Zymosan-triggered tyrosine phosphorylation in mouse bone-marrow-derived macrophages is enhanced by respiratory-burst priming agents

Simon P. GREEN, John A. HAMILTON and Wayne A. PHILLIPS

University of Melbourne, Department of Medicine, Royal Melbourne Hospital, Parkville 3050, Australia

We have investigated the relationship between tyrosine phosphorylation and respiratory-burst activity in mouse bone-marrow-derived macrophages (BMM). We demonstrate that zymosan, an agent known to trigger the macrophage respiratory burst, also triggers the activation of tyrosine kinase activity, resulting in rapid tyrosine phosphorylation on numerous proteins, and provide evidence for the role of tyrosine phosphorylation in the triggering of the BMM respiratory burst. Agents, such as tumour necrosis factor α (TNF α), interferon- γ (IFN- γ) or lipopolysaccharide (LPS), which prime the macrophage for an enhanced zymosan-triggered respiratory burst, increase tyrosine phosphorylation triggered by zymosan. The zymosan-triggered tyrosine phosphorylation and respiratory-burst activity were partially suppressed by the tyrosine kinase inhibitors α -cyano-3-ethoxy-4-hydroxy-5-phenylmethylcinnamide (ST638) and herbimycin A. In addition, pre-exposure of BMM to vanadate, a phosphotyrosine phosphatase inhibitor, greatly enhanced the ability of zymosan to induce tyrosine phosphorylation and trigger the respiratory burst. These data highlight the importance of the balance between tyrosine kinase and phosphotyrosine phosphatase activity in determining the ultimate level of tyrosine phosphorylation in BMM and suggest that zymosan-triggered tyrosine phosphorylation is an important biochemical signal for triggering of the respiratory burst.

INTRODUCTION

One of the major functions of differentiated phagocytic cells, such as macrophages and neutrophils, is to provide the body with an immediate innate defence mechanism against pathogenic micro-organisms. The ability of macrophages to protect the body against these micro-organisms is largely dependent on the respiratory burst, a process that generates oxygen radicals required for killing these pathogens [1]. The potential of mature macrophages to generate oxygen radicals can be enhanced by priming. Primed cells are able to respond to a subsequent stimulus with a greatly increased respiratory burst. *In vitro*, it is possible to induce this primed state by exposure of macrophages to cytokines [2,3].

We have previously demonstrated that zymosan, a cell-wall component of the yeast *Saccharomyces cerevisiae* and which consists of the carbohydrate polymers β -glucan and α -mannan, is able to trigger the respiratory burst in unprimed mouse bone-marrow-derived macrophages (BMM) [2]. Although zymosan has been shown to stimulate diacylglycerol generation [4], phosphoinositol turnover and the induction of *c-fos* and *c-myc* mRNA levels [5] in BMM, the signal-transduction pathways involved in the activation of the respiratory burst are not understood.

Previous studies, primarily in the neