Effects of macrophage colony-stimulating factor and phorbol myristate acetate on 2-D-deoxyglucose transport and superoxide production in rat peritoneal macrophages

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2-D-Deoxyglucose (2-dGlc) uptake and accumulation into rat peritoneal macrophages was increased by colony-stimulating factor (mCSF) by stimulating the coupling between endofacial hexokinase activity and the sugar transporter. The evidence for this is as follows: (1) mCSF significantly decreased the K_m for *zero-trans* uptake ($P < 0.05$), without altering V_{max} ; (2) the accumulation of free 2-dGlc was increased by mCSF ($P < 0.05$); (3) mCSF retarded the rate of exit of accumulated free 2-dGlc. The mCSF-dependent increase in 2-dGlc uptake by macrophages was enhanced by preincubation of the cells in mCSF-free solution. The activity of the hexose monophosphate shunt (HMPS) measured by the differential uptake of 2-d[1-³H]Glc and 2-d[2,6-³H]Glc was not stimulated by mCSF. Also, in quiescent cells, superoxide production, as determined by cytochrome *c* reduction, was unaffected by mCSF. Phorbol myristate acetate (PMA; 40 nM) stimulated both the HMPS activity and superoxide production. Both these effects were dependent on the uptake of external sugar (2-dGlc). Incubation of the macrophages with mCSF enhanced the sugar transport and PMA-dependent stimulation of HMPS activity and superoxide production, indicating a role for mCSF in the 'priming' of macrophage functions. Both HMPS activity and superoxide production are entirely dependent on uptake of exogenous sugar, since the potent sugar-transport inhibitor cytochalasin B competitively inhibited 2-dGlc uptake, HMPS activity and superoxide generation in PMA-activated cells ($K_i \approx 0.3 \mu\text{M}$ for all three processes). Over a wide range of 2-dGlc concentrations, 4 mol of superoxide were generated/mol of 2-dGlc metabolized in the HMPS pathway, indicating coupling between these processes. The K_m of 2-d[2,6-³H]Glc uptake in PMA-treated cells was $0.45 \pm 0.07 \text{ mM}$, and V_{max} was $1.32 \pm 0.05 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml of cell water}^{-1}$. It is evident that there is a large degree of slippage between HMPS activity and membrane-associated hexokinase activity, since the K_m for HMPS activity was $0.06 \pm 0.02 \text{ mM}$ and the V_{max} was $0.10 \pm 0.03 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml of cell water}^{-1}$.

INTRODUCTION

Phagocytes are an essential part of the natural immunity system, protecting the body against opportunistic infection of bacteria and fungi by producing toxic chemicals, including superoxide radicals, via a membrane-bound enzyme, NADPH oxidase (Klebanoff, 1980; Babior *et al.*, 1981; Fantone & Ward, 1982; Babior, 1984). The production of free radicals is associated with an increase in oxygen consumption and C-1 oxidation of sugar via the hexose monophosphate shunt (Arthur *et al.*, 1986, 1988) and is known as the 'respiratory burst'. A correlation between increased 2-deoxyglucose (2-dGlc) transport and immunological activation with microbes has been reported (Bonventre *et al.*, 1977), and work on the macrophage cell line J774.C3C showed that the production of free radicals was totally dependent on extracellular glucose (Kiyotaki *et al.*, 1984). This finding suggests that agents which influence the uptake of hexoses into phagocytes will directly affect the ability of these cells to perform their physiological roles.

Macrophage colony-stimulating factor (mCSF) is a lineage-specific haemopoietin that stimulates proliferation and supports differentiation and survival of cells in the mononuclear phagocyte series (Sherr, 1990). It also enhances the ability of the mature macrophages and monocytes to kill micro-organisms (Lee & Warren, 1987), and tumour cells (Wing *et al.*, 1982; Sampson-Johannes & Carlino, 1988). These effects may, at least in part, be explained by the observation that mCSF stimulates the pro-

duction of free radicals in murine macrophages (Wing *et al.*, 1985).

One of the most rapid effects of mCSF is to increase hexose transport (Hamilton *et al.*, 1986, 1988). It is not known in detail what biochemical events are associated with the differentiation and proliferation of macrophages and related cell types, but the stimulation of sugar transport by mCSF may be important. Studies on an interleukin 3-dependent haemopoietic precursor cell line indicate that interleukin 3 promotes survival and proliferation by its ability to increase sugar uptake (Dexter *et al.*, 1986).

Recently, it was proposed that the stimulation of 2-dGlc uptake in rat peritoneal macrophages by mCSF was due to increased coupling between sugar transport and hexokinase activity at the endofacial surface of the glucose transporter (Rist *et al.*, 1990). Previously, it was shown that the rate of uptake of 2-dGlc and the accumulation of free 2-dGlc are reduced in a hexokinase-deficient mutant of a Chinese-hamster ovary cell line (Faik *et al.*, 1989), and also that the rate of uptake of 2-dGlc is coupled to hexokinase activity in rat thymocytes treated with phorbol myristate acetate (PMA) (Naftalin & Rist, 1989, 1990). Similar suggestions have been made to explain insulin-dependent stimulation of sugar transport in adipocytes (Thompson & Kleinzeller, 1989).

In the present paper the kinetic parameters of 2-dGlc uptake and exit in rat macrophages were studied in order to characterize further mCSF stimulation of sugar transport in PMA-activated

Abbreviations used: mCSF, macrophage colony-stimulating factor; 2-dGlc, 2-D-deoxyglucose; PMA, phorbol myristate acetate; HMPS, hexose monophosphate shunt; DMEM, Dulbecco's modification of Eagle's medium; SOD, superoxide dismutase.

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cells. Also, the effects of mCSF and PMA on the activity of the hexose monophosphate shunt (HMPS) were correlated with the sugar-dependent production of superoxide radicals. It is shown that PMA-dependent superoxide production in rat peritoneal macrophages is correlated with activity of the HMPS. mCSF alone, although stimulating 2-dGlc uptake and accumulation, has no effect on either superoxide generation or HMPS activity, but when added together with PMA it has a synergistic effect on PMA-dependent superoxide production and HMPS activity.

The relationship between transport, HMPS and superoxide-radical production in PMA-stimulated macrophages is examined, and it is shown that there is close coupling between 2-dGlc transport and hexokinase activity and between HMPS activity and superoxide production; however, there is no evidence of close coupling between superoxide production (and hence HMPS activity) and 2-dGlc transport.

MATERIALS AND METHODS

Cell preparation

Rat peritoneal macrophages were prepared as described previously (Rist *et al.*, 1990). Briefly, Wistar rats (150–200 g body wt.) were anaesthetized with diethyl ether and then killed by cervical dislocation. Immediately, a 10 ml sample of Hepes-buffered saline (Hepes, 5 mM; NaCl, 140 mM; KCl, 5 mM; MgCl₂, 1.2 mM), pH 7.3 at 37 °C, containing 0.85 % (w/v) NH₄Cl to lyse erythrocytes, was injected into the peritoneal cavity of the rats. After 5–10 min of gentle massage of the abdomen, the cell suspension was withdrawn and centrifuged (300 g for 3 min), before being washed twice with saline. Each experiment required three or four rats, with each animal yielding approx. $(1-2) \times 10^7$ macrophages.

Microscopy and cell counting

The preparation described did not result in death or damage to the cells. There was little or no debris detectable by optical microscopy, and 95 % of cells excluded Evans' Blue stain. Differential counts of stained preparations of the peritoneal washings showed > 90 % macrophages and monocytes.

The mean cell volume of the macrophages was estimated by measurement of the 3-O-methyl-D-glucose space and by using an Elzone 280PC particle analyser (Particle Data Ltd., Kingstone, Hereford, U.K.). The estimate obtained for macrophage volume was 330 fl, and thus 3×10^9 cells $\approx$ 1 ml of cell water. All sugar-uptake rates (etc.) are normalized to 3×10^9 cells. Cell numbers were analysed using the Elzone 280PC instrument with macrophage suspensions diluted to between 5×10^5 and 1×10^6 cells/ml.

