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TWO FUNCTIONALLY AND CHEMICALLY DISTINCT IMMUNOMODULATORY COMPOUNDS IN THE GEL OF *ALOE VERA*

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Summary

An aqueous extract of *Aloe vera* gel was analyzed guided by modulatory activity with regard to the in vitro activation of human complement and of human polymorphnuclear leucocytes (PMN). Upon ultrafiltration a high (h-M_r) and a low (l-M_r) molecular mass fraction were obtained. Pre-incubation of human pooled serum with the h-M_r fraction resulted in a depletion of classical and alternative pathway complement activity. In contrast, only the l-M_r fraction could inhibit the production of free oxygen radicals by activated PMNs. The latter activity cannot be attributed to non-specific effects like toxicity, interference with stimulant binding or scavenger activity.

Introduction

Historically, the mucilagenous parenchymous tissue (gel) of the leaves of *Aleo vera* (L.) Burm.f. (Liliaceae) has been used as a therapeutic agent for a plethora of medical and cosmetic purposes. These uses have stimulated studies on its clinical activity, its biochemistry and mode of action. The results of such studies have recently been discussed in a review paper by Grindlay and Reynolds (1986). Despite the overwhelming amount of data, there is as yet no clear correlation between the reported pharmacological properties of the gel and the identified components. This has prompted us to start a systematic study of the gel focussed on its application in the treatment of disorders such as bacterial infections (Solar et al., 1979) and arthritis (Saito et al., 1982). The study is aimed first at a characterization of the influence of a partially purified aqueous gel extract on the human immune system. In the present paper we report the interaction of gel

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compounds with the human complement system and the activation of human polymorphonuclear leukocytes (PMN). These two parts of the immune system play an important role in the antigen non-specific defense to infections.

Analysis of an aqueous extract of the gel guided by assays revealed two functionally different compounds, namely macromolecular compounds (h-M_r) with anti-complementary activity and low molecular mass (l-M_r) compounds which inhibit the biosynthesis of free oxygen radicals by activated PMN. The possible relation between our findings and the therapeutical use of the crude gel is discussed.

Materials and methods

Buffers

Five times concentrated veronal-saline buffer, containing 25 mM veronal (barbital) and 750 mM NaCl at pH 7.35 (VSB, 5×) was used. This served as a stock solution for VSB^{2+}, containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (Mayer, 1961) and for EGTA-VB, containing 5 mM Mg^{2+} and 8 mM ethyleneglycol-bis (2-aminoethyl)tetraacetic acid (EGTA).

Hanks balanced salt solution (HBSS) was prepared by dissolving the commercially available powdered medium (Gibco Ltd., Paisley, Scotland) in demineralized (demi) water. The pH of this solution was adjusted to pH 7.4 by the addition of $NaHCO_3$ according to the manufacturer's instructions. Before use, 0.1% (w/v) gelatin was added (HBSS-gel).

Preparation of plant extracts

The leaves of *Aloe vera* plants were harvested in Sri Lanka (Kurunegala and Badulla district). The mucilagenous parenchymous tissue was excised from fresh leaves, lyophilized and kept dry until use.

To obtain about 280 mg of an aqueous extract, 400 mg of the lyophilized gel was swollen in 100 ml HBSS under gentle stirring at room temperature. The yellowish extract was centrifuged for 15 min at 3000 rev./min to remove solid parts. The supernatant was decanted and subsequently fractionated by ultrafiltration under nitrogen pressure in an Amicon chamber Amicon Corp., Lexington MA, U.S.A.). Using a Diaflo YM-10 membrane (M_r cut-off value 10,000), a low molecular weight fraction was obtained with a dry weight of 190 mg and a high molecular weight fraction of 30 mg (l-M_r and h-M_r, respectively). The residue on the filter was dissolved in HBSS under gentle stirring. All fractions were kept frozen at −20 °C until use.

Hemolytic assay for human complement activity

Human pooled serum (HPS) from five healthy volunteers was used as a source of complement (C). Classical (CP) and alternative pathway (AP) C activities were determined in a microtiter assay (Klerx et al., 1983). The test was performed in V-welled microtiter plates (no. 651101; Greiner

Labortechnik, Nürtingen, F.R.G.). By use of a multichannel pipet (Titertek; Flow Laboratories, Solna, Finland), the samples to be tested on the ability to modulate CP and AP C activation were logarithmically diluted in 100 μl VSB^{2+} or EGTA-VB, respectively. Subsequently, 25 μl of a 4×10^{-2} and $4 \times 10^{-2.2}$ dilution of HPS in VSB^{2+} (CP) or a $4 \times 10^{-0.4}$ and $10^{-0.6}$ dilution of HPS in EGTA-VB (AP) were added per well. After a standard pre-incubation at 37 °C for 30 min, 25 μl of a 2×10^8 suspension of sensitized sheep erythrocytes (CP) or a 2×10^8 suspension of uncoated rabbit erythrocytes in EGTA-VB (AP) were added per well. The plates were incubated at 37 °C for 60 (CP) or 30 (AP) min. After the final incubation step, the plates were centrifuged for 5 min at 3000 rev./min (Labofuge) Heraeus-Christ GmbH, Osterode am Harz, F.R.G.) to precipitate intact cells and cell-ghosts. To quantify the hemolysis, 50 μl of the supernatants were mixed with 200 μl water in 96-well flat-bottom microtiter plates (no. 655101; Greiner). The absorbance at 405 nm was measured in an automatic Elisa reader (Titertek). Controls in this assay consisted of similarly treated supernatants of erythrocytes incubated in water (100% hemolysis), in VSB^{2+} or EGTA-VB (0% hemolysis) or in buffer supplemented with the appropriate HPS dilution (0% inhibition). Since the optimal serum concentration (giving rise to 40–60% lysis) showed some slight variation in the different experiments, two serum concentrations were tested routinely.

Isolation of human polymorphonuclear leukocytes (PMNs)

PMNs were isolated from the peripheral blood of healthy volunteers by dextran sedimentation and Ficoll density gradient centrifugation as described by Verhoef et al. (1977).

Radiolabelling of bacteria

Bacteria were radiolabelled by growing overnight in Mueller Hinton broth (Difco, Detroit MI, U.S.A.) with 4 μCi/ml [^{3}H]thymidine (Radiochemical Centre, Amersham, U.K.). The labelled cells were washed three times in phosphate-buffered saline (PBS, pH 7.4) and subsequently suspended in HBSS gel to a concentration of 5×10^8 colony-forming units (CFU) per ml using a spectrophotometric method.

Opsonization of zymosan and radiolabelled bacteria

To opsonize zymosan (from *Saccharomyces cerevisiae*, Sigma Chemical Co, St. Louis MO, U.S.A.), 1 ml of a 50-ml suspension in HBSS was mixed with 3 ml undiluted HPS. After incubation for 30 min at 37 °C, the suspension was centrifuged for 5 min at 3000 rev./min. The pellet was resuspended in 3 ml HBSS-gel.

For the opsonization of encapsulated *Escherichia coli* O8K43 or the unencapsulated mutant strain $O8K^-$, 200 μl of a bacterial suspension (2.5×10^8 CFU/ml) were mixed with 800 μl of a 20% or 1% (v/v) dilution of HPS in HBSS gel, respectively. After incubation at 37 °C for 30 min the bacteria

were washed with ice-cold PBS and pelleted at 3000 rev./min for 15 min and resuspended in 1 ml ice-cold HBSS gel.

Production of free oxygen radicals by PMN

The production of free oxygen radicals by activated PMN was determined by luminol-enhanced chemiluminescence (Aniansson et al., 1984). Briefly, 5×10^5 PMN were suspended in 550 µl ice-cold HBSS gel in 2-ml flat bottom vials (Sterilin Ltd., Feldham, Middlesex, U.K.). After addition of 100 µl of a 30 µM solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) in HBSS gel and a metal spinbar, the vials were placed in a picolite luminometer (Packard United Technologies, Downers Grove IL, U.S.A.) to equilibrate at 37 °C under gentle stirring. Subsequently, 50 µl of the opsonized zymosan suspension was added to each vial. The chemiluminescence was counted for 6 s at 2-min intervals over a total period of 16 min. Test samples were dissolved in HBSS.

The production of superoxide anion in vitro was measured by the reduction of succinyl-cytochrome *c* (Kuthan et al., 1982). The reaction mixture had a total volume of 1 ml, containing 100 mM PBS, 100 mM EDTA, 400 µg/ml rat liver microsomes, 32 µM cytochrome *c* and 100 µM NADPH. Menadione (50 µM) was added as substrate for superoxide production (Thor et al., 1982). Paired reactions with and without 10 µl speroxide dismutase (1 mg/ml) were performed. The kinetics of succinyl-cytochrome *c* reduction was determined in a double-beam spectrophotometer (Aminco DW-2a UV-Vis, American Instrument Company, Silver Spring MA, U.S.A.) at 550 nm.

Quantitation of phagocytosis

Phagocytosis by PMN was studied as described by Verhoef et al. (1977). Briefly, 200 µl of a suspension of 5×10^6 PMNs were mixed in triplicate with 200 µl of the radiolabelled bacterial suspension obtained after opsonization. Incubation was performed in snap-cap Bio-Vials (Beckman, Chicago IL, U.S.A.) placed in a shaking water-bath at a constant temperature of 37 °C. The incubation was stopped after 2, 6, or 12 min by adding 2 ml ice-cold PBS. Non-attached bacteria were removed by washing three times at 160 g with the same buffer. After addition of 3 ml of scintillation cocktail (Aqua Luma Plus, Lumac/3M bv, Schaesberg, The Netherlands), ^{3}H-incorporation was measured in a liquid scintillation counter.

Results

Immuno-modulatory activity

The crude aqueous extract of *Aloe vera* gel (50 ml) was passed through an ultrafiltration membrane. The residue was washed once with 50 ml of HBSS-gel. The final volumes of the thus obtained 1-M_r and h-M_r fractions were adjusted to 100 ml. Equal volumes of the three extracts were tested for modulation of C activation and of the zymosan-induced chemiluminescence of human peripheral blood PMN.

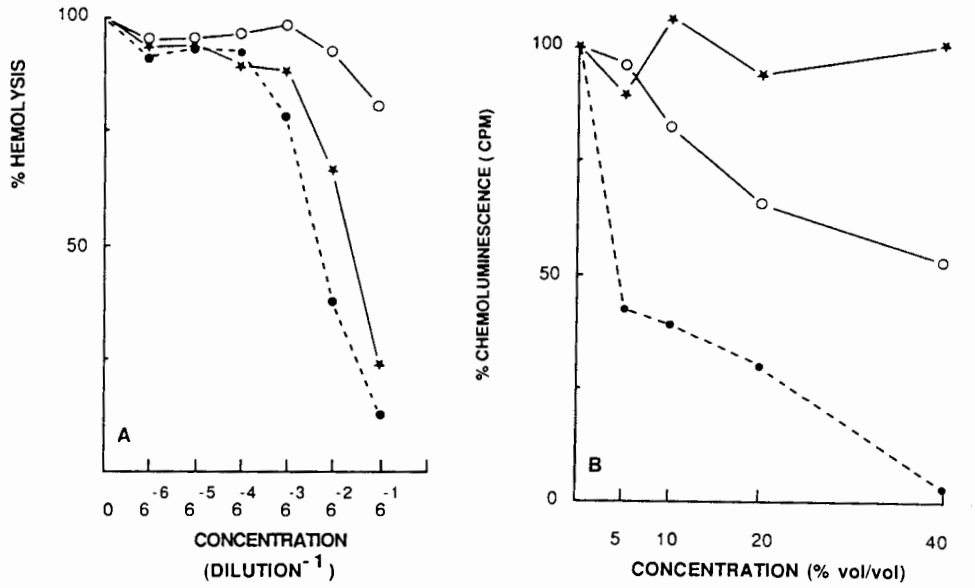

Fig. 1. Modulatory activity of the l-M_r and h-M_r fraction on CP complement activity and PMN activation. Fifty millilitres of an aqueous extract (●- - - - -●) of *Aloe vera* gel was passed through a YM-10 ultra-filtration membrane. The residue (h-M_r; ★———★) and the filtrate (l-M_r; O———O) were dissolved in 100 ml HBSS. (A) Logarithmic dilutions in VSB^{2+} of a 20% solution (v/v) of each fraction were incubated for 30 min at 37 °C with HPS. The chosen HPS dilution gave rise to 40–60% lysis. After a subsequent incubation for 60 min at 37 °C with sensitized sheep erythrocytes, hemolysis was quantified by the absorbance at 405 nm. The results are normalized by defining the control values (0% inhibition) at 100%. (B) Human PMNs were stimulated with opsonized zymosan particles. The production of free oxygen radicals was quantified by the luminol-enhanced chemiluminescence. The results are expressed as the percentage cpm from a control incubation (without gel extract). In both assays, the standard error for the results was within ± 10%.

Incubation of HPS with the crude extract and with the h-M_r fraction resulted in a marked dose-dependent depletion of CP C activity, as determined by the lysis of sensitized sheep erythrocytes. The l-M_r fraction, however, did not affect C activity (Fig. 1A). Conversely, apart from the crude extract, only the l-M_r fraction inhibited the chemiluminescence of PMN, whereas the h-M_r fraction was inactive in this assay (Fig. 1B). We concluded from these findings that the aqueous extract of *Aloe vera* gel contains two classes of chemically and functionally distinct immunomodulators.

Anti-complementary activity

In the next set of experiments the mechanism of C depletion during incubation of HPS with the h-M_r fraction of the gel was investigated. The results in Fig. 2 show the CP C activity in HPS when pre-incubation with

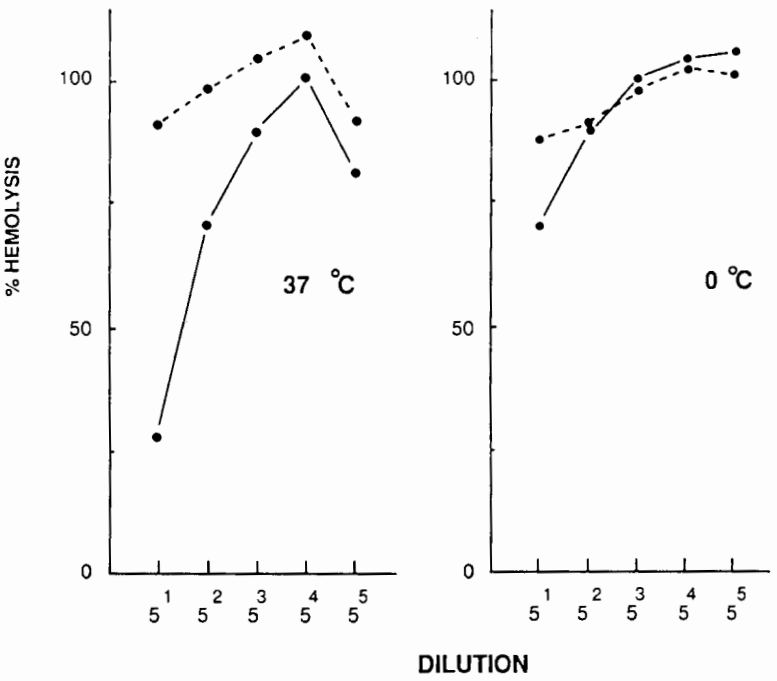

Fig. 2. Mode of depletion of CP complement activity. Logarithmic dilutions of the h-M_r fraction (●——●) or the solvent (●- - - - - - -●) in VSB^{2+} were incubated for 30 min at 37 °C or at 0 °C with HPS. The chosen HPS dilution gives rise to 40–60% lysis. After a subsequent incubation for 60 min at 37 °C with sensitized sheep erythrocytes, hemolysis was quantified by the absorbance at 405 nm. Results are normalized by defining the control values (0% inhibition) at 100%. The standard error for the results was within ± 10%.

the h-M_r fraction was performed at 0 and 37 °C, respectively. A negligible depletion of C activity (18%) was found when the plates were kept at 0 °C. However, at 37 °C a marked (up to 60%) dose-dependent depletion of C-activity was observed.

To find out at which part of the C cascade the h-M_r components exerted their inhibitory activity, terminal route activation in h-M_r treated HPS was effected also via the alternative pathway (AP). The results in Fig. 3 show a decrease of hemolytic activity. However, in the latter case the sample concentration required for inhibition is higher than for CP C activation.

Inhibitory effect on PMN

To exclude possible non-specific effects causing the inhibition of chemiluminescence of activated PMNs, the following activities of the l-M_r fraction were investigated: (i) cytotoxicity, (ii) interference with the binding of the stimulatory agent, and (iii) scavenger activity (capture of free oxygen radicals).

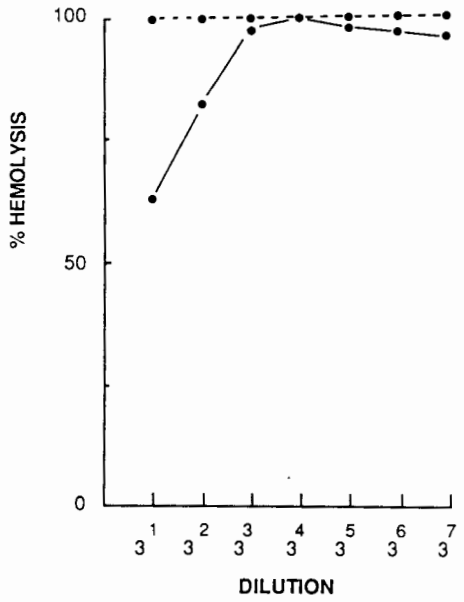

Fig. 3. Depletion of AP complement activity. Logarithmic dilutions of the h-M_r fraction (●——●) in EGTA-VB (solvent, ●- - - - - - -●) were incubated for 30 min at 37 °C with HPS. The chosen HPS dilution gave rise to 40–60% lysis. After a subsequent incubation for 30 min at 37 °C with uncoated rabbit erythrocytes, hemolysis was quantified by the absorbance at 405 nm. Results are normalized by defining the control values (0% inhibition) at 100%. The standard error for the results was within ± 10%.

The chemiluminescence in zymosan-activated PMNs reaches a peak level after 8–10 min. Addition of the l-M_r fraction resulted in a decrease of the peak level, but the reaction kinetics did not alter. The results in Fig. 4 demonstrate that incubation of PMN for 30 min with the l-M_r fraction did not influence the level of chemiluminescence after activation. Therefore, the inhibitory activity of the l-M_r fraction is not likely to be due to toxicity.

Next the possible interference of l-M_r components with the binding of the stimulant was studied. In this study, sensitized and radiolabelled bacteria were used instead of sensitized zymosan. Since both stimulants were used in an opsonized form, binding to the PMN occurs most likely via the same sites, namely complement and Fc receptors. As is shown in Table 1 the presence of the l-M_r fraction did not influence the uptake of radiolabel by the PMN.

Finally the interference of the l-M_r fraction with superoxide anion production in vitro was determined. The results in Table 2 show that the reduction of succinyl-cytochrome *c* was unaffected by the l-M_r fraction. Therefore, the presence of scavenger molecules in this fraction which could explain the reduction of superoxide anion production, is not likely.

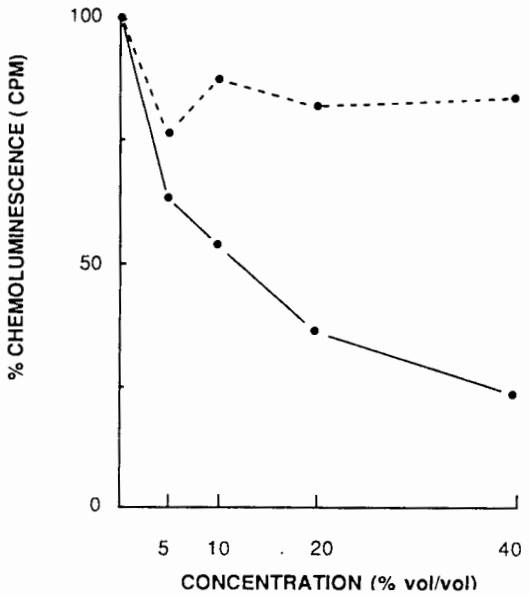

Fig. 4. Cytotoxicity of the l-M_r fraction. PMNs are incubated for 30 min at 37 °C in the absence (●——●) or presence (●- - - - - - -●) of the l-M_r fraction, diluted in HBSS. After thorough washing of the cells, they were tested for zymosan-induced chemiluminescence in the absence (●- - - - - - -●) or presence (●——●) of similar concentrations of the l-M_r fraction. Results are normalized by defining the control values (incubation without gel extrct) at 100%. The standard error for the results was within ± 10%.

TABLE 1

QUANTITATION OF THE BINDING BY PMN OF OPSONIZED STIMULANTS

PMNs were incubated at 37 °C in the presence of various concentrations of the 1-M_r fraction. The binding and subsequent phagocytosis of particulate antigens was quantitated by incubation with [^{3}H]thymidine-labelled bacteria. (A) encapsulated *E. coli* strain O8K43; (B) unencapsulated mutant O8K$^-$, which were opsonized in HPS. At the indicated time intervals, the phagocytotic process was arrested by the addition of ice-cold PBS. After thorough washing of the cells to remove unattached bacteria, ^{3}H-incorporation was determined in a liquid scintillation counter. The results are given as the percentage incorporation of the total amount of available radiolabel (dpm ± S.E.). NT = not tested.

Strains	Time (min)	Cell-bound ^{3}H (%, dpm) at l-M_r concentrations						
		0	0.5	1.0	2.0	5.0	10.0	25.0
A	2	51	47	53	29	53	55	59
	6	69	68	67	56	75	62	68
	12	66	61	65	58	71	65	63
B	2	48	45	54	NT	44	43	43
	4	83	76	98	NT	77	79	76
	12	80	75	85	NT	108	77	77

Mean total counts/vial = 9919 dpm ± 621.
Mean total counts/vial = 7304 dpm ± 572.

TABLE 2

SCAVENGER ACTIVITY OF THE l-M_r FRACTION

Free oxygen radical production in vitro by menadione was assayed by the reduction of succinylated cytochrome *c*. The possible scavenger activity of the l-M_r fractions was deduced from the modulation of the shift in light absorption at 550 nm (given in arbitrary units). Controls in this test consisted of incubations with the solvent (HBSS).

	Change at 550 nm
Control	0.044
10% l-M_r	0.038
10% Solvent	0.053
20% l-M_r	0.022
20% Solvent	0.022

Discussion

The results of the present study indicate that the aqueous extract of *Aloe vera* gel contains at least two classes of substances with immuno-modulatory activity in vitro. Incubation of HPS with the macromolecular h-M_r fraction at 37 °C resulted in a dose-dependent depletion of CP C activity. C depletion, however, did not take place when pre-incubation was performed at 0 °C (Fig. 2). These observations suggest that C consumption rather than (in)activation of (a single) C component(s) is involved. The inhibition of AP C activity points to the consumption of C components in the terminal route. The isolation and characterization of the active compounds is the subject of our current investigations. Preliminary results indicate that we may be dealing with a polysaccharide. In accordance with literature data (Gowda et al., 1979), hydrolysis of the h-M_r fraction contains, among neutral monosaccharides, also traces of glucuronic acid, which might contribute to the anionic character of the molecule. These findings favour a similar mechanism of C depletion in human serum as found in murine serum by the synthetic polyanion dextran-sulphate, which interferes with AP C activation (Klerx et al., 1985).

Only minor anti-complementary activity was detected in the l-M_r fraction, which contains substances smaller than 10,000. This fraction, however, was more potent in the modulation of zymosan-induced PMN activation. The complexity of this assay prompted us to exclude various artifacts. The binding of a stimulant to membrane receptors induces a transmembrane signal resulting in activation enzyme-systems which catalyze the production of free oxygen-radicals (Allen et al., 1972; McPhail et al., 1979).

Our results demonstrate that the activity of the l-M_r fraction could not be explained by the presence of compounds with a toxic or scavenger effect (Fig. 4). Moreover, since incubation with the l-M_r fraction did not interfere with the incorporation of radio-labelled bacteria, the attachment to

membrane receptors remains most likely unaffected. Therefore, it seems that some l-M_r substance(s) in the gel interfere(s) with those intracellular processes involved in receptor-mediated PMN activation pathways leading to the biosynthesis of oxygen radicals.

The isolation and characterization of the active l-M_r components are the focus of our present research. Preliminary results show that the inhibitory activity is reduced by passage of this fraction through a Con A-sepharose column, pointing at the presence of glycosidic structures. Analysis by thin-layer chromatography of the chloroform extractable residue from an acid hydrolysate suggests the presence of various anthracene derivatives.

Extrapolation of the results of the present in vitro study to the healing capacity of *Aloe vera* gel is a tempting but delicate matter. Our results, however, may provide better insight into the therapeutic value of the gel. Various authors have reported that polyanionic molecules possess adjuvant activity for cell-mediated and humoral immune responses to different antigens in rodents (Diamantstein et al., 1971; McCarthy et al., 1977; Kishima et al., 1983). Therefore, one might speculate that polyanions, leaking from the gel after topical application to an infected wound, could enhance the local immune response to pathogens. In addition, l-M_r substances might reduce local toxic effects of free oxygen radicals (Lunec et al., 1981) which are released in the pericellular tissue by the activated PMNs (Babior et al., 1973). This seems to be of value since the phagocytotic capacity of the PMNs remains unaffected.

Acknowledgements

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