Non-Specific Immune Responses Elicited By Phagocytes On The Dermatophyte:

Trichophyton verrucosum

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Abstract

Trichophyton verrucosum is a zoophilic dermatophyte that causes ringworm in man and animals worldwide. Phagocytosis was demonstrated to be an effectual defence mechanism against few dermatophytes. Yet, the effect of this mechanism on *T. verrucosum* has not been established. The objective of the study was to determine the cytotoxic effects of polymorphic neutrophils (PMN) and mononuclear leukocytes (MNL) collected from normal, experimentally infected or vaccinated guinea pigs on *T. verrucosum* spores and germlings.

In general, PMNs and MNLs exerted similar cytotoxic effects on both spores and germlings.

Cytotoxic activities exerted by PMNs on spores and germlings of *T. verrucosum* were found to be higher than that exerted by MNLs. Increasing target/effector cell ratio and time in culture also increased cytotoxic effects of both phagocytes. Furthermore, it was demonstrated that previous priming by infection or vaccination had no effect on PMNs-cytotoxic activities but enhanced the cytotoxic effects exerted by MNLs.

Results indicated that PMNs and MNLs were effective in killing *T. verrucusum* spores and germlings in-*vitro.*

Introduction

The dermatophytes are taxonomically related fungi causing different skin infections referred to as tineas in man or ringworm in man and animals. The zoophilic dermatophyte *Trichophyton verrucosum* is associated principally with cattle and camel ringworm (FadlElmula et al., 1994), but it has been reported to infect a wide range of animal hosts together with man (Emmons et al. 1977),. In animals, the lesions start with thickening of skin, alopecia and scaliness. They may involve small circular areas or become confluent in extensive areas. The exudate of the inflammatory process glues hair together into thick grey asbestos-like crusts which reveal bleeding ulcerated areas on removal. Ringworm in animals in the Kingdom of Saudi Arabia (KSA) was reported. In an outbreak of a mixed skin infection in camels, *Dermatophilus congolensis* and *Microsporum gypsum* were isolated (Gitao et al., 1998). Clinical ringworm in camels was also reported (Al-Hendi et al. 1998). In man the disease has also been described in the K.S.A. (Venugopal and Venugopal, 1992; 1993).

Cell-mediated immune responses are considered to play a major role in combating ringworm (Grappel, 1981). Lepper (1972) demonstrated by skin tests, that cellular immunity was effective in elimination of ringworm infection caused by *T. verrucosum* in calves. Although circulating antibodies have been demonstrated in the sera of humans and animals with natural or experimental dermatophyte infections, evidence that antibodies have any protective role is largely inconclusive.

An invasion of the human and animal skin by microorganisms elicits host defence mechanisms. Phagocytosis is an essential defence mechanism against foreign matter in the body(Roitt, et al., 1993). Ingestion and killing of microorganisms is achieved by polymorphonuclear neutrophills (PMN) and mononuclear leukocytes (MNL). Engulfment of microorganisms, initiates metabolic changes that include an increase in oxygen uptake with production of hydrogen peroxide, superoxide anion, hydroxyl radicals and singlet oxygen. These products are highly toxic to microorganisms and the process is called respiratory burst (Babior, 1978).

Human neutrophils and monocytes exerted significant fungicidal effect on *Trichophyton quinckeanum* and *T. rubrum* suggesting that the respiratory burst of neutrophils and monocytes may be an important mechanism in the in vivo killing of dermatophytes (Calderon and Hay, 1987). This study is intended to see if *T. verrucosum* is susceptible to phagocytic cells and whether prior priming of an animal by vaccination could affect the fungicidal activity of the phagocytes.

Materials And Methods

Experimental Animals:

For experimentation, Dunkin-Hartley outbred guinea pigs were used. Peripheral leukocytes were prepared from blood of experimentally infected, immunized and control animals. Experimental infection and immunization were done using microconidia suspension of a heavily-sporulating strain of *T. verrucosum* which is known to be a slow-grower with poor harvest (Emmons et al. 1977).

Preparation of spores and germlings:

T. verrucosum strain (NCPF 684) originally isolated from a patient with tinea corporis in Belfast (U.K.), was used in this study. Spores and germlings were compared because as spores grow to become germ-tubes, more antigens are exposed and immunogens are released. A technique was developed to prepare spore suspension. This was done by inoculating the strain onto lactritmel agar (Borelli, 1962) which was reported by few workers to be useful for growth promotion (Badillet et al. 1975) and then incubated at 28^{o}C for three weeks. Surface growth was removed gently and kept in bottles of sterile distilled water (D.W.) which were vigorously shaken in a whirlimixer (Jenco) for 2-3 min, filtered through glass wool to remove mycelial fragments and reconstituted in sterile D.W. Viability was tested by plating onto brain heart infusion (BHI) agar. Germlings were obtained by mixing 250 µl of a spore suspension containing 1x 10^{7} spores/ml with 1 ml BHI broth. The culture was incubated at 28^{o}C for 18-24h to produce germ tubes approximately 2-3 times the length of the spores. This was determined by pilot experiments. The spores and germlings were

suspended in Eagle's minimum essential medium (EMEM) (Gibco) at concentration of 3 x 10^4/ml.

Experimental Infection and Vaccination:

Primary infection was done in ten animals and five animals served as control. The animals were prepared for primary infection by shaving an area approximately 4 x 4 cm at the back region. The inoculum site was gently scratched with a sterile scalpel until redness but no bleeding was observed, then disinfected with alcohol. The standard inoculum of 0.2 ml spore suspension (1 x 10^7 spores) in phosphate buffer saline (PBS) + 0.05% Tween 80 was applied and spread evenly over the site. Control animals were prepared in the same way but were inoculated with 0.2 ml of PBS + 0.05 % Tween 80 alone.

The spore suspension was used as live vaccine as reported by Sarkisov et al. (1971); however the preparation methods were different. In the present study, lactritmel agar and pure live spores were used while the other workers used live attenuated vaccine (Sarkisov et al. 1971). The vaccine was tested in ten guinea pigs and ten animals served as control. A total dose of 1 ml containing 1 x 10^7 spores per animal was inoculated intramuscularly divided into two doses at seven-days interval. Similarly, control guinea pigs were inoculated using sterile D.W. All animals were provided with dry diet in form of pellets and water ad libitum.

Preparation of Phagocytes:

Peripheral leukocytes were collected four weeks post-infection and vaccination as described by Weir (1986). Briefly, heparinized blood (10 ml) was mixed with 6% dextran (Sigma) at 5:1 ratio in 25 ml bottles. The bottles were allowed to stand at 37^{o}C for 1 hr. The leukocyte-rich cloudy supernate was treated with 0.83% ammonium chloride to lyse erythrocytes and then washed three times in EMEM at 500 g for 5 min. The leukocyte suspension was layered over Histopaque (density 1.077; Sigma) in a ratio of 2:1 in centrifuge tubes. The tubes were centrifuged at 1500 g for 10 min. Cells collected from the interface were mononuclear cells. These were washed twice in balanced salt solution. The pelleted cells, mainly neutrophils, were

collected and introduced in 0.83% ammonium chloride bottles for 10 min at room temp to lyse the remaining erythrocytes. PMN and MNL were resuspended in EMEM containing 5% autologous guinea pig serum.

Cytotoxic Assays:

PMNs and MNLs from control, infected or vaccinated animals were mixed with spores and germlings in ratio of 1:1 and 1:25 in small tubes in a total volume of 1 ml. The control tubes were set by incubating spores or germlings (3×10^4/ml) without leukocytes. The mixture was shaken at 100 rpm for 5 min in a Rotatest shaker (model R100, Luckham). The cells were incubated at 37°C in an atmosphere of 5-10% CO^2 for either three or 24 h. After incubation, cells were treated with cold DW to disrupt leukocytes. They were then washed three times at 3000 g/10 min and resuspended in PBS. The mixture was vigorously shaken on a whirlimixer (Jenco) and clumps were broken by repeated passing through a fine pipettes. A volume containing approx. 150-200 spores or germling was mixed with molten BHI agar cooled to 37°C – 40oC and distributed into 5 mm petri dishes (Sterilin). The dishes were incubated at 30°C for 5-7 days and then colony-forming units (CFU) per plate were counted. Viability percentage (V%) was calculated as follows:

$$V\% = \frac{\text{CFU in test solution}}{\text{CFU in control solution}} \times 100$$

to indicate cytotoxic perecentage. The mean cytotoxic percentage was calculated for each type of animal treatment. Statistical analysis was done usig x^2 (chi square) test (Clarke, 1984).

Results

The cytotoxic activity of PMNs and MNLs from control, infected and vaccinated guinea pigs was tested against spores and germlings. In general, PMNs, and MNLs exerted similar cytotoxic effects on both spores and germlings (Figs. 1-4).

Increasing the target: effector cell ratio from 1:1 to 1:25 increased cytotoxic percentage in PMNs from control animals by 6.4% in sproes and 6.1% in germling (Figs. 1 and 3a).

An increase in target: effector cell ratio in MNLs from controls increased killings by 1.8% in spores and 2.8% in germlings (Figs. 2 & 4a). PMNs from experimentally infected animals, increased cytotoxic percentage by 10.5% in sproes and 5.9% in germlings (Figs. 1 & 3a). The comparable figures for cytotoxicity by MNLs was 3.7% in sproes and 3.4% in germlings (Figs. 2 and 4a).

In immunized animals, increase of the target: effector cell ratio increased cytotoxicity by PMNs by 8% in spores and 11.4% in germlings (Fig. 1 and 3a) while the cytotoxic effect of MNLs increased by 1.5% in spores and 1% for germlings (Figs. 2 and 4a).

The time duration of culture also affected the cytotoxic activity of the cells. After 3 hr. incubation, PMNs and MNLs from the three groups showed less than 20% cytotoxicity at target: effector cell ratio 1:1 (Fig.s 1 and 2). Incubation for 24 h at target: effector cell ratio 1:25 was tested. PMNs from the three groups of animals showed a cytotoxic percetnage of about 40% for spores and germlings (Fig. 3b). MNLs in general showed a reduced cytotoxic effect on spores and germlings. Cells from experimentally infected and immunized guinea pigs exerted a higher cytotoxic percentage (26% cytotoxicity on average) comapred to MNLs from control animals (17% cytotoxicity) (Fig. 4b).

Discussion

The possible role of PMNs and MNLs in the cytotoxicity of spores and germlings of *T. verrucosum* was investigated. Few workers have studied cytotoxic activity of phagocytic cells on dermatophytes. Calderon and Hay (1987), examined the cytotoxic effect of human neutrophils and monocytes on dermatophytes. They showed that neutrophils had substantial effects against germlings of *T. quinckeanum* and *T. rubrum*, while monocytes showed less cytotoxic activity but lymphocytes had none. In the present study, PMNs or MNLs from guinea pigs generally exerted similar cytotoxic activity against spores or germlings and there was no significant difference ($P<0.05$). This point needs further investigation as sproes and germlings differ in size and possibly in immunogenic stimuli as well.

Cytotoxic effects increased with increasing target: effector cell ratio from 1:1 to 1:25 ($P<0.05$). There was a difference in cytotoxic activity of PMNs and MNLs on spores or germlings at 1:25 ratio after 3 hrs incubation ($P<0.1$) and after 24 hrs ($P<0.05$). For instance, after 3 hr. incubation at target: effector cell ratio of 1:25, the cytotoxic percentage of PMN was between 20 and 30% and after 24 hr. the percentage was about 40%. The cytototoxic percetnage of MNLs after 3 hr. was between 10 and 20% and after 24 hrs was between 20 and 30%. Phagocytic cells from the three groups of guinea pigs showed some differences in their cytotoxic activities. Almost no difference was observed in cytotoxic percentage of PMNs from control, infected or vaccinated guinea pigs after 3 or 24 hrs culture ($P<0.5$). However, MNLs from control, infected or vaccinated guinea pigs displayed different cytotoxic effects. After incubation for 3 hrs, MNLs from the three groups showed similar cytotoxic percentages, but after 24 hrs some differences were observed. MNLs from infected guinea pigs had higher cytotoxic percentage than MNLs from control aninmals and MNLs from vaccinated guinea pigs showed higher cytotoxicity than those from infected and control animals. However, these arithmatical differences were not statistically significant ($P<0.05$) These results may indicate that previous priming by infection or vaccination had no effect on PMN cytotoxic effect but affected the cytotoxic activity of MNLs. This may be explained by the fact that polymorphonucler leucocytes do not show specificity for antigens(Roitt et al., 1993). Exposure to microbial products could activate

macrophages and monocytes (Roitt et al., 1993). Previous priming must have sensitized lymphocytes to *T. verrucosum* antigens and re-exposure to the same antigens enhanced their phagocytic and cytotoxic activities as demonstrated in the present study. Natural killer (NK) cells, being lymphocytic cells, are cytotoxic and have been reported to play a role in resistance to fungal infection. Recent evidence suggests that NK cells and some T-lymphocytes may be responsible for antibody-dependent cell-mediated cytotoxicity (ADCC). (Hyde, 1995). Our results may give further evidence that cell mediated immunity is important tin fighting dermatophytoses as reported by other investigators (Grappel, 1981; Sparke et al. 1995). The findings of the present experiment showed some resemblance to those reported by Calderon and Hay (1987). They further suggested that products of respiratory burst of neutrophils may be important in killing dermatophytes. Other killing mechanisms of phagocytes may contribute to their fungicidal effects. Oxygen-independent killing mechanisms against dermatophytes need to be investigated. It was reported that nitric oxide pathway is effective against the yeast Cryptococcus (Roitt et al., 1993). It could be important efficient mechanisms as well in killing of *T. verrucosum*.

In conclusion, PMNs and MNLs were demonstrated to play a role in cytotoxicity to *T. verrucosum* in-vitro. Cell-mediated immune responses are important in defence against *T. verucosum*. Previous priming of animals by infection or vaccination by *T. verrucosum* spores enhanced the immune responses on reexposure, thus, afforded a degree of protection.

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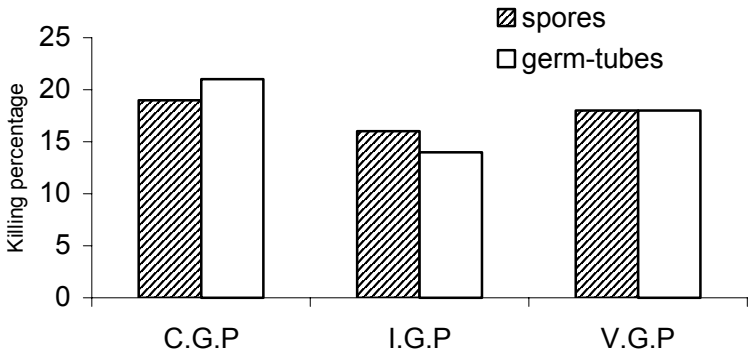


Fig. 1 Fungicidal effect of guinea pig PMNs on spores and germlings of *T. verrucosum* at target-effector cell ratio of 1:1 after incubation for 3h.