Radioactive-sugar uptake studies

Sugar uptake into macrophages was measured using 2-deoxy-D-[1-³H]glucose (Amersham International; initial sp. radioactivity 17 Ci/mmol; final concn. 0.5 μ Ci/ml) or 2-deoxy-D-[2,6-³H]glucose (Amersham International; initial sp. radioactivity 42.5 Ci/mmol; final concn. 0.5 μ Ci/ml), with or without various concentrations of recombinant human mCSF (Cetus Corporation; supplied by Sera Labs Ltd., Crawley, Sussex, U.K.) and PMA (Sigma). After incubation, sugar uptakes were halted by adding iso-osmotic ice-cold stopping solution, consisting of saline containing phloretin and HgCl₂ at final concentrations of 100 μ M and 1 μ M respectively. The background passive uptake of sugar was determined by measuring uptake in the presence of stopping solution. All uptake rates are expressed as phloretin-sensitive uptakes. After adding stopping solution, the macrophages were centrifuged for 3 min at 3000 g, the supernatants removed, and

a sample was retained for the determination of the extracellular radioactivity. The cells were then washed twice by suspension and re-centrifugation in fresh ice-cold stopping solution to remove extracellular isotope. Finally, the cell pellets were lysed by vortex-mixing in distilled water, and the lysate was used to determine total uptake and, when required, free sugar and hexose phosphate concentrations within the cell pellet. Radioactivity was counted with a scintillation fluid of the following composition: 1 litre of toluene (BDH), 1 litre of Synperonic NX (Durham Chemicals Distributors Ltd., Birtley, Tyne and Wear, U.K.) and 5 g of 2,5-diphenyloxazole (Sigma).

Estimation of phosphorylated and non-phosphorylated sugar

A Whatman DE81 anion-exchange filter was washed with 2 ml of ice-cold distilled water by suction via a Buchner filtration tube and the water discarded; then 1 ml of cell lysate (at 4 °C) was filtered and the filtrate retained. Next the filter was washed with 2 ml of cold distilled water and this second filtrate was added to the first. The combined filtrate was sampled for free sugar and the remainder discarded. The sugar phosphate retained on the filter was removed by washing with 2 ml of 0.5 mM-HCl, and a sample was counted for radioactivity. Total, free sugar and hexose phosphates per 3×10^9 macrophages were estimated after correction for quench and dilution. This method of separation was previously shown to give accurate results (Naftalin & Rist, 1989).

Radioactive sugar exit from preloaded cell suspensions

These exit experiments were carried out as described previously with rat thymocytes (Naftalin & Rist, 1989). Macrophage suspensions containing approx. 5×10^5 cells/ml were incubated with 2-d[2,6-³H]Glc (0.1 mM) for 90 min with or without mCSF (110 pM). At the end of this incubation, the cells were loosely packed by centrifugation at 1500 g for 3 min and the supernatants removed. Next, the macrophages were washed in saline at 20 °C, re-centrifuged and finally resuspended in fresh saline with nominally zero sugar at 37 °C (2-dGlc < 0.01 mM).

Zero-trans net exit was measured by observing the time-dependent loss of radioactivity from the cell sugar and hexose phosphate pools into the nominally sugar-free solutions.

Macrophage cell culture

Macrophages were isolated as described above and then cultured in bacteriological-grade Petri dishes with DMEM (Dulbecco's modification of Eagle's medium), which contains approx. 5.5 mM-Glc, but no mCSF. After 24 h the cells were removed from the Petri-dish surface with vigorous agitation and washed twice in saline by repeated centrifugation and re-suspension. Finally, the macrophages were re-suspended in saline containing 2-d[2,6-³H]Glc (0.1 mM) in the presence or absence of mCSF (110 pM), and the uptake measured for 60 min at 37 °C.

Measurement of superoxide production

Superoxide-anion production by macrophages was measured using a method adapted from that described by Weening *et al.* (1975). 20 μ M-Cytochrome *c* (horse heart; Sigma) was present in the Hepes buffer during incubation of the cells and the reaction was stopped by placing a 1.5 ml sample of the suspension in an Eppendorf tube on ice. After centrifugation at 3000 g for 3 min at 4 °C, the supernatant was removed and stored on ice. For determination of the amount of reduced cytochrome *c* produced during the incubation, the absorbance spectrum was measured from 540 to 560 nm in a Unicam SP8000 dual-beam spectrophotometer.

Reduced cytochrome *c* in the sample, and thus superoxide

generated, was calculated using an absorbance coefficient of 21.1 mm⁻¹·cm⁻¹ (Van Gelder & Slater, 1962) and expressed as μmol·h⁻¹·3 × 10⁹ cells⁻¹. Each condition had an identical control containing 10 μg of superoxide dismutase (SOD)/ml, and every value is expressed as the SOD-inhibitable superoxide production.

Measurement of HMPS activity

HMPS activity of macrophages was measured by using the ‘double-label’ method previously reported (Rist *et al.*, 1990), with cells being incubated in 2-d[1-³H]Glc or 2-d[2,6-³H]Glc. In the HMPS, the 1-position carbon atom is cleaved from the hexose. When cells with an active HMPS are incubated with 2-d[1-³H]Glc, the label is lost as ³H₂O, but label at other positions is retained within the cytosol. It follows that the difference between the net uptake of 2-d[1-³H]Glc and 2-d[2,6-³H]Glc indicates the activity of the HMPS pathway, whereas the rate of uptake of 2-d[2,6-³H]Glc measures the true rate of sugar uptake via the plasma-membrane sugar-transport system, and the accumulation of unphosphorylated 2-d[1-³H]Glc within the cytosol measures the level of free 2-dGlc unaltered by HMPS activity.

RESULTS

Concentration-dependent effects of mCSF on 2-d[1-³H]Glc accumulation within macrophages

The mCSF-concentration-dependence for 2-d[1-³H]Glc (0.1 mM) uptake into rat peritoneal macrophages incubated for 2 h is shown in Table 1. It was previously reported that mCSF stimulated 2-dGlc uptake by rat peritoneal macrophages at a concentration of 1000 units/ml or 440 pM (Rist *et al.*, 1990). Table 1 shows that, at concentrations below this, mCSF significantly enhances 2-dGlc uptake and the effect is concentration-dependent. At all concentrations of mCSF, uptake of 2-dGlc (0.1 mM) was significantly increased (*P* < 0.05 at 22 and 44 pM; *P* < 0.001 at 110, 220 and 440 pM; *n* = 7), with maximum stimulation between 220 and 440 pM. Hamilton *et al.* (1988), using mCSF purified from cultures, showed that rat peritoneal macrophages respond in a similar way by increased uptake of 2-dGlc. The data reported here are included in order to confirm that these cells respond in a dose-dependent way to pure human recombinant mCSF. In subsequent experiments, mCSF was used at 110 or 220 pM, since these concentrations gave a statistically significant stimulation of 2-dGlc uptake.

Effects of preincubation on mCSF-dependent stimulation of 2-dGlc uptake

Freshly isolated macrophages, preincubated for various times in mCSF-free DMEM culture medium (5.5 mM-Glc) (see the Materials and methods section), were incubated in 2-d[1-³H]Glc (0.1 mM) with or without mCSF (110 pM) and the uptake stopped after 60 min. The uptake of 2-d[1-³H]Glc (0.1 mM) in the presence of mCSF (110 pM) is expressed as a fraction of the uptake without mCSF at each time point (Fig. 1). In freshly isolated cells, mCSF (110 pM) increased 2-dGlc uptake by a factor of approx. 1.3-fold (*P* < 0.05; *n* = 5), and with increasing preincubation time without mCSF, the relative mCSF stimulation of 2-dGlc uptake was enhanced. After 24 h preincubation, mCSF stimulated 2-dGlc uptake approx. 2-fold (*P* < 0.001; *n* = 5). These results are consistent with the finding that macrophages cultured in the absence of growth factor up-regulate mCSF receptors with time (Guilbert *et al.*, 1986) and indicate that freshly isolated macrophages are nearly optimally stimulated with native mCSF. However, the main aim of the present investigation was to show the regulatory effects of mCSF on

2-dGlc transport, HMPS activity and superoxide production in quiescent and PMA-stimulated macrophages.

Effects of mCSF and PMA on the zero-trans influx kinetics of 2-dGlc uptake

Macrophages were incubated for 90 min at 37 °C, with or without mCSF (110 pM), washed in saline and then transferred into various concentrations (0.01–5 mM) of 2-d[2,6-³H]Glc, with or without mCSF (110 pM) and PMA (40 nM). Total uptake of 2-d[2,6-³H]Glc was measured for 10 min at 37 °C, before being stopped and the internal sugar determined (see the Materials and methods section).

Fig. 2 shows the uptake of 2-d[2,6-³H]Glc, (μmol·10 min⁻¹·ml of cell water⁻¹), plotted against sugar concentration (mM) after subtraction of the phloretin-insensitive uptake. The *K_m* for zero-trans uptake of 2-dGlc into macrophages without mCSF or PMA was 0.73 ± 0.11 mM, and the *V_{max}* was 1.28 ± 0.06 μmol·min⁻¹·ml of cell water⁻¹ (*n* = 8) and are esti-

Table 1. Concentration-dependent effects of mCSF on 2-d[1-³H]Glc (0.1 mM) accumulation

Uptake of 2-d[1-³H]Glc (0.1 mM) into rat peritoneal macrophages incubated at 37 °C with various concentrations of mCSF (0–440 pM) was measured over 2 h. Results are expressed as means ± S.E.M. At all concentrations, mCSF significantly increased the uptake of 2-dGlc above control (**P* < 0.05 and ****P* < 0.001); *n* was 7 in each case.

[mCSF] (pM)	2-d[1- ³ H]Glc uptake (μmol·2 h ⁻¹ ·ml of cell water ⁻¹)
0	3.51 ± 0.06
22	3.72 ± 0.07*
44	3.75 ± 0.09*
110	4.01 ± 0.06***
220	4.30 ± 0.10***
440	4.32 ± 0.16***

Fig. 1. Effects of preincubation on mCSF-dependent stimulation of 2-d[1-³H]Glc (0.1 mM) uptake

Results are expressed as means ± S.E.M. Macrophages were preincubated for various times in mCSF-free culture medium and then the uptake of 2-d[1-³H]Glc (0.1 mM) with or without mCSF (10 pM) was measured over 60 min at 37 °C. mCSF-dependent uptake is expressed as a fraction of uptake without mCSF at each time point. Initially, mCSF significantly increased 2-dGlc uptake by a factor of 1.3 (*P* < 0.05, *n* = 5). This factor increased significantly with increased preincubation time, and at 24 h it was 2-fold (*P* < 0.001, *n* = 5).

Fig. 2. Effects of mCSF (110 pM) and PMA (40 nM) on the zero-trans influx kinetics of 2-d[2,6-³H]Glc uptake

Macrophages were incubated for 90 min at 37 °C in the presence or absence of mCSF (110 pM), washed and then the uptake of various concentrations of 2-d[2,6-³H]Glc (0.01 to 5 mM) with (■) or without (□) mCSF (110 pM) and PMA (40 nM) (○) was measured over 10 min at 37 °C. Phloretin-sensitive uptake of 2-d[2,6-³H]Glc ($\mu\text{mol} \cdot 10 \text{ min}^{-1} \cdot \text{ml of cell water}^{-1}$) is plotted against concentration of 2-dGlc and fitted by the ENZFITTER program of the Michaelis-Menten equation. The control K_m for zero-trans uptake of 2-dGlc was $0.73 \pm 0.11 \text{ mM}$, and the V_{max} was $1.28 \pm 0.06 \cdot \text{min}^{-1} \cdot \text{ml of cell water}^{-1}$ ($n = 8$). mCSF (110 pM) significantly decreased the K_m for zero-trans uptake to $0.44 \pm 0.04 \text{ mM}$ ($P < 0.05$, $n = 8$), but did not significantly alter the V_{max} ($1.31 \pm 0.03 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml of cell water}^{-1}$). PMA (40 nM) significantly decreased the K_m for zero-trans uptake to $0.45 \pm 0.07 \text{ mM}$ ($P < 0.05$, $n = 7$), without significantly altering the V_{max} ($1.32 \pm 0.05 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml of cell water}^{-1}$).

mated by non-linear least-squares regression. In mCSF-treated cells the K_m was $0.44 \pm 0.04 \text{ mM}$ and the V_{max} was $1.31 \pm 0.03 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml of cell water}^{-1}$ ($n = 8$). mCSF significantly decreased the K_m for zero-trans uptake of 2-d[2,6-³H]Glc ($P < 0.05$), without significantly altering the V_{max} . In the presence of PMA (40 nM) the K_m for zero-trans uptake was $0.45 \pm 0.07 \text{ mM}$ and the V_{max} was $1.32 \pm 0.05 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml of cell water}^{-1}$ ($n = 7$). PMA decreased the K_m for zero-trans uptake significantly ($P < 0.05$), without significantly altering the V_{max} .

Effects of mCSF (110 pM) on the zero-trans net exit of 2-d[2,6-³H]Glc

Macrophages were incubated with 2-d[2,6-³H]Glc (0.1 mM) for 90 min with or without mCSF (110 pM). Exit of total sugar was then monitored by measuring the loss of radioactivity from the cells into nominally sugar-free medium, in the presence or absence of mCSF (110 pM), over the subsequent 40 min. The decrease in total sugar is expressed as the percentage loss of radioactivity, with the zero-time value as the 100 % reference value. Fig. 3 shows that the rate of loss of 2-dGlc is decreased by mCSF (110 pM). The gradient of the linear regression line of log(percentage of cell sugar remaining) is decreased by mCSF (110 pM) ($-0.013 \pm 0.0006 \text{ min}^{-1}$, control; $-0.01 \pm 0.0003 \text{ min}^{-1}$, mCSF; $P < 0.01$, $n = 6$).

Effects of mCSF (220 pM) on the steady-state accumulation of 2-d[1-³H]Glc (0.1 mM)

mCSF (220 pM) caused a significant increase in accumulation of total sugar, free sugar and hexose phosphate (Table 2), as previously reported (Rist *et al.*, 1990). In control macrophages

Fig. 3. Fractional loss of 2-d[2,6-³H]Glc from macrophages treated with or without mCSF (110 pM)

Cells preloaded for 90 min at 37 °C with 2-d[2,6-³H]Glc (0.1 mM) in the presence (□) or absence (■) of mCSF, were washed to remove external sugar, transferred to sugar-free medium and the loss of radioactivity monitored over 40 min. Results (means $\pm$ S.E.M.) are expressed as the logarithm of the percentage of the 100 % zero-time control and then fitted by least-squares regression. The rate of loss of 2-d[2,6-³H]Glc is decreased significantly by mCSF (110 pM) from $0.013 \pm 0.0006 \text{ min}^{-1}$ (control) to $0.01 \pm 0.0003 \text{ min}^{-1}$ ($P < 0.01$, $n = 6$). The extracellular concentration of 2-dGlc did not exceed 0.2 % of the intracellular concentration over the period of the experiment.

Table 2. Effect of mCSF (220 pM) on total and free sugars and hexose phosphate accumulation in macrophages after 2 h incubation in 2-d[1-³H]Glc (0.1 mM)

Results are expressed as means $\pm$ S.E.M. and n is the number of separate determinations. *** $P < 0.001$, ** $P < 0.05$ for test versus control value on the same row (Student's t test). Freshly isolated macrophages were incubated with 2-d[1-³H]Glc (0.1 mM) in the presence or absence of mCSF (220 pM). After 2 h the uptakes were stopped and total, free and phosphate sugar accumulation determined by anion-exchange chromatography (see the Materials and methods section). mCSF (220 pM) increased the accumulation of total and free 2-dGlc and hexose phosphate above control. Over 2 h the extracellular concentration of 2-dGlc decreased $< 2\%$, both in control and mCSF-treated cells.

Sugar	Accumulation ($\mu\text{mol} \cdot 2 \text{ h}^{-1} \cdot 3 \times 10^9 \text{ cells}^{-1}$)	
	Control	mCSF (220 pM)
Total	3.56 ± 0.06	$4.57 \pm 0.04^{***}$
Free	2.25 ± 0.08	$2.56 \pm 0.09^{**}$
Hexose phosphate	1.12 ± 0.04	$1.73 \pm 0.17^{***}$
$n \dots$	10	4

there was an approx. 20-fold higher concentration of free 2-dGlc in the cytosol ($2.25 \pm 0.08 \text{ mM}$, $n = 10$) than in the external saline (0.1 mM). This free-sugar accumulation was significantly increased by mCSF (220 pM) to a 25-fold stimulation ($P < 0.05$). The values shown here are higher than we previously reported (Rist *et al.*, 1990) owing to improved methods for cell counting and cell-size discrimination and to the exclusion of erythrocytes from the cell preparation.

The increase in uptake of low concentrations of 2-dGlc, the decrease in exit and the increased accumulation of free 2-dGlc are all consistent with an mCSF-dependent increase in the

Table 3. Effect of mCSF (110 pM) and PMA (40 nM) on superoxide production in various 2-dGlc concentrations

Macrophages were preincubated with or without mCSF (110 pM) and various concentrations of unlabelled 2-dGlc for 30 min at 37 °C, before being challenged or not with PMA (40 nM) and incubated for another 30 min. Superoxide production was measured over 1 h as the SOD-inhibitable reduction of 20 μM -cytochrome *c* ($\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{ml}$ of cell water $^{-1}$). mCSF (110 pM) alone had no significant effect on the superoxide production at any concentration of 2-dGlc, but PMA (40 nM) significantly increased the free-radical generation at all sugar concentrations ($*P < 0.001$; in all cases $n = 9$), and this stimulation was enhanced by increasing sugar concentration. mCSF preincubation before PMA activation, significantly increased superoxide production above that with PMA alone at 0.01, 0.1 and 1.0 mM-2-dGlc ($\dagger P < 0.05$ at 0.01 and 1.0 mM; $\dagger\dagger P < 0.01$ at 0.1 mM; $n = 9$), but not at 0 or 10 mM-2-dGlc ($n.s.$, not significant).

[2-dGlc] (mM)...	Superoxide production ($\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{ml}$ of cell water $^{-1}$)				
	0	0.01	0.1	1	10
Control	1.40 $\pm$ 0.30	1.97 $\pm$ 0.35	2.88 $\pm$ 0.37	3.61 $\pm$ 0.78	3.52 $\pm$ 0.67
PMA (40 nM)	5.36 $\pm$ 0.71*	5.88 $\pm$ 0.38*	11.41 $\pm$ 0.59*	13.53 $\pm$ 0.69*	16.62 $\pm$ 1.01*
mCSF (110 pM)	2.07 $\pm$ 0.25	2.61 $\pm$ 0.31	3.82 $\pm$ 0.53	4.03 $\pm$ 0.62	3.24 $\pm$ 0.35
PMA + mCSF	7.00 $\pm$ 0.18 ^{n.s.}	9.28 $\pm$ 1.08 $\dagger$	14.15 $\pm$ 0.71 $\dagger\dagger$	15.68 $\pm$ 0.72 $\dagger$	17.46 $\pm$ 1.17 ^{n.s.}
<i>n</i>	9	9	9	9	9

Table 4. Effects of mCSF (110 pM) and PMA (40 nM) on HMPS activity

Cells were incubated for 60 min at 37 °C with or without mCSF (110 pM) and PMA (40 nM) (for the last 30 min) in various concentrations of 2-d[1- ^3H]Glc or 2-d[2,6- ^3H]Glc (0.01–1 mM). Uptake of 2-dGlc at the different concentrations is shown in $\mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9$ cells $^{-1}$. mCSF (110 pM) significantly increased the uptake of both isotopes at all 2-dGlc concentrations ($*P < 0.05$, in all cases $n = 6$), but there was no significant difference between the uptake of the two isotopes in control or mCSF-treated cells. PMA significantly increased the difference between 2-d[1- ^3H]Glc and 2-d[2,6- ^3H]Glc uptake at all 2-dGlc concentrations ($\dagger\dagger\dagger P < 0.001$, in all cases $n = 6$), without significantly increasing the uptake of 2-d[2,6- ^3H]Glc. mCSF (110 pM) and PMA (40 nM) together significantly increased the uptake of 2-d[2,6- ^3H]Glc at all 2-dGlc concentrations, when compared with the control ($\dagger P < 0.05$ at 0.01 mM; $\dagger\dagger P < 0.01$ at 1.0 mM-2-dGlc, $n = 6$), but not mCSF alone. mCSF and PMA significantly increased the difference between the uptake of the two isotopes at all sugar concentrations, when compared with a control ($P < 0.001$, in all cases $n = 6$) and when compared with PMA alone ($P < 0.05$, in all cases $n = 6$).

[2-dGlc] (mM)	Carbon atoms	HMPS activity ($\mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9$ cells $^{-1}$)			
		Control	PMA	mCSF	PMA + mCSF
0.01	C-1	0.27 $\pm$ 0.01	0.14 $\pm$ 0.01	0.39 $\pm$ 0.04*	0.11 $\pm$ 0.01
	C-2,6	0.28 $\pm$ 0.03	0.30 $\pm$ 0.03 $\dagger\dagger\dagger$	0.43 $\pm$ 0.04*	0.44 $\pm$ 0.05 $\dagger$
0.1	C-1	2.59 $\pm$ 0.20	1.29 $\pm$ 0.04	3.44 $\pm$ 0.29*	1.16 $\pm$ 0.04
	C-2,6	2.71 $\pm$ 0.23	2.79 $\pm$ 0.13 $\dagger\dagger\dagger$	3.57 $\pm$ 0.30*	3.27 $\pm$ 0.10 $\dagger$
1.0	C-1	9.54 $\pm$ 0.58	7.77 $\pm$ 0.14	11.93 $\pm$ 0.50*	8.25 $\pm$ 0.15
	C-2,6	10.22 $\pm$ 0.45	10.47 $\pm$ 0.14 $\dagger\dagger\dagger$	12.42 $\pm$ 0.81*	11.83 $\pm$ 0.10 $\dagger\dagger$

coupling between sugar transport and hexokinase activity (Rist *et al.*, 1990).

Effect of mCSF (110 pM) and PMA (40 nM) on superoxide production at various 2-dGlc concentrations

Isolated macrophages were incubated in Hepes buffer containing 20 μM -cytochrome *c* and various concentrations (0–10 mM) of unlabelled 2-dGlc, with or without mCSF (110 pM) for 60 min at 37 °C. PMA (40 nM) was added after 30 min when required. Superoxide production was assayed by the rate of reduction of cytochrome *c* (see the Materials and methods section).

Table 3 shows superoxide production ($\mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9$ cells $^{-1}$) at various concentrations of external 2-dGlc, with or without mCSF (110 pM) and PMA (40 nM). mCSF (110 pM) alone had no significant effect on superoxide generation at any 2-dGlc concentration. In the absence of PMA there is a small, but insignificant, 2-dGlc-dependent effect on superoxide generation. PMA (40 nM) and the PMA/mCSF (110 pM) treatment significantly increased superoxide production at all 2-dGlc concentrations tested, including zero ($P < 0.001$, in all cases; $n = 9$). The PMA-dependent stimulation was dependent on the external 2-dGlc concentration, with significantly more superoxide production at the higher sugar concentrations.

There was no significant effect of mCSF preincubation (110 pM) on PMA-dependent superoxide production at 0 or 10 mM-2-dGlc. However, at all the intermediate concentrations of sugar, mCSF (110 pM) pretreatment before PMA activation significantly increased the generation of superoxide above the levels observed with PMA alone ($P < 0.05$ at 0.01 and 1 mM-2-dGlc; $P < 0.01$ at 0.1 mM-2-dGlc; $n = 9$).

Experiments using the same protocol as described above, but substituting glucose for 2-dGlc, showed that glucose is a better substrate for superoxide production than 2-dGlc. The production of superoxide radicals was approximately three times higher with glucose at all sugar concentrations, e.g. at 10 mM-2-dGlc with PMA, superoxide production was $16.62 \pm 1.01 \mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9$ cells $^{-1}$ ($n = 9$) and at 10 mM-Glc with PMA, superoxide production was $51.5 \pm 2.38 \mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9$ cells $^{-1}$ ($n = 4$). Although not an ideal substrate, 2-dGlc has the important advantage that it allows the measurement of sugar transport and HMPS activity in isolation from further metabolism.

Effects of mCSF (110 pM) and PMA (40 nM) on HMPS activity at various 2-dGlc concentrations

Macrophages were incubated for 60 min with or without mCSF (110 pM) in various concentrations (0.01–1 mM) of either 2-d[1- ^3H]Glc or 2-d[2,6- ^3H]Glc at 37 °C. PMA (40 nM) was

Fig. 4. Concentration-dependence of PMA on HMPS activity

Freshly isolated macrophages were incubated for 45 min at 37 °C in either 2-d[1-³H]Glc (□) or 2-d[2,6-³H]Glc (■) (0.1 mM) with various concentrations of PMA (0–100 nM). PMA had no significant effect on 2-d[2,6-³H]Glc (0.1 mM) uptake at any concentration, but 2-d[1-³H]Glc (0.1 mM) uptake was significantly decreased at 40 and 100 nM-PMA ($P < 0.001$; in both cases $n = 4$). There was no significant difference in uptake of the two labels at 40 or 100 nM-PMA.

added or not added after 30 min of the incubation. Table 4 shows that mCSF (110 pM) significantly increased the rate of uptake of both 2-d[1-³H]Glc and 2-d[2,6-³H]Glc at sugar concentrations in the range 0.01 to 1 mM ($P < 0.05$, in all conditions; $n = 6$). There was no significant difference between uptake of the two forms of labelled sugar in either control or mCSF-treated macrophages, indicating that mCSF increased hexokinase activity, but did not stimulate the HMPS. This confirms and extends previous work (Rist *et al.*, 1990).

PMA (40 nM) alone did not significantly increase the uptake of 2-d[2,6-³H]Glc at any sugar concentration over 60 min. This contrasts with the stimulatory effect of PMA (40 nM) on the initial rate of 2-dGlc transport (Fig. 2). PMA significantly enhanced the difference between the uptake of 2-d[1-³H]Glc and 2-d[2,6-³H]Glc ($P < 0.001$, at all 2-dGlc concentrations; $n = 6$), and this result indicates that PMA stimulates the activity of the HMPS pathway, but without concurrent stimulation of sugar transport. The amount of 2-dGlc metabolized by the HMPS (i.e. the difference between the uptake of the isotopes) was increased significantly as the concentration of sugar was increased ($P < 0.001$ at all concentrations), indicating that the PMA stimulation of the HMPS is sugar-dependent. Treatment of macrophages with the combination of mCSF (110 pM) and PMA (40 nM) significantly increased the uptake of 2-d[2,6-³H]Glc at all sugar concentrations when compared with the control ($P < 0.05$ at 0.01 and 0.1 mM-2-dGlc; $P < 0.01$ at 1.0 mM 2-dGlc; $n = 6$). However, there was no significant difference between 2-d[2,6-³H]Glc uptake in cells treated with the mCSF/PMA combination and those stimulated with mCSF (110 pM) alone.

PMA (40 nM) and mCSF (110 pM) together significantly increased the difference between the uptake of 2-d[1-³H]Glc and 2-d[2,6-³H]Glc at all sugar concentrations ($P < 0.001$ in all cases). The difference between the uptake of the two isotopes with PMA and mCSF was significantly greater than the difference induced with PMA alone at each 2-dGlc concentration ($P < 0.05$, in all cases). This result indicates that the mCSF/PMA treatment stimulates the activity of the HMPS more than observed with PMA alone. The amount of 2-dGlc metabolized by the HMPS

was increased significantly as the concentration of 2-dGlc was increased ($P < 0.001$ at all concentrations), and the mCSF/PMA-stimulated HMPS activity is also sugar-dependent.

Fig. 4 shows the effect of varying the PMA concentration on the uptake of 2-d[1-³H]Glc or 2-d[2,6-³H]Glc (0.1 mM) for 45 min at 37 °C. Increasing the PMA concentration had no significant effect on the uptake of 0.1 mM-2-d[2,6-³H]Glc, but at 40 and 100 nM-PMA the uptake of 0.1 mM-2-d[1-³H]Glc was significantly reduced ($P < 0.001$ in both cases; $n = 4$). However, there was no significant effect of increasing the concentration of PMA from 40 to 100 nM on the difference in uptake of the two labels. Thus the mCSF-dependent increase in HMPS activity is due to an enhancement of the maximal response elicited by PMA. This finding is consistent with the view that mCSF acts synergistically with PMA to generate superoxide production indirectly by stimulating hexokinase activity.

Correlation between superoxide production and HMPS activity in PMA (40 nM) and PMA with mCSF (110 pM)-treated cells

Macrophages were incubated for 1 h at 37 °C with or without mCSF (110 pM) and in the presence of PMA (40 nM) during the last 30 min. Parallel experiments measured the production of superoxide and the uptake of 2-d[1-³H]Glc or 2-d[2,6-³H]Glc at 0, 0.01, 0.1 and 1 mM-2-dGlc. Table 5 shows the PMA- and PMA with mCSF-stimulated superoxide production ($\mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9 \text{ cells}^{-1}$) at each 2-dGlc concentration after the subtraction of the zero-sugar value, and the HMPS activity ($\mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9 \text{ cells}^{-1}$) was calculated from the differential uptake of the labelled forms of sugar and multiplied by 4. There was no significant difference between the superoxide production and the HMPS activity in PMA-treated macrophages at 0.01, 0.1 or 1.0 mM-2-dGlc. Similarly, there was no significant difference between these parameters in the PMA and mCSF-stimulated cells at any 2-dGlc concentration. These data indicate that there is a 1:4 correlation between the amount of transported sugar being metabolized by the HMPS and the production of superoxide by the macrophages.

Effects of cytochalasin B on PMA-stimulated 2-dGlc uptake, HMPS activity and superoxide production

To study sugar uptake and HMPS activity, macrophages were incubated for 15 min at 37 °C in various concentrations of 2-d[1-³H]Glc or 2-d[2,6-³H]Glc (0–10 mM) with PMA (40 nM) and with various concentrations (0–1 μM) of cytochalasin B (Sigma Chemical Co.). To measure superoxide production, the cells were preincubated for 60 min at 37 °C in various concentrations of unlabelled 2-dGlc (0.1–10 mM) and cytochalasin B (0–10 μM), with 20 μM -cytochrome *c* in the buffer. PMA (40 nM) was then added for a further 60 min, and superoxide production was measured as described in the Materials and methods section.

Fig. 5 shows a Dixon plot of the effect of cytochalasin B on the PMA-activated uptake of 2-d[2,6-³H]Glc and HMPS activity at 0.1 and 1.0 mM-2-dGlc. The lines were fitted by linear least-squares regression, and it can be seen that increasing the concentration of 2-dGlc reduces the inhibition by cytochalasin B of both processes. This indicates that cytochalasin B competitively inhibits the uptake of 2-d[2,6-³H]Glc and HMPS activity. The intersection of the lines at the different concentrations of 2-dGlc gives the $-K_i$ of cytochalasin B for HMPS activity and for the uptake of 2-d[2,6-³H]Glc. Therefore, the K_i of cytochalasin B for the uptake of 2-d[2,6-³H]Glc can be estimated as $0.28 \pm 0.11 \mu\text{M}$ and the K_i for HMPS activity is $0.35 \pm 0.10 \mu\text{M}$ ($n = 4$).

Fig. 6 shows a Dixon plot of the effect of cytochalasin B on the PMA-stimulated production of superoxide radicals with 0.1, 1.0

Table 5. Correlation between superoxide production and HMPS activity in PMA and PMA-mCSF treated macrophages

The production of superoxide and the activity of the hexose monophosphate shunt by PMA- (40 nM) and mCSF- (110 pM) treated peritoneal macrophages were measured in parallel experiments under identical conditions (see the Materials and methods section). Superoxide production at each 2-dGlc concentration is expressed as $\mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9 \text{ cells}^{-1}$ after subtraction of the zero-sugar value, and the HMPS activity (same units) is calculated from the differential uptake of 2 + d[1- ^3H]Glc and 2-d[2,6- ^3H]Glc, multiplied by a factor of 4. At each 2-dGlc concentration there is no significant difference between superoxide production and HMPS activity (multiplied by 4).

[2-dGlc] (mM)	Superoxide production ($\mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9 \text{ cells}^{-1}$)		HMPS activity ($\mu\text{mol} \cdot \text{h}^{-1} \cdot 3 \times 10^9 \text{ cells}^{-1}$)	
	PMA	PMA + mCSF	PMA	PMA + mCSF
0.01	0.5 $\pm$ 0.1	1.3 $\pm$ 0.2	0.6 $\pm$ 0.2	1.3 $\pm$ 0.2
0.1	6.0 $\pm$ 0.3	8.0 $\pm$ 0.5	5.9 $\pm$ 0.6	8.4 $\pm$ 0.5
1.0	10.0 $\pm$ 0.4	13.0 $\pm$ 0.8	10.8 $\pm$ 1.0	14.3 $\pm$ 1.0
n ...	9	9	10	10

Fig. 5. Dixon plot of the effect of cytochalasin B on PMA-stimulated 2-d[2,6- ^3H]Glc uptake and HMPS activity at various 2-dGlc concentrations

Macrophages were incubated for 15 min at 37 °C with 0.1 or 1.0 mM-2-d[1- ^3H]Glc or 2-d[2,6- ^3H]Glc, with PMA (40 nM) and various concentrations of cytochalasin B (0–1 μM). The uptake of 2-d[2,6- ^3H]Glc and the activity of the HMPS were calculated as $\mu\text{mol} \cdot 15 \text{ min}^{-1} \cdot \text{ml}$ of cell water $^{-1}$, plotted as 1/v against the concentration of cytochalasin B and lines were then fitted by linear least-squares regression. The $-K_i$ for cytochalasin B on 2-d[2,6- ^3H]Glc uptake and HMPS activity are given by the intersection of the lines at the different concentrations of 2-dGlc. The K_i for cytochalasin B on 2-d[2,6- ^3H]Glc uptake was 0.28 ± 0.11 ($n = 5$), and the K_i for cytochalasin B on HMPS activity was 0.35 ± 0.10 ($n = 5$). Symbols: HMPS activity: $\square$, 0.1 mM-2-dGlc; $\blacksquare$, 1.0 mM-2-dGlc; 2-d[2,6- ^3H]Glc uptake: $\circ$, 0.1 mM-2-dGlc; $\bullet$, 1.0 mM-2-dGlc.

and 10 mM-2-dGlc present in the bathing solutions. The lines are fitted by linear least-squares regression to five data points, although only three are shown on these axes. Cytochalasin B competitively inhibits the 2-dGlc-dependent generation of superoxide radicals by PMA-stimulated macrophages. The $-K_i$ of cytochalasin B is given by the intersection of the three lines,

Fig. 6. Dixon plot of the effects of cytochalasin B on PMA-induced superoxide production at various 2-dGlc concentrations

PMA-dependent (40 nM) SOD-inhibitable superoxide production was measured from cells incubated for 60 min at 37 °C in 0.1, 1.0 and 10 mM unlabelled 2-dGlc and cytochalasin B (0–10 μM). The five data points for each sugar concentration (only three shown on these axes) were fitted with lines by linear least-squares regression. The intersection of the three lines for the different concentrations ($\square$, 0.1 mM; $\blacksquare$, 1 mM; $\circ$, 10 mM) of 2-dGlc gives the $-K_i$ for inhibition of superoxide production by cytochalasin B. Therefore the K_i for cytochalasin B on PMA-stimulated superoxide production was $0.28 \pm 0.12 \mu\text{M}$ ($n = 5$).

Table 6. Kinetic parameters of PMA-dependent 2-dGlc transport and superoxide production

Experiments were performed over a range of 2-d[2,6- ^3H]Glc concentrations (0–10 mM) and cytochalasin B (cyto B) concentrations (0–5 μM), with PMA (40 nM) present throughout, and the uptake of 2-dGlc and superoxide production was assayed over 1 h at 37 °C. The data obtained were plotted using the ENZFITTER program of the Michaelis–Menten equation, and the kinetic parameters were calculated. $K_i = [\text{cytochalasin B}] / [(K_p/K_m) - 1]$. The results are expressed as means $\pm$ S.E.M. for n separate determinations. The units are: K_m and K_p , mM; K_i , μM ; V_{max} , $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}$ of cell water $^{-1}$.

	PMA		PMA + cyto B	
	K_m	V_{max}	K_p	K_i
2-d[2,6- ^3H]Glc uptake	0.45 ± 0.07	1.32 ± 0.05	1.80 ± 0.43	0.33 ± 0.11
Superoxide production	0.06 ± 0.02	0.40 ± 0.02	1.32 ± 0.48	0.24 ± 0.12
n ...	5	5	5	

and thus the K_i of cytochalasin B for superoxide production is $0.28 \pm 0.12 \mu\text{M}$ ($n = 5$).

The data were also plotted by least-squares regression using the ENZFITTER program of the Michaelis–Menten equation (not shown), and the kinetic parameters for PMA-stimulated 2-d[2,6- ^3H]Glc uptake and superoxide production calculated for control and cytochalasin B-treated macrophages (Table 6). The K_i for these processes was calculated from the formula:

$$K_i = [\text{cytochalasin B}] / [(K_p/K_m) - 1]$$

The K_m ($0.45 \pm 0.07 \text{ mM}$, $n = 5$) and the V_{max} ($1.32 \pm 0.05 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}$ of cell water $^{-1}$, $n = 5$) for PMA-stimulated 2-d[2,6- ^3H]Glc uptake are both significantly higher than the K_m ($0.06 \pm 0.02 \text{ mM}$, $n = 5$) and V_{max} ($0.40 \pm 0.02 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}$ of cell water $^{-1}$, $n = 5$) for PMA-dependent superoxide production

($P < 0.001$, in both cases). Treatment of cells with cytochalasin B significantly increases the K_m for both 2-dGlc uptake and superoxide generation ($P < 0.001$ in both cases), but the calculated K_i values for inhibition by cytochalasin B are not significantly different for the two processes.

DISCUSSION

Evidence of coupling between transport and metabolism of 2-D-deoxyglucose in rat peritoneal macrophages

Role of mCSF. The results indicate, as previously reported (Rist *et al.*, 1990), that mCSF increases sugar uptake by stimulating hexokinase activity coupled to sugar movement. The evidence supporting this conclusion is as follows: firstly, mCSF decreased the K_m of *zero-trans* 2-dGlc uptake without altering the V_{max} (Fig. 2); secondly, the exit of accumulated 2-dGlc was decreased by mCSF (Fig. 3); and thirdly, mCSF increases the steady-state accumulation of free 2-dGlc into the cytosolic pool (Table 2). These data are inconsistent with the conventional explanation of the changes in sugar transport, which contends that the enhanced uptake is due to an increase in the number of hexose transporters (Christopher *et al.*, 1976; Yamada *et al.*, 1983; Kuroda *et al.*, 1987), because we observe that, during the early phase of mCSF activation of macrophages, the V_{max} for 2-dGlc uptake is unaltered.

An alternative model for activation of sugar transport has been proposed where uptake is controlled by alterations in hexokinase activity at the endofacial surface of the sugar transporter (Naftalin & Smith, 1987). This 'loose-coupling' model envisages that increased coupling of hexokinase activity to sugar transport may result from adhesion of the enzyme to the cell membrane in the vicinity of the transporter. The predicted effects of this would be (a) a decrease in the K_m for *zero-trans* uptake of sugar, as a consequence of decreased bound free sugar activity at the endofacial surface of the transporter, (b) a decrease in the rate of free sugar exit from the cells, because any cytosolic free sugar which binds to the endofacial surface would be rephosphorylated by bound hexokinase, instead of escaping from the cell via the transporter, and (c) accumulation of free sugar within the cytosol to a steady-state concentration above that seen in control macrophages, as a result of the combined effects of (a) and (b), and the presence of sugar phosphatases.

The observation of all three of these predicted effects of the loose-coupling model in macrophages indicates that there is an mCSF-dependent coupling of sugar transport with hexokinase activity. A similar loose-coupling system between hexokinase and sugar transport has been reported in rat thymocytes and Chinese-hamster ovary cells (Naftalin & Rist, 1989; Faik *et al.*, 1989).

The data indicate that mCSF alone increases 2-dGlc uptake into rat macrophages without activation of the HMPS pathway or superoxide production (Tables 3 and 4). In contrast, PMA increases both the activity of the HMPS (Table 4) and production of superoxide (Table 3), as previously reported (Arthur *et al.*, 1988; Mohsensin & Latifpour, 1990). The stimulation of superoxide generation by PMA was sugar-dependent, and similarly the level of activity of the HMPS was higher as external 2-dGlc concentrations were raised. Although exposure to mCSF in the absence of PMA did not increase superoxide production and HMPS activity, it enhanced the effects of PMA. This 'priming' effect of mCSF on PMA-stimulated superoxide generation was previously reported in murine macrophages (Wing *et al.*, 1985), and synergistic activation of the HMPS pathway by these two agents was shown in rat peritoneal macrophages (Rist *et al.*, 1990).

Increasing the duration of preincubation before treatment with mCSF enhances the stimulatory effect of the cytokine on the uptake of 2-dGlc (Fig. 1). This may be due to the presence of endogenous cytokines in the freshly isolated macrophages which mask the effect of exogenous mCSF. Furthermore, it has been reported that incubation in the absence of growth factor leads to an up-regulation of mCSF receptors (Guilbert *et al.*, 1986; Boocock *et al.*, 1989; for a review, see Sherr, 1990), and this will result in greater stimulation of sugar transport on re-addition of mCSF.

Phillips & Hamilton (1990) reported that prolonged incubation of macrophages with high concentrations of mCSF results in a reduction in the respiratory burst and inhibits the priming of murine macrophages by a variety of agents, including interferon and bacterial lipopolysaccharide. This result apparently contradicts previous reports, including those of Hamilton *et al.* (1988) and Rist *et al.* (1990), where it was shown that mCSF and granulocyte-macrophage CSF stimulate glucose uptake. These recent results suggest that prolonged incubation, i.e. for 48 h, with very high mCSF concentrations, i.e. 5000 units/ml, leads to mCSF-receptor down-regulation. This does not negate the present findings, where only low concentrations of mCSF were used for relatively short periods, and these treatments lead to enhanced 2-dGlc uptake and to synergistic effects on PMA- and sugar-dependent superoxide production.

Role of HMPS activity in activation of macrophage sugar transport

The correlation between HMPS activity and superoxide generation has been shown in hepatic macrophages (Arthur *et al.*, 1986, 1988), and in rat alveolar macrophages, NADPH has been demonstrated to be a substrate for the reduction of oxygen to superoxide (Forman *et al.*, 1980; Mohsensin & Latifpour, 1990). Indeed, all phagocytic cell types, during the 'respiratory burst', metabolize glucose via the HMPS, producing NADPH, which is then utilized by the membrane-bound superoxide-generating enzyme (Babior, 1984).

The HMPS in transforming glucose into ribose 5-phosphate generates 2 mol of NADPH per mol of glucose (Landau & Wood, 1983). Each mol of NADPH is then utilized by the respiratory-burst enzyme to produce 2 mol of superoxide from the reduction of 2 mol of oxygen (Babior & Peters, 1981; DiGregorio *et al.*, 1989). Comparison of the difference between the C-1 and C-2,6 during labelled 2-dGlc uptakes in this series of experiments (i.e. sugar utilized by the HMPS) and the superoxide generated at each sugar concentration (after subtraction of zero-sugar value) shows a 1:4 correlation for PMA and mCSF/PMA-stimulated macrophages (Table 5). Thus, although 2-dGlc-6-P is a poorer substrate than glucose 6-phosphate for glucose-6-phosphate dehydrogenase (superoxide production with equimolar concentrations of glucose is approx. 3-fold higher than with 2-dGlc), it is nevertheless metabolized via this route in rat peritoneal macrophages. The major advantage in use of 2-dGlc over glucose for study of regulation of sugar transport and HMPS metabolism is that a clear concentration-dependent increase in superoxide production and HMPS metabolism can be established in activated macrophages.

Role of cytochalasin B in inhibition of reactive oxygen intermediates production. Cytochalasin B is a specific inhibitor of sugar binding at the endofacial surface of the human erythrocyte sugar transporter (Basketter & Widdas, 1978; Devés & Krupka, 1978), thereby acting as a competitive inhibitor of processes mediated at the endofacial surface, e.g. facilitated *zero-trans* exit. However, cytochalasin B acts as a non-competitive inhibitor of processes that are mainly mediated by the exofacial sugar-binding site, e.g. facilitated *zero-trans* uptake. In the present

Scheme 1. Interactions between the transport of sugar, HMPS activity and superoxide production and the possible sites of action of PMA, mCSF and cytochalasin B in rat peritoneal macrophages

study cytochalasin B is shown, paradoxically, to act as a competitive inhibitor of *zero-trans* 2-dGlc uptake and, less surprisingly, as a competitive inhibitor of 2-dGlc-dependent HMPS activity and superoxide production.

The quasi-competitive inhibition of *zero-trans* 2-dGlc by cytochalasin B implies that net uptake of 2-dGlc into macrophages is rate-limited by endofacial rather than by exofacial processes, i.e. sugar uptake is rate-limited by either the low rate of dissociation of sugar from the endofacial site or the catalysed rate of transformation of endofacially bound sugar to 2-dGlc 6-phosphate. These data are consistent with the view that sugar transport in macrophages is coupled to endofacial hexokinase activity. The competitive inhibition of both HMPS and superoxide production by cytochalasin B shows that these processes are also dependent on exogenous sugar uptake into the cells. Preincubation of the cells with 2-dGlc does not reduce the inhibition by cytochalasin B of superoxide production (R. J. Rist, G. E. Jones & R. J. Naftalin, unpublished work).

