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Communication

Microcalorimetry does not predict the cellular phagocytosis of latex microspheres☆

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Abstract

Current literature highlights the potential suitability of microcalorimetry for the investigation of cell–drug interactions. Previous work using bacteria or antigens derived from infectious organisms yielded conclusions that heat production is a quantitative means of measuring phagocytosis. In this study we evaluated the potential of flow-through microcalorimetry as a method of quantifying the phagocytosis of microsphere particulates. The technique avoids the need to incorporate radioactive or fluorescent markers into the particulate formulation, and would be widely applicable in biopharmaceutical research. Using the monocyte cell line Mono Mac 6 a power output of 9.00 μW per million cells was increased significantly on addition of zymosan, lipopolysaccaride (LPS) and phorbol myristate acetate but not following exposure to FITC labelled latex microspheres (LM). TNFα production increased on exposure to zymosan, LPS and LPS–phorbol myristate acetate, though not on exposure to LB. An assay was developed which allowed the quantification of internalised particulates in phagocytic cells using fluorescent activated cell sorting (FACS). In contrast to the microcalorimetric and TNFα data FACS revealed that 20% of the MM6 population phagocytosed a mean of 1.35 LM. Microcalorimetry and measurements of TNFα production are assays of cellular activation a phenomenon not necessarily associated with phagocytosis. FACS, however, serves as a specific and quantitative measure of phagocytosis. Microcalorimetry may not be a suitable technique for the quantitative assessment of the phagocytosis of drug delivery particulates.

Keywords: Phagocytosis; Microcalorimetry; FACS; Cellular activation

1. Introduction

Microcalorimetry refers to the measurement of heat transfers of approximately 1 μJ. Current literature points to the potential suitability of microcalorimentry for the investigation of cell–drug interactions (Beezer et al., 1995; Wadso, 1995). It has been accepted that cellular processes are associated with a heat output since energy

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released by nutrient oxidation to fuel cellular activities is ultimately transformed into heat (Bottcher and Furst, 1997). A number of cellular parameters such as cell cycle, respiratory burst (Kemp et al., 1995) and indeed phagocytosis (Hayatsu et al., 1986, 1988; Shimoyama et al., 1991; Ding et al., 1994) have been successfully measured by microcalorimetry. However, previous phagocytic investigations performed with microcalorimetry have been limited to bacteria or antigens derived from infectious agents, leading to the conclusion that heat production is a good quantitative measure of phagocytosis. Indeed, it has been suggested that microcalorimetry could be utilised as a technique to assess cytotoxicity (Thoren et al., 1990; McGuinness and Barisas, 1991) or reticuloendothelial function in certain pathologies (Ding et al., 1994).

Fluorescent activated cell sorting (FACS) has been used as a technique to quantify the phagocytosis of fluorescent particles, allowing measurements of single cells or particulates to be made at high speed (Steinkamp et al., 1982; Schurmann et al., 1997 Torche et al., 1999). Its application in the pharmaceutical sciences has previously been highlighted (Ramanathan, 1997). However, in using FACS it is essential to distinguish between internalised particulates and those remaining in a loose external attachment with the cell membrane (Evora et al., 1998; Torche et al., 1999). Other methods utilised to assess the phagocytic processes include microscopic techniques such as confocal, fluorescence and bright-field microscopy. However, such analyses are arduous to perform and do not provide an objective measure of quantitation.

In this study we evaluated the potential of flow-through microcalorimetry as a method of quantifying the phagocytosis of microsphere particulates. The technique avoids the need to incorporate radioactive or fluorescent markers into the particulate formulation, and as such would be widely applicable in biopharmaceutical research. Using latex microspheres as a model system we found that phagocytosis by mononuclear cell populations, as quantified by FACS, was not associated with cellular activation as denoted by an increased heat output (microcalorimetry) or indeed biochemical measures such as upregulated TNFα production. Microcalorimetry may not be a suitable technique for the quantitational assessment of the phagocytosis of drug delivery particulates.

2. Materials and methods

2.1. Materials

The human monocyte cell line Mono Mac 6 (MM6) was cultured in RMPI-1640 supplemented with penicillin–streptomycin antibiotic solution, 10% foetal bovine serum (FBS) and 1% glutamine. (Life Technologies, Paisley, UK). Lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate, zymosan A and fluorescein-isothiocyanate labelled latex microspheres (LM; diameter 2.73 μm) were all purchased from Sigma Chemical Company (Poole, UK).

2.2. Microcalorimetry

A Thermometric 2277 Thermal Activity Monitor (TAM) in conjunction with the operating and analytical software Digitam 3.0 was used throughout. A 10 ml volume of cell suspension (2 million cells/ml) was mounted in an external waterbath and circulated through the TAM at a flow rate of 40 ml/h. A stable baseline of metabolism could be maintained for approximately 4 h. LPS (150 ng/ml), phorbol myristate acetate (15 ng/ml), zymosan (11 mg/ml) and LM (40 million/ml) were added to the circulating media outside the TAM avoiding disruption to the measuring chamber.

2.3. Fluorescent activated cell sorting (FACS) assay

Cells were exposed to LM (40 million/ml) for 2 h after which cells and LM were analysed using a FACScan flow cytometer (Becton Dickinson). The fluorescence intensity (FI) of single cell and LM events were recorded. Fluorescence associated with microspheres alone or with cells alone (autofluorescence) was isolated from that of cell associated microspheres. This latter population

was then divided according to degrees of associated fluorescence. Results were collected as the median FI (MFI) of 5000 cells ($n = 3$) and expressed as the mean MFI for each of three experiments.

To isolate fluorescence derived from internalised LM the cells were subjected to an acid wash to remove microspheres bound externally to the cell membrane. Cells were washed in a buffer of 28 mM sodium acetate, 117 mM sodium chloride adjusted to pH 5 by centrifugation (2 min at 550 g). The effectiveness of this washing procedure was verified by the use of inhibitors in the medium to block fluorescent microsphere internalisation by the cells, i.e. the only associated LM would be those externally bound to the plasma membrane. Specifically, the inhibitors were sodium azide (845 ng/ml) an inhibitor of the electron transport chain and cytochalasin D (15 ng/ml) an actin inhibitor. In cells treated with inhibitors and subsequent to the acid wash FIs were reduced to approximately autofluorescence. As further controls LPS (150 ng/ml) and phorbol myristate acetate (15 ng/ml) were included in some cultures throughout the experimental duration including a preincubation stage of 1 h.

2.4. Enzyme linked immunoabsorbant assay (ELISA) studies

TNFα production was assayed by ELISA (Amersham Life Science). MM6 cells were seeded at 0.5 million/ml in 24 well plates at a volume of 1 ml/well. The plates were then left for 24 h under usual culture conditions then incubated for a further three hours with the test substrates, LPS (100 ng/ml), phorbol myristate acetate (10 ng/ml), zymosan (5.5 mg/ml) and LM (40 million/ml). The cells were pelleted by centrifugation and the supernatant used for assay.

3. Results

3.1. Microcalorimentry

A resting baseline of 9.00 ± 0.683 μW per million cells ($n = 12$, mean ± s.d.) was reproduced at the beginning of each experiment. The addition of zymosan increased the output to a peak of 11.9 ± 0.426 μW per million cells ($n = 3$, mean ± s.d.) (Fig. 1a). Addition of LPS (Fig. 1b) and phorbol myristate acetate (Fig. 2a) resulted in smaller, short lived raises ($n = 3$, mean ± s.d.) of 0.339 ± 0.155 and 0.533 ± 0.233 μW per million cells, respectively, and which were maintained for approximately 10 min. Following LPS (below 450 ng/ml) mediated elevations in heat output, readings recovered to baseline values. At cumulative LPS levels of 450 ng/ml the heat output continually declined below the original baseline readings. Following exposure to phorbol myristate acetate, baseline readings did not stabilise, displaying a sharp declining profile. Additions of phorbol myristate acetate beyond 30 ng/ml did not provoke any further activation. No deviation from the baseline of metabolism was observed upon addition of LM (Fig. 2b).

3.2. FACS

Cultures incubated with sodium azide and cytochalasin D had a FI similar to autofluorescence (Fig. 3). For monocytes not incubated with inhibitors, $20.3 \pm 1.79\%$ of the MM6 population phagocytosed a mean of 1.35 ± 0.05 LM ($n = 4$, mean ± s.d.). Both values were increased significantly by preincubations with LPS and phorbol myristate acetate (Fig. 3).

3.3. ELISA

LPS–phorbol myristate acetate and zymosan upregulated the production of TNFα approximately 100-fold whilst after the addition of LM alone, TNFα levels were equal to background (Table 1).

4. Discussion

A rise in heat output as assessed by microcalorimetry was observed upon addition of zymosan, LPS, phorbol myristate acetate, but not upon addition of LM, to monocytic cell populations. As a non-specific measure of cellular pro-

cesses microcalorimetry alone could not determine whether the phagocytosis of LM elicited no change in metabolism, or the LM were infact not phagocytosed. However, a FACS assay was developed that isolated the fluorescence of internalised LM (verified by the use of inhibitors). Assayed by FACS, MM6 cultures proved phagocytic suggesting that microcalorimetry is not a good measure of phagocytosis. The phagocytic capacity of the MM6 populations was increased by exposure to

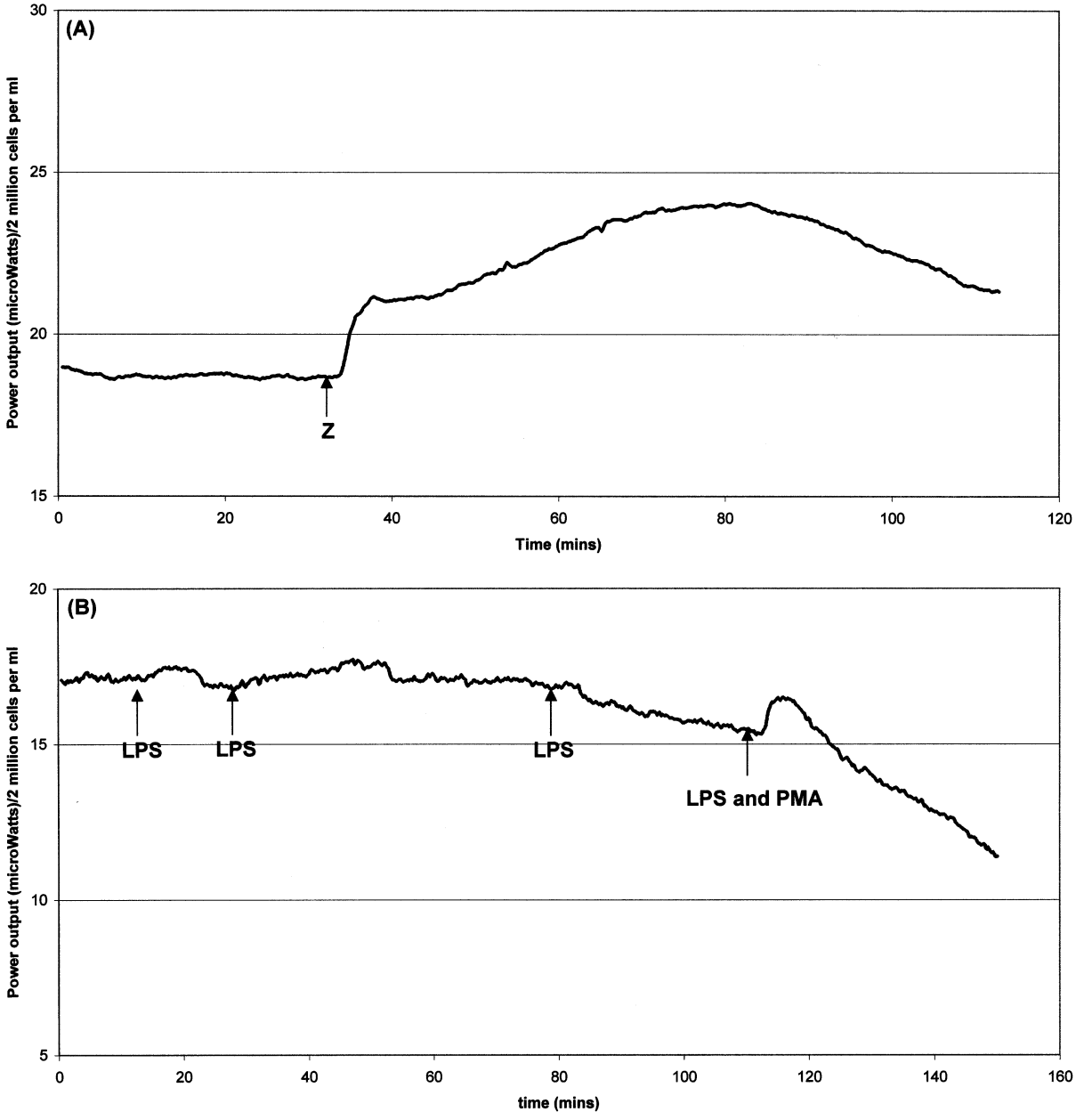

Fig. 1. Representative graph showing the effect of zymosan A (11 mg/ml) addition (A); and LPS and phorbol myristate acetate (PMA), 150 and 15 ng/ml, respectively, addition (B); on the energy output of Mono Mac 6 cells (2 million/ml).

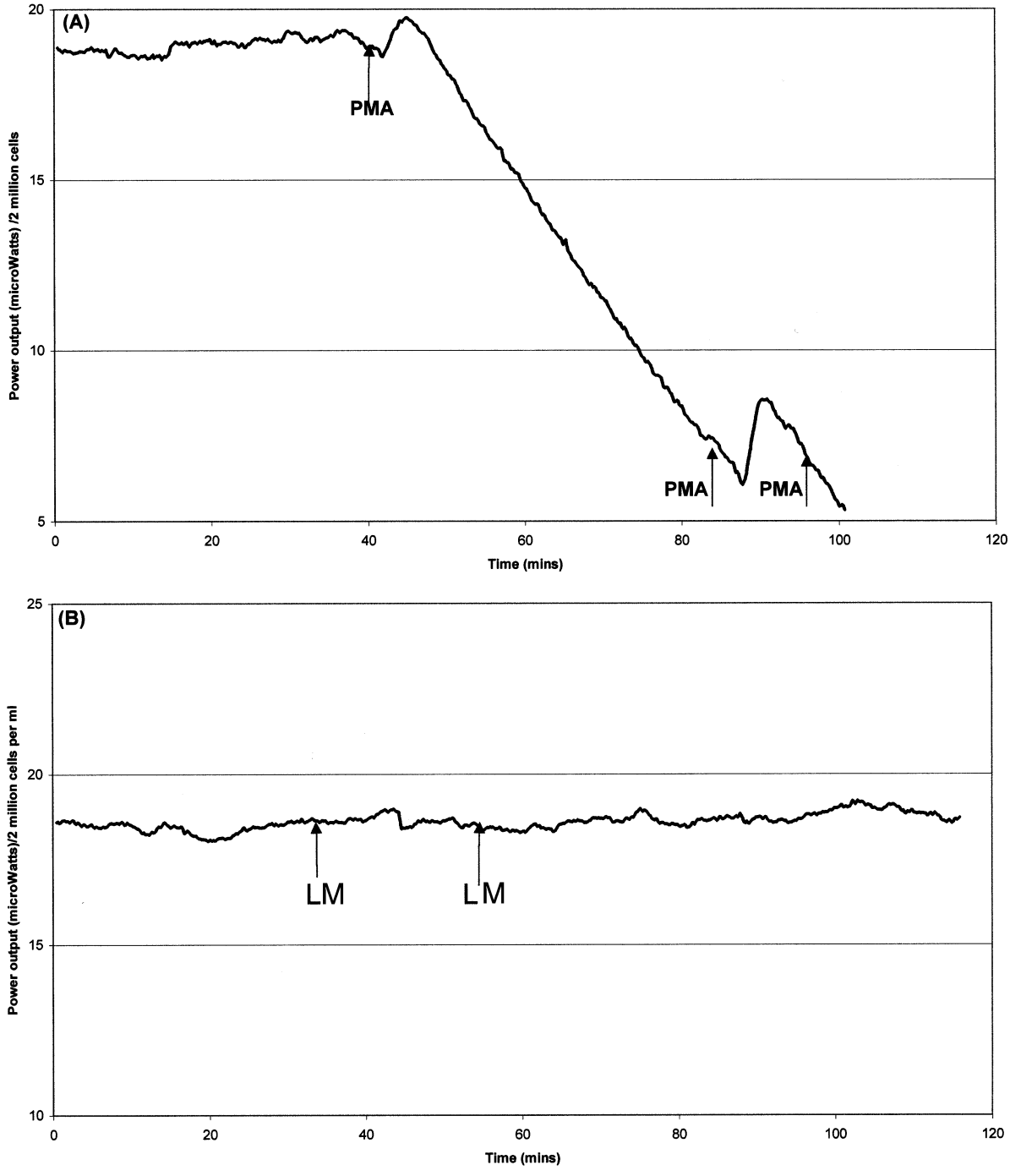

Fig. 2. Representative graph showing the effect of phorbol myristate acetate (PMA) (15 ng/ml) addition (A); and latex microsphere (LM), 40 million/ml, addition (B); on the energy output of Mono Mac 6 cells (2 million/ml).

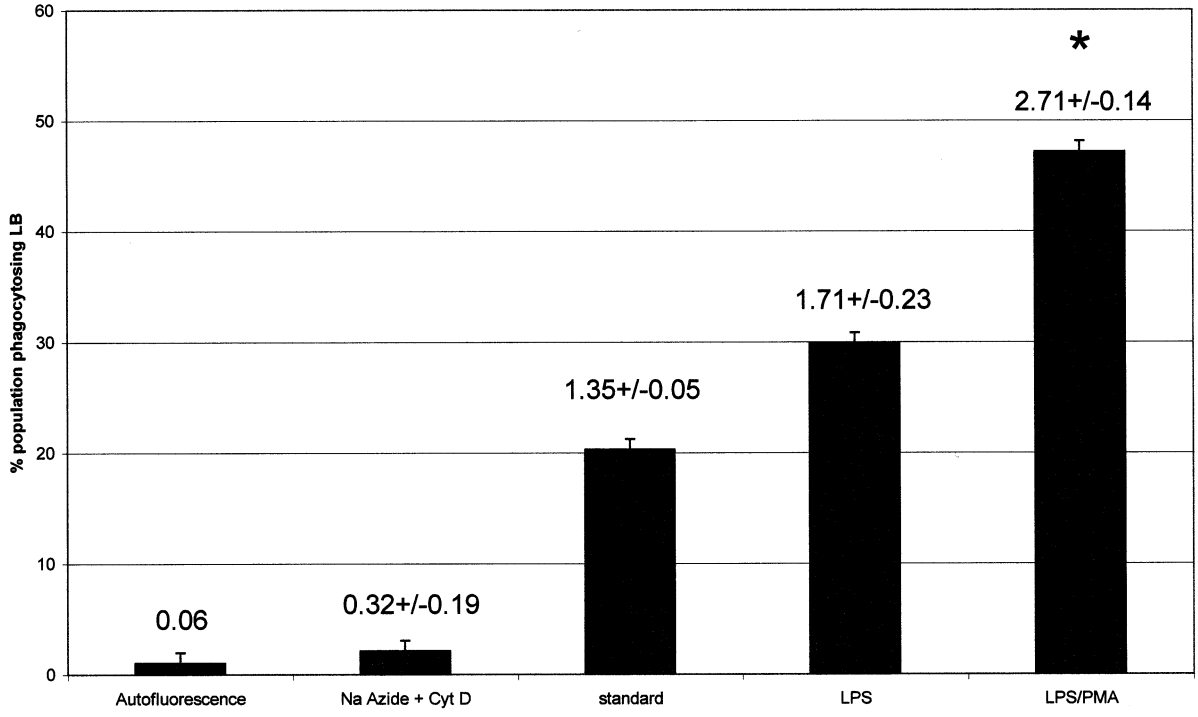

Fig. 3. Graph of the percentage of MM6 populations uptaking LM added to cells at 40 million/ml under varying culture conditions. Figures above bars refer to the median FI (expressed as number of LM uptaken). For MM6 cells alone (autofluorescence) results have been standardised and expressed in terms of the fluorescence of LM. ($n = 3$; *, denotes a significant difference from standard).

LPS–phorbol myristate acetate, most probably inducing differentiation to a more mature lineage (Zieglerheitbrock et al., 1994).

As stated additions of zymosan, LPS and LPS–phorbol myristate acetate caused a rise in the heat output of cell populations a finding that correlated with TNFα production. It would seem that where heat output is increased so TNFα production is upregulated. Both indices are measures of cellular activation, however, no activated response by MM6 cells was detectable by either technique upon addition of LM. It would seem that although LM are phagocytosed they induce no other cellular responses at least as judged by microcalorimetry and TNFα production.

Successive additions of LPS, a mitochondrial toxin (Glover et al., 1996), caused an eventual decline in metabolism as measured by microcalorimetry. Phorbol myristate acetate addition subsequent to causing an initial activation also proved toxic. These data support the application of microcalorimetry as an index of toxicity (Thoren et al., 1990; McGuinness and Barisas, 1991), but not as a measure of the phagocytosis of particulate drug delivery systems.

Table 1
TNFα production by MM6 cells following exposure to varying culture conditions for a 3 h time period

Treatment	TNFα production (pg per 0.5×10^6 cells)
Background	10.1 ± 1.53
LM	11.5 ± 1.32
LPS/PMA	873 ± 118
Zymosan A	>1000[a]

[a] TNFα produced exceeded the range of the assay.

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