

Colophony: an *in vitro* model for the induction of sensitization

J. ELMS, L. J. ALLAN, I. PENGELLY, D. FISHWICK, P. N. BECKETT and A. D. CURRAN

Health and Safety Laboratory, Sheffield, UK

Summary

Background The potential of colophony fumes from soldering flux to induce asthma has been known since the 1970s, however, no direct *in vitro* or *in vivo* evidence has been reported. The present study investigated the potential of colophony to stimulate human phagocytic cells to produce reactive oxygen species.

Methods The human cell line HL-60 was differentiated to produce cells with a monocyte-like and a neutrophil-like phenotypes. A number of procedures were used to confirm the phenotype of these differentiated cells including morphology, esterase activity, flow cytometry and phagocytosis. The potential of colophony to stimulate human phagocytic cells to produce reactive oxygen species was monitored using flow cytometry.

Results We were able to show that intracellular peroxide levels were increased in both monocyte-like and neutrophil-like cells, but not in undifferentiated HL-60 cells following the addition of colophony.

Conclusions The resin acid epoxides and hydroperoxides which have been suggested to be sensitizers in contact allergy, are degraded during the soldering process. However, conditions for the oxidation of colophony may occur *in vivo* as a result of the colophony-induced oxidative burst from neutrophils and monocytes. These oxidation products may then interact with body proteins to further initiate immune responses. Therefore for the preparation of low molecular weight chemical (LMWC)–protein conjugates, consideration must be taken to determine whether the LMWC is undergoing a reaction *in vivo* before it is interacting with body proteins.

Keywords: colophony, HL-60, monocytes, neutrophils, occupational asthma, oxidative burst

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Introduction

Low molecular weight chemicals (LMWCs) can cause a variety of occupational health problems including respiratory tract irritation and occupational asthma in susceptible individuals. It has been assumed that LMWCs interact with body proteins in the lung, and this LMWC–protein conjugate induces an immune response. For this reason LMWC–modified human serum albumin conjugates have been synthesized and used in the radioallergosorbent test (RAST) for the detection of specific immunoglobulin (Ig) E. However, for many LMWCs there is a poor correlation between specific IgE and clinical allergic symptoms. This has been documented for isocyanate-induced occupational

asthma, where the presence of specific IgE to isocyanate is apparent in only 10–20% of symptomatic subjects [1], and in the case of glutaraldehyde [2] where specific IgE was detected in only 18.2% subjects with clinically diagnosed occupational asthma. The measurement of specific IgE in individuals reporting occupational asthma caused by LMWCs does not therefore appear to be an appropriate or beneficial tool for monitoring exposed workers, as often the diagnosis of LMWC-induced occupational asthma does not correlate with the presence of specific IgE antibodies. The explanation for this observation may lie in the production of inappropriate LMWC–protein conjugates for the detection of specific IgE, or that a non-IgE mediated mechanism is responsible for inducing the respiratory symptoms.

In this study we have investigated the *in vitro* potential of colophony, as an example of a LMWC, to interact

Correspondence: J. Elms, Health and Safety Laboratory, Broad Lane, Sheffield, S3 7HQ, UK.

with immune cells and provide the conditions required to enable binding to body proteins and the induction of sensitization.

Colophony (rosin) is an example of a mixture of LMWCs which has been reported to cause allergic skin [3] and respiratory reactions. It is a natural product, which due to its stickiness, emulsifying and insulating properties is a common ingredient in a diverse range of products such as adhesives, ink, paints and soldering fluxes. The potential of colophony fumes to induce asthma has been recognized since the 1970s, and in 1994 the SWORD project reported some 5% of all new cases of occupational asthma to this cause [4]. Consequently there is an abundance of clinical evidence to suggest that individuals can become sensitized by colophony but there appears to be little immunological evidence to support this finding. It is well recognized that lung disorders caused by inhalable dusts [5] involve the production of reactive oxygen species by macrophages as a by-product of phagocytosis, and subsequent destruction of neighbouring cells. This observation accompanied with the previous lack of demonstrable specific antibodies to colophony in sensitized individuals [6] led us to investigate the mechanism of colophony-induced respiratory disorders using an *in vitro* challenge system.

Materials and methods

Colophony preparation

A small piece of the solid colophony was broken off to expose a fresh surface. Fifty milligrams of the freshly exposed colophony was placed in a 25-mL volumetric flask and made up to a 2-mg/mL solution with diethyl ether. An aliquot of this solution was taken and analysed as per MDHS83 [7] gas chromatographic method to determine the total resin acid (TRA) concentration. A 1-mg TRA aliquot of the colophony solution was transferred to the gas chromatographic vial and evaporated in a stream of dry nitrogen. The resulting residue was redissolved to give a final concentration containing 1 mg/mL TRA.

The absence of any measurable quantities of resin acid epoxides was confirmed by gas chromatographic analysis.

Cell culture

HL-60 cell line (obtained from the European Collection of Animal Cell Cultures) was cultured at 37 °C in RPMI-1640 (Life Technologies Ltd, Paisley, UK) supplemented with 10% v/v foetal calf serum (Life Technologies Ltd) and 0.1% v/v Gentamycin (Life Technologies Ltd) (RPMI/10%FCS/Gen) in a humidified incubator with 5% CO₂. The cells were suspended at 2×10^5 cells/mL, and the viability was monitored at each passage.

Induction of cell differentiation – differentiation to neutrophils

All-trans-retinoic acid (RA; Sigma, Poole, UK) was used to induce the differentiation of HL-60 cells to neutrophils [8]. DMSO has also been shown to induce differentiation, but due to antioxidant [9] effects was not employed in this investigation. RA was dissolved in ethanol, and was diluted in RPMI/10%FCS/Gen to concentrations of 1.7×10^{-8} , 1.7×10^{-7} and 1.7×10^{-6} mol/L with a final concentration of ethanol 0.1%. Cells were cultured in the above media for up to 6 days to promote their differentiation. Control cells were cultured with 0.1% ethanol in media to determine the effect of ethanol on cell differentiation and viability.

Induction of cell differentiation – differentiation into monocytes

1 α ,25-dihydroxyvitamin D (calcitriol, VD₃; a gift from Roche Discovery, Wellwyn Garden City, UK) was used to induce the differentiation of HL-60 cells to monocytes [10]. Phorbol 12-myristate 13-acetate has previously been demonstrated to induce monocyte differentiation but was not used as it would interfere with oxidative burst reactions. VD₃ was dissolved in ethanol to give final concentrations of 2.4×10^{-9} , 2.4×10^{-8} , 2.4×10^{-7} mol/L, respectively. Cells were cultured in the above media for up to 6 days to promote their differentiation.

Monitoring of differentiation – morphological changes

Control and treated cells were cytocentrifuged onto slides and examined using a light microscope (Leitz, Wetzlar, Germany) with attached light microscope and Frame Grabber software (Media Cybernetics, Silver Spring, MD, USA). Each slide was examined by two separate investigators (J. E. and L. A.).

Monitoring of differentiation – esterase activity

Cytocentrifuge slide preparations of control and treated cells were fixed with citrate-acetone-formaldehyde. The cell preparations were incubated with either α -naphthyl acetate (NAE) (Sigma) or naphthol AS-D chloroacetate (NCAE) (Sigma) in the presence of diazonium salts. Sites of NCAE activity stained as bright red granulation which identify cells of a neutrophil phenotype, whereas NAE activity appeared as black granulation, which is prominent in monocytes. This activity is most prominent in monocytes. Each slide was examined by two separate investigators (J. E. and L. A.).

Monitoring of differentiation – measurement of cellular markers by flow cytometry

One hundred microlitres of differentiated, undifferentiated and challenged cells (2×10^5 cell/mL suspended in PBS)

were added to tubes containing combinations of labelled antibodies (CD14, CD45, CD16 and CD66b and appropriate isotype controls). Tubes were gently mixed and left in the dark for 20 min. Before analysis 250 μ L of Isoton II (Coulter Electronics, Luton, UK) was added to each tube and vortexed. Cells were identified by CD45⁺ staining, and the expression of CD14 and CD66b was determined.

Challenge with colophony

Undifferentiated and differentiated cells were grown in 24-well plates (2.5×10^5 cell/mL) and incubated for up to 1 h with 0, 5 and 50 μ g/mL colophony.

End points – measurement of oxidative burst

The base level of peroxide within cells was measured using the DCFH-Peroxide Cell Probe (Coulter Electronics). All buffers were made with pyrogen/endotoxin-free water. Differentiated, undifferentiated and challenged cells were washed in PBS and resuspended at $3 \pm 0.5 \times 10^6$ cell/mL in PBS. Fifty microlitres of cells were added to appropriately-labelled LP4 tubes and placed in a water bath at 37 °C for 10 min. Before use, the cell probe reagent was reconstituted with 0.25 mL water and 25 μ L was added to the appropriate tubes, 25 μ L of PBS was added to the blank tube. After a gentle mix the tubes were incubated at 37 °C for exactly 5 min and then immediately placed on ice. Prior to analysis on a calibrated Epics XL flow cytometer, the cells were suspended in 1 mL of PBS.

Results

Induction of cell differentiation

The HL-60 cell line established in culture maintained a viability of greater than 90%.

Retinoic acid induced the differentiation of the HL-60 cell line into a neutrophil-like phenotype, whereas the

addition of VD₃ to the cell culture media induced significant differentiation of HL-60s to monocyte-like cells.

Monitoring cell differentiation

The esterase staining demonstrated the appropriate esterase content for each route of differentiation, compared with the control HL-60 cell line. However, it was very difficult to distinguish between red and black granulation when a purple counter-stain had been used. The time course data showed the maximum CD14 expression for HL-60 cell with VD₃ was day 4, with VD₃ at a concentration of 2.4×10^{-7} mol/L. The maximum expression of CD66b for HL-60 cell with RA was day 5, with RA at 1.7×10^8 mol/L. Ethanol did not affect the expression of any cell surface markers as compared with the control cells. Undifferentiated, differentiated cells and cells in ethanol maintained a viability of greater than 85% until day 6.

Challenge with colophony

Treated and untreated cell lines exhibited 85% viability when challenged with colophony at 5 μ g/mL and 50 μ g/mL for up to 1 h.

End points – measurement of oxidative burst

Base line peroxide levels were increased in VD₃- and RA-treated cell lines when colophony had been added to media for 1 min (Table 1, Fig. 1.) However, when colophony was added for 1 h, only background peroxide levels were detected (Fig. 2). This pattern was not observed in the undifferentiated HL-60 cell line or when the treated or untreated cells were challenged with the inert substance HSA (results not shown).

Discussion

Occupational asthma induced by the inhalation of fumes from heated colophony as part of the soldering flux has been

Table 1. Intracellular peroxide levels in HL-60, RA and VD₃ treated cells after colophony addition into cell culture media for 1 minute and 1 h

Cell type	Challenge	% increase in mean linear fluorescence	
		1 min	1 h
HL-60 cells	5 μ g/mL colophony	12.7 $\pm$ 5.2	12.9 $\pm$ 8.3
	50 μ g/mL colophony	15.9 $\pm$ 4.7	7.6 $\pm$ 2.4
RA cells	5 μ g/mL colophony	40.8 $\pm$ 9.4	8.2 $\pm$ 4.4
	50 μ g/mL colophony	75.9 $\pm$ 5.0	9.4 $\pm$ 2.2
VD ₃ cells	5 μ g/mL colophony	45.4 $\pm$ 3.3	9.4 $\pm$ 10.9
	50 μ g/mL colophony	122.7 $\pm$ 9.2	12.0 $\pm$ 8.0

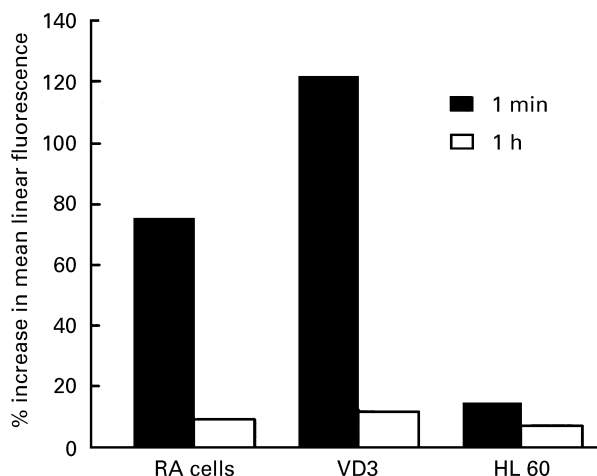


Fig. 1. Intracellular peroxide levels in HL-60-, RA- and VD₃-treated cells after colophony addition into cell culture media

well documented in the electronics industry. Abietic acid is one of the main constituents of colophony, which has been traditionally implicated in occupational asthma [11] and contact allergy. It has been demonstrated that abietic acid can become oxidized in air to form resin acid epoxides and hydroperoxides, which have now been suggested to be sensitizers in contact allergy [12,13]. During the soldering process, colophony is heated to temperatures which we have shown would result in the degradation of any existing epoxides or hydroperoxides. It is unlikely that the pyrolysis fumes inhaled by the exposed workers would contain the oxidation products of abietic acid, which would reasonably suggest that these oxidative products are not the causative agents of the colophony-induced asthma. During an oxidative burst reaction, reactive oxygen species (ROS) are produced that are thought to be mandatory for the effective defence against bacterial infections, but play a role in

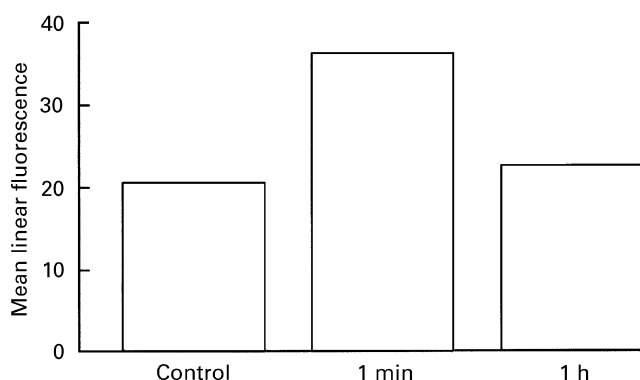


Fig. 2. Peroxide levels in RA-treated cells following challenge with 50 µg/mL colophony

the subsequent damage to host tissues. These ROSs are essentially superoxide, hydrogen peroxide, hydroxyl free radical and possible singlet oxygens which are released either after cell surface stimulation or phagocytosis. An increased intracellular peroxide level was demonstrated within the immune cells challenged with colophony. The stimulation of an oxidative burst by colophony has led us to hypothesize the method by which colophony induces respiratory problems. We propose that the oxidation of abietic acid may occur *in vivo* due to the oxidative products produced by the stimulation of monocytes and macrophages in the lung. If this oxidation process is occurring *in vivo* after the inhalation of colophony, then these oxidation products, which may be epoxides or hydroperoxides may then interact with body protein to further initiate immune responses. We conclude that for the preparation of LMWC-protein conjugates, consideration must be taken to determine whether the LMWC is undergoing a reaction *in vivo* before it is interacting with body proteins.

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