The quasi-competitive inhibition of the sugar-dependent component of superoxide production and of the HMPS by cytochalasin B may explain some apparently contradictory data in the literature. Kiyotaki *et al.* (1984), using 10 mM-glucose, observed a negligible inhibition of glucose transport into a cloned macrophage cell line. At this high concentration of sugar the competitive effects of cytochalasin B are insignificant. Kiyotaki *et al.* (1984) also observed a cytochalasin B-dependent enhancement of superoxide production. A cytochalasin B-dependent increase in superoxide has also been observed in rat polymorphonuclear leucocytes when incubated in sugar-free suspensions and stimulated by chemotactic peptide (Edwards *et al.*, 1984). Since it is known that the oxygen-burst response is entirely sugar-dependent (Kiyotaki *et al.*, 1984), it seems likely that the cytochalasin B-evoked increase in superoxide production is unrelated to the inhibitor effects on sugar transport. Significantly, cytochalasin B has been reported to increase superoxide production with a concurrent decrease in the formation of phagosomes in activated human granulocytes (Root & Metcalf, 1977). This finding suggests that cytochalasin B treatment of phagocytes allows the externalization of free radicals which would otherwise remain trapped in the cells and fail to be measured.

The identical K_i for cytochalasin B-dependent inhibition of

superoxide production and *zero-trans* 2-dGlc uptake ($\approx 0.30 \mu\text{M}$; Table 6) suggests that the generation of superoxide is entirely dependent on transport of 2-dGlc. However, the lower K_m and $V_{\max}$ for 2-dGlc-dependent free-radical production than for *zero-trans* 2-dGlc uptake (superoxide production, $K_m = 0.06 \pm 0.02 \text{ mM}$, $V_{\max} = 0.40 \pm 0.02 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}$ of cell water $^{-1}$, $n = 5$; *zero-trans* 2-dGlc uptake, $K_m = 0.45 \pm 0.07 \text{ mM}$, $V_{\max} = 1.32 \pm 0.05 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}$ of cell water $^{-1}$, $n = 5$) indicate that these processes are not tightly coupled, in that a large proportion of the transported sugar is not metabolized to generate superoxide radicals. The observation that there is a 1:4 correlation between the amount of sugar metabolized by the HMPS pathway and the production of superoxide in PMA and mCSF-PMA-stimulated macrophages (Table 5) suggests that there is a high degree of coupling between these two processes. The relationship between the transport of sugar, HMPS activity and free-radical production and the possible sites of action of PMA, mCSF and cytochalasin B can be represented by a flow diagram (Scheme 1).

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REFERENCES

- Arthur, M. J. P., Kowalski-Saunders, P. & Wright, R. (1986) *Gastroenterology* **91**, 174–181
- Arthur, M. J. P., Kowalski-Saunders, P. & Wright, R. (1988) *Gastroenterology* **95**, 1588–1594
- Babior, B. M. (1984) *J. Clin. Invest.* **73**, 599–601
- Babior, B. M. & Peters, W. A. (1981) *J. Biol. Chem.* **256**, 2321–2323
- Babior, G. L., Rosin, R. E., McMurrich, B. J., Peters, W. A. & Babior, B. M. (1981) *J. Clin. Invest.* **67**, 1724–1728
- Basketter, D. A. & Widdas, W. F. (1978) *J. Physiol. (London)* **278**, 389–401
- Bonventre, P. F., Straus, D., Baughn, R. E. & Imhoff, J. (1977) *J. Immunol.* **118**, 1827–1835
- Boockock, C. A., Jones, G. E., Stanley, E. R. & Pollard, J. W. (1989) *J. Cell. Sci.* **93**, 447–456
- Christopher, C. W., Kohlbacher, M. S. & Amos, H. (1976) *Biochem. J.* **158**, 439–450
- Devés, R. & Krupka, R. M. (1978) *Biochim. Biophys. Acta* **510**, 339–348
- Dexter, T. M., Whetton, A. D. & Hayworth, C. M. (1986) in *Oncogenes and Growth Control* (Kahn, P. & Graf, T., eds.), pp. 163–169, Springer-Verlag, Berlin
- DiGregorio, K. A., Cilento, E. V. & Lantz, R. C. (1989) *Am. J. Physiol.* **256**, 405–412
- Edwards, S. W., Hallett, M. B. & Campbell, A. K. (1984) *Biochem. J.* **217**, 851–854
- Faik, P., Morgan, M. J., Naftalin, R. J. & Rist, R. J. (1989) *Biochem. J.* **260**, 153–155
- Fantone, J. C. & Ward, P. A. (1982) *Am. J. Pathol.* **107**, 397–418
- Forman, H. J., Nelson, J. & Fisher, A. B. (1980) *J. Biol. Chem.* **255**, 9879–9883
- Guilbert, L. J., Tynan, P. W. & Stanley, E. R. (1986) *J. Cell. Biochem.* **31**, 203–216
- Hamilton, J. A., Vairo, G. & Lingelbach, S. R. (1986) *Biochem. Biophys. Res. Commun.* **138**, 445–454
- Hamilton, J. A., Vairo, G. & Lingelbach, S. R. (1988) *J. Cell. Physiol.* **134**, 405–412
- Kiyotaki, C., Peisach, J. & Bloom, B. R. (1984) *J. Immunol.* **132**, 857–866
- Klebanoff, S. J. (1980) *Ann. Intern. Med.* **93**, 480–489
- Kuroda, M., Honnor, R. C., Cushman, S. W., Londres, C. & Simpson, I. A. (1987) *J. Biol. Chem.* **262**, 245–253
- Landau, B. R. & Wood, H. G. (1983) *Trends Biochem. Sci.* **8**, 292–296
- Lee, M. T. & Warren, M. K. (1987) *J. Immunol.* **138**, 3019–3022
- Mohsensin, V. & Latifpour, J. (1990) *J. Appl. Physiol.* **68**, 2384–2390
- Naftalin, R. J. & Rist, R. J. (1989) *Biochem. J.* **260**, 143–152
- Naftalin, R. J. & Rist, R. J. (1990) *Biochem. J.* **265**, 251–259

- Naftalin, R. J. & Smith, P. M. (1987) *Biochim. Biophys. Acta* **897**, 93–111
- Phillips, W. A. & Hamilton, J. A. (1990) *J. Cell. Physiol.* **144**, 190–196
- Rist, R. J., Jones, G. E. & Naftalin, R. J. (1990) *Biochem. J.* **265**, 243–249
- Root, R. K. & Metcalf, J. A. (1977) *J. Clin. Invest.* **60**, 1266–1279
- Sampson-Johannes, A. & Carlino, J. A. (1988) *J. Immunol.* **141**, 3680–3686
- Sherr, C. J. (1990) *Blood* **75**, 1–12
- Thompson, K. A. & Kleinzeller, A. (1989) *Am. J. Physiol.* **257**, 214–222
- Van Gelder, B. F. & Slater, E. C. (1962) *Biochim. Biophys. Acta* **58**, 593–595
- Weening, R. S., Wever, R. & Roos, D. (1975) *J. Lab. Clin. Med.* **85**, 245–252
- Wing, E. J., Waheed, A., Shadduck, R. K. & Nagle, L. S. (1982) *J. Clin. Invest.* **69**, 270–276
- Wing, E. J., Ampel, N. M., Waheed, A. & Shadduck, R. K. (1985) *J. Immunol.* **135**, 2052–2056
- Yamada, K., Tillotson, L. G. & Isselbacher, K. J. (1983) *J. Biol. Chem.* **258**, 9786–9792

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