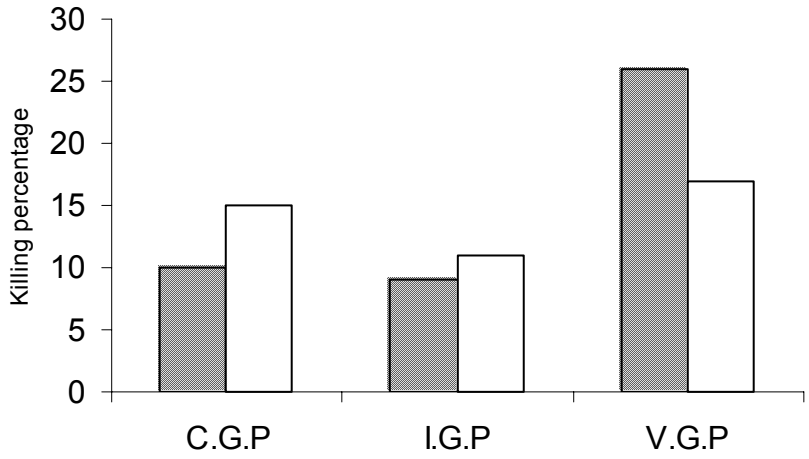
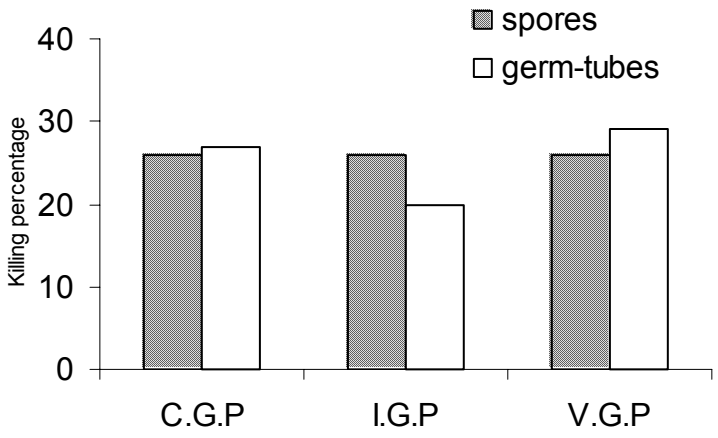


Fig. 2 Fungicidal effect of guinea pig mononuclear leucocytes (MNLs) on spores and germlings of *T. verrucosum* at target-effector cell ratio of 1:1 after incubation for 3h.
C.G.P. = Control guinea pig,
I.G.P. = Infected guinea pig And
V.G.P. = Vaccinated guinea pig.

(a)



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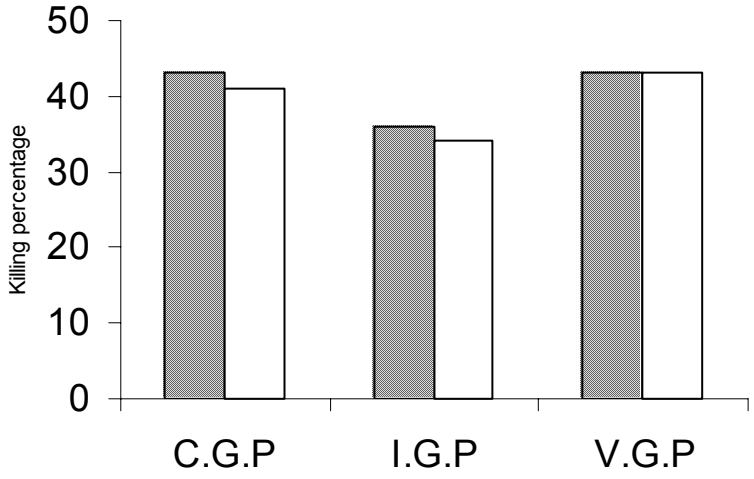
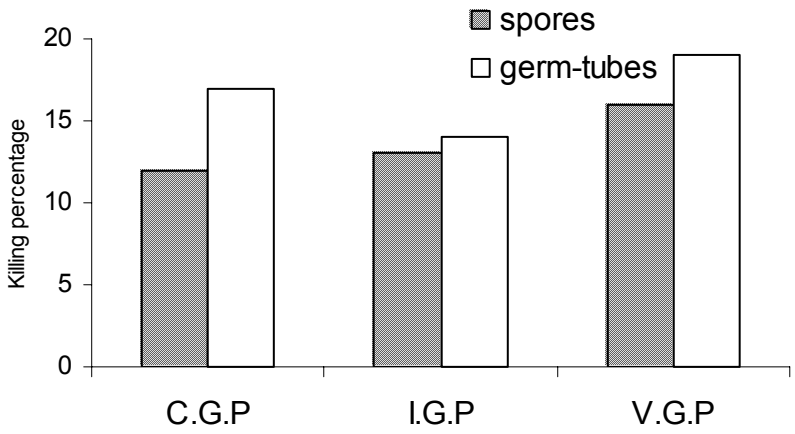


Fig. 3 Fungicidal effect of guinea pig (PMNs) on spores and germlings of *T. verrucosum* at target-effector cell ratio of 1:25 after incubation for 3h (a) and 24h (b).

C.G.P. = Control guinea pig,
I.G.P. = Infected guinea pig And
V.G.P. = Vaccinated guinea pig.

(a)



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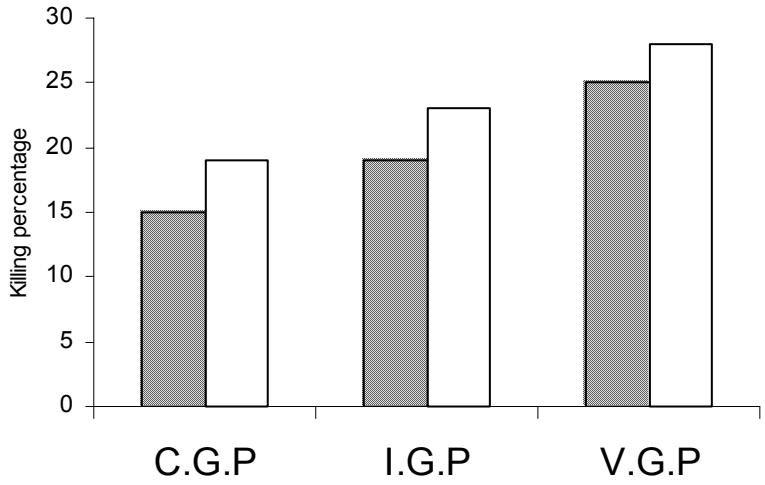

Fig 4 Fungicidal effect of guinea pig (MNLs) on spores and germlings of *T. verrucosum* at target-effector cell ratio of 1:25 after incubation for 3h (a) and 24h (b).

C.G.P. = Control guinea pig,
I.G.P. = Infected guinea pig And
V.G.P. = Vaccinated guinea pig.

الاستجابة المناعية غير النوعية من البلاعم للفطر الجلدي تراكوفايتون فيروكوزم

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الملخص :

يهدف هذا البحث الى تحديد الاثار السمية للعدلات مفصصة النوى و وحيدات النوى على غبيرات و انابيب الانبات للفطر الجلدى تراكوفايتون فيروكوزم.

إستعملت القبيعة كحيوان تجارب و تمت اصابته بالخمج الفطرى فى جلده و تلقيحة بواسطة غبيرات الشعروية المبرقة. بعد ذلك إستخلصت الخلايا البلعمية من نوع العدلات مفصصة النوى و وحيدات النوى من هذه الحيوانات و من حيوانات سليمة و تمت دراسة آثارها القاتلة على غبيرات و أنابيب الانبات من تراكوفايتون فيروكوزم.

أظهرت الدراسة إن سمية العدلات مفصصة النوى أكثر من سمية وحيدات النوى على الغبيرات و انابيب الانبات كما أثبت إن زياده نسبة الخلايا المستهدفة / الخلايا المستفعلة و زيادة زمن التجربة فى وسط الاستزراع أدت الى زيادة الآثار القاتلة للنوعين من البلاعم.

و اكثر من ذلك أوضحت الدراسة إن خبرة الجسم السابقة بالفطر عن طريق الخمج او التلقيح لم تؤ ثر على سمية العدلات المفصصة النوى فى حين إن لها أثر علىالأنشطة البلعمية لوحيدات النوى.

البلعمة و السمية بواسطة العدلات و وحيدات النوى كأحد آليات الدفاع النوعية وغير النوعية ثبت انها مجدية فى التخلص من أحماج الفطر الجلدى تراكوفايتون فيروكوزم.