stained sections.

to the increased number of intratumoral injections or the different preparation of BCG used in this study. In humans, despite good clinical efficacy, intravesical BCG treatment of bladder is associated with occurrence of various adverse effects including pneumonia and fever¹⁷. No side effects were observed with intratumoral BCG treatment of CTVT.

In this study we showed that the regression time in tumours subjected to immunochemotherapy was significantly shorter than that of vincristine or BCG alone ($P < 0.05$). Shin and colleagues demonstrated that vincristine can be used as the optimal chemotherapeutic agent for combined chemotherapy with dendritic cell based immunotherapy of murine fibrosarcoma models²². No previous studies have been done for combined

vincristine and BCG therapy.

Vincristine is a naturally occurring vinca alkaloid that inhibits cell mitosis by inhibiting tubulin polymerisation and thus preventing metaphase spindle formation⁹. In this study we showed that vincristine caused reduction in tumour cell mitosis followed by apoptosis. However, vincristine also resulted in the reduction of TILs compared with controls, suggesting that vincristine interferes with the host immune response to the tumour, probably by causing bone marrow suppression⁹ or by causing reduced clonal expansion of local monocytes and cytotoxic T-cells.

The mechanism by which BCG causes tumour regression is not clear. Previous in vivo animal models of BCG's antitumour effect suggest the critical role of cell-

mediated immune response²⁶. In this study, treatment of tumours with BCG was associated with appearance of numerous macrophages and TILs, suggesting that various mechanisms are involved in BCG-induced tumour regression. The multipronged response was also reflected in the observation of both necrosis and apoptosis of tumour cells during the 2nd week after treatment with BCG.

The role of tumour macrophages has been investigated by several scientists. Macrophages have been reported to effectively target and kill cancer cells through apoptosis primarily through the production of reactive nitrogen species⁷ and reactive oxygen species¹⁶.

CTVT cells during the progressive phase do not express major histocompatibility complex (MHC) class I antigens and therefore cannot be recognised by cytotoxic lymphocytes¹³. Macrophages could also have secreted cytokines which caused tumour cells to express MHC class I antigens. Cytokines such as gamma-interferon can activate several cancer cell lines to express MHC antigens²⁷. In addition, the contribution of tumour associated macrophages to the induction of major MHC I expression in vivo has been reported¹⁸.

On the other hand, macrophages could have produced cytokines which attracted TILs. Bacteria-stimulated macrophage supernatants have been shown to induce the migration of T-helper cell type 1 cells²⁵. BCG was also shown to cause proliferation of natural killer (NK) cells and BCG activated NK cells were shown to be cytotoxic to bladder cancers cells in a MHC unrestricted manner²³. In the present study we did not characterise the TILs that appeared after local BCG therapy. The 'effector' cell population responsible for the antitumour activity in human bladder cancers is currently believed to be NK cells and/or cytotoxic T-cells^{2,3}.

This study, based on a limited number of study cases, showed that immunochemotherapy (BCG and vincristine) of CTVT is more effective than vincristine therapy alone. Histological studies of the treated tumours suggest additive effects of vincristine and BCG and these results showed that CTVT may be used as a suitable experimental model for pharmacological studies on immunochemotherapy of tumours.

In conclusion, we have reported the clinical course, the histological findings and the outcome of dogs with progressing CTVT that were treated with intratumoral BCG, vincristine and combined BCG/vincristine. Vincristine caused tumour cell apoptosis while BCG stimulated the local

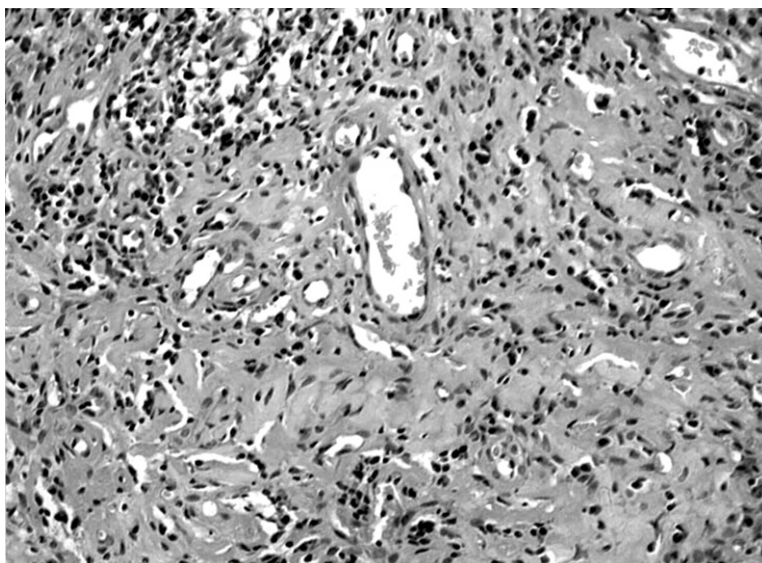


Fig. 4: Histology of CTVT 3 weeks after BCG/vincristine combination therapy. Replacement of tumour parenchyma by fibrovascular tissue. Representative photomicrograph ($\times 400$ magnification) of H&E-stained sections.

host immune system resulting in an increase in macrophages and TILs that induce tumour cell necrosis and apoptosis. These findings might provide rationale for decrease in the number of vincristine injections needed for treatment of CTVT. Further studies using more cases are warranted to address this issue. The role of specific of NKs, cytotoxic lymphocytes, T-helper cells and other inflammatory cells needs to be investigated.

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