Investigation of the Immunostimulatory Properties of Oxihumate

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A unique process has been developed to convert bituminous coal by controlled wet oxidation followed by base treatment to a water-soluble humate called oxihumate. The effects of oxihumate on the proliferative response of lymphocytes has been studied *in vitro* and *ex vivo*. Oxihumate increased the proliferative response of phytohaemagglutinin-stimulated human lymphocytes, from a concentration of 20 μg/ml and upwards. This response was even more striking in the case of lymphocytes from HIV-infected patients and was not limited to the *in vitro* setting since similar effects were observed *ex vivo* following administration of a non-toxic dosage of 4 g oxihumate per day to HIV-positive individuals for two weeks. Mechanistic studies revealed that stimulation of the proliferative response of lymphocytes by oxihumate is associated with an increased production of IL-2, as well as expression of the IL-2 receptor in the setting of decreased production of IL-10. Oxihumate therefore holds promise for the treatment of immunocompromized patients.

Key words: Oxihumate, Immunostimulation, IL-2

Introduction

Humic substances are widely spread in nature. They occur mainly in heavily degraded peat but also in all natural environments in which organic materials and microorganisms are, or have been present (Visser, 1973; Hartenstein, 1981). Peat extracts have been used from ancient times in therapeutic baths for the treatment of various conditions for many years (Brandt, 1964; Eichelsdörfer, 1976; Klöcking, 1994). The antiseptic properties of peat were first recognized during World War I when it was applied directly on to wounds to prevent infection (Haanel, 1924).

More recently, humate has been used in the treatment of Von Willebrand disease (Lopez-Fernandez *et al.*, 1992). Patients were treated with an infusion of 35 mg/kg body weight after which normal factor VIII levels were achieved.

A unique process has been developed to convert bituminous coal by controlled wet oxidation, followed by base treatment to water-soluble humates, called oxihumate (the potassium salt of oxihumic acid) (Bergh *et al.*, 1997). The possible application of coal-derived humic and fulvic acid as antimicrobials, has been described by Cloete *et al.* (1990) and Van Rensburg *et al.* (2000) whereas the anti-inflammatory properties of coal-derived fulvic acid has been reported recently by Van Rensburg *et al.* (2001) and Snyman *et al.* (in press).

Antiviral properties, at a concentration of 100 μg/ml of ammonium humate (the ammonium salt of humic acid) *in vitro* has been described by Thiel *et al.* (1981) resulting in the successful use of this agent as a topical treatment for herpes virus-induced skin diseases (Klöcking *et al.*, 1983). Schneider *et al.* (1996) reported on the anti-HIV activity of synthetic humate analogues derived from hydroquinone. These compounds inhibited HIV-1 infection of MT-2 cells with an impressively low IC_{50} of 50–300 ng/ml. The infectivity of HIV particles was inhibited by interference of a V3 loop-mediated step of virus entry. Similar results were found with oxihumate (Van Rensburg *et al.*, 2002). In this study we investigated the effects of oxihumate on human lymphocyte functions.

Abbreviations: PHA, Phytohaemagglutinin A; MNL, mononuclear leukocytes.

Materials and Methods

Oxihumate

Oxihumate was provided as a 1% solution in water by Enerkom (Pty) Ltd, Pretoria, South Africa. Average values for the elemental composition of oxihumate are 40%, 2.5% and 1% for carbon, hydrogen and nitrogen respectively whereas the approximate molecular weight of humic acid obtained from oxidized coal is between 57 and 70 kD (Piccolo *et al.*, 2000).

Mononuclear leukocytes (MNL)

These were prepared as described previously (Anderson *et al.*, 1993) by density centrifugation on Histopaque-1077 (Sigma Diagnostics, St Louis, MO, USA) of blood taken either from healthy adult human volunteers or HIV-infected individuals (with a CD4 count between 209 and 504 X 10^6/l) before treatment or after a 2-week treatment of either placebo, 4 g or 6 g oxihumate taken orally per day. These patients participated in a pilot study to evaluate the therapeutic efficacy of oxihumate in HIV-infected individuals. This trial was carried out in accordance with the World Medical Association Declaration of Helsinki. All ethical and legal standards were followed as determined by the University of Pretoria, as well as the Medicine Control Council of South Africa.

The cells were then either resuspended to 2×10^6/ml in complete RPMI 1640 medium (supplemented with 1% glutamine, penicillin and streptomycin at 100 μg/ml and 10% heat inactivated fetal calf serum obtained from Bio Whittaker, Walkersville, Maryland) or incubated first in complete medium in 5 ml tissue culture flasks for 30 min to remove adherent cells from the suspension before re-suspending to 2×10^6/ml.

Mitogen-activated MNL proliferation

Fifty microliters of MNL suspension (1×10^5 cells/well), were added to 110 μl of complete RPMI 1640 medium in the wells of microtiter culture plates (96 wells) followed by 20 μl of various oxihumate concentrations (5–100 μg/ml) and 20 μl of the mitogen phytohaemagglutinin (PHA, 5 μg/ml final concentration). Control systems without oxihumate and/or mitogen were included and the final volumes of all the wells were adjusted to 200 μl. After 72 hours incubation (37 °C in an atmosphere of 5% CO_2) the extent of lymphocyte proliferation was assayed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reactivity which detects only viable cells (Mosman, 1983). The plates were read on a Ceres UV 900 micro-ELISA reader using a test wavelength of 540 nm and a reference wavelength of 620 nm.

Measurement of cytokines

These experiments were set up as described above. MNL suspensions (1 ml) obtained from healthy donors were treated in the presence of PHA (5 μg/ml) with 60, 80 and 100 μg/ml oxihumate for 72 h in 5 ml plastic tubes and the supernatants collected and stored at – 70 °C for subsequent IL-2 and IL-10 determination using the relevant Biotrak TM human ELISA systems from Amersham TM (Amersham International Plc, Buckinghamshire, England).

Expression of CD25

MNL suspensions obtained from healthy donors were treated in the presence of PHA (5 μg/ml) with or without 100 μg/ml oxihumate for 72 h and CD25 expression measured with an FITC-conjugated monoclonal antibody against CD25. Flow cytometric analysis was performed using a Coulter Epics XL-MLC flow cytometer (Coulter Corp, Miami, Florida, USA) equipped with a 488 nm air-cooled argon laser.

Results

MNL proliferation

Oxihumate had no effect on resting lymphocytes up to a concentration of 100 μg/ml, but increased the proliferative response of PHA-stimulated monocyte depleted lymphocytes at 20 μg/ml and upwards in a dose-related manner (Fig. 1A). Similar effects were seen when monocyte rich MNL were used (Fig. 1B). This response was even more striking in the case of monocyte rich lymphocytes (MNL) from HIV-infected patients (Fig. 1B).

Significant ($p < 0.05$) increases in PHA-stimulated proliferation of MNL of HIV-infected individuals were also observed *ex vivo* following

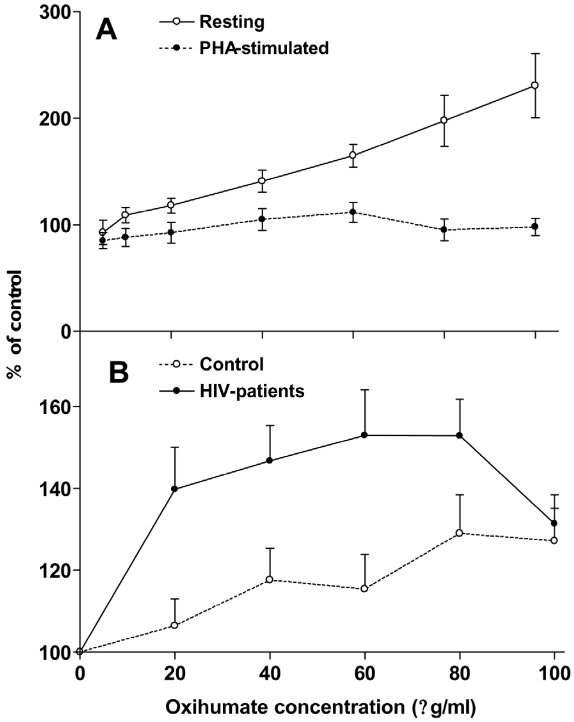

Fig. 1. The effects of a 72 h treatment with various concentrations of oxihumate on **[A]** resting and phytohaemagglutinin (PHA)-stimulated, monocyte-depleted, human lymphocyte proliferation and **[B]** PHA-stimulated mononuclear lymphocyte (MNL) cultures from normal donors and HIV-infected individuals. Results expressed as percentage of control ± SEM of 5–18 different experiments.

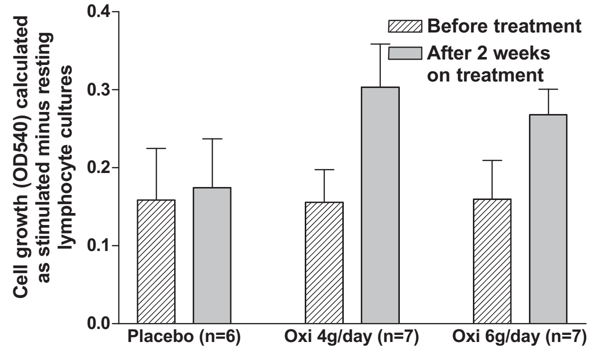

Fig. 2. The effects of a two week treatment of HIV-positive individuals with oxihumate on phytohaemagglutinin-stimulated mononuclear lymphocyte cultures *ex vivo*.

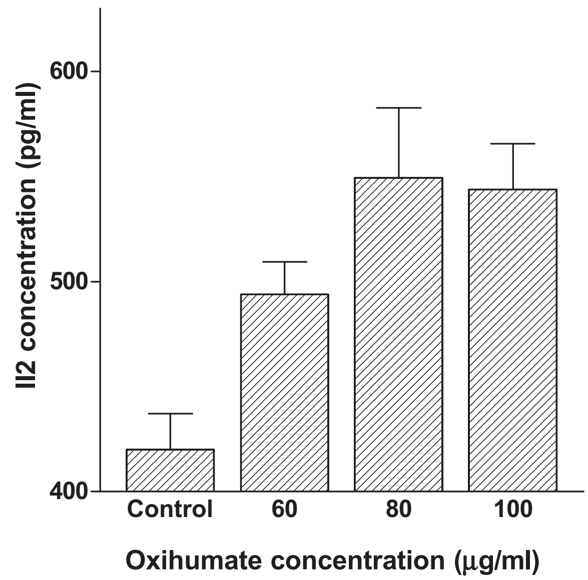

Fig. 3. The effects of a 72 h treatment of various concentrations of oxihumate on IL-2 production by phytohaemagglutinin-stimulated mononuclear lymphocyte cultures. Results expressed as the mean ± SEM of 4 different experiments.

administration of 4 g (but not 6 g) oxihumate per day for 2 weeks, compared to the placebo-treated group (Fig. 2).

IL-2 secretion

Oxihumate increased the secretion of IL-2 by PHA-stimulated MNL significantly ($p < 0.05$) at all three concentrations tested (Fig. 3).

IL-10 secretion

The effects of oxihumate treatment on IL-10 secretion by PHA-stimulated MNL are shown in Fig. 4. Oxihumate decreased the secretion of IL-10 at all three concentrations to the level observed in resting cells ($p < 0.001$).

CD25 expression

Oxihumate (at 100 µg/ml) increased the expression of the IL-2 receptor CD25 by PHA stimulated MNL significantly ($p < 0.05$) but had no effect on the levels of CD25 on resting MNL. Control values (median) for PHA stimulated MNL were 17.3 ± 0.3, whereas the values for MNL treated with 100 µg/ml oxihumate were 27.6 ± 0.2.

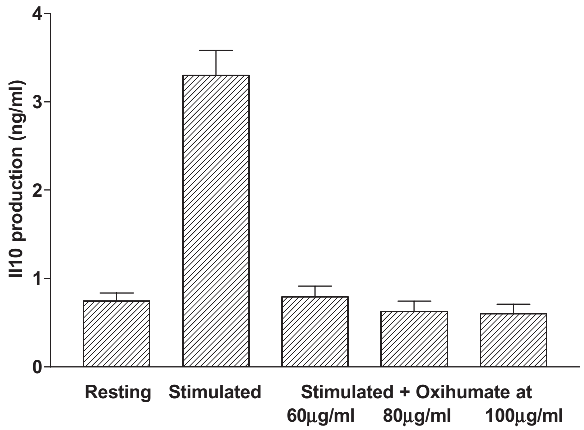

Fig. 4. The effects of a 72 h treatment of various concentrations of oxihumate on IL-10 production by phytohaemagglutinin-stimulated mononuclear lymphocyte cultures. Results expressed as the mean ± SEM of 4 different experiments.

Discussion

Although the HIV disease is associated with an increased rate of T-cell turnover, the loss of $CD4^+$ cell numbers exceeds the capacity to replenish with the resultant loss of cellular immune function (Losso *et al.*, 2000). A function of interleukin-2 (IL-2), a T-cell-derived cytokine, is to promote T-cell growth and maturation. Although IL-2 does not reduce viral replication *in vitro* (Kovacs *et al.*, 1996) it might counteract the virus-induced loss of $CD4^+$ cells in HIV infected individuals by increasing the proliferation of T-cells. IL-2, given in conjunction with a combination of highly active anti-retroviral therapy (HAART), causes dramatic increases in mean CD4 counts compared to HAART alone (Davey *et al.*, 2000; Shearer *et al.*, 1998).

Oxihumate, a water-soluble humate derived from coal, increased the proliferative response of PHA-stimulated MNL as well as monocyte depleted human lymphocytes, at 20 µg/ml and upwards. This response was even more striking in the case of lymphocytes from HIV-infected patients and was therefore not limited to the *in vitro* setting. Similar effects were observed *ex vivo* following administration of 4 g oxihumate per day to HIV positive individuals for two weeks. This increase can be attributed to increased production of IL-2, as well as expression of the IL-2 receptor (CD25) on lymphocytes. Oxihumate therefore seems to enhance the activity of T_{H1} cells (IL-2 producing cells) whilst decreasing, at the same time, IL-10, a T_{H2}-associated cytokine. The ability of some HIV-positive individuals to maintain normal T_{H1} type responses has long term protective effects on the survival of these patients as disease progression is attributed to a T_{H1} to T_{H2} cytokine shift (Shearer *et al.*, 1998; Altfeld *et al.*, 2000).

Oxihumate therefore possesses both immunostimulatory, as well as anti-viral activity (Van Rensburg *et al.*, 2002) and did not produce any measurable toxicity in experimental animals during either sub-chronic or acute oral or dermal exposure (Progress Report: Biochon (Pty) Ltd, Pretoria, South Africa, July 1999), nor did it produce any measurable toxicity in HIV-infected individuals treated with oral doses of up to 8 g per day for two weeks (Botes *et al.*, 2002). This combination of properties in one compound appears to be unique and merits further evaluation in immunocompromized patients such as those infected with HIV.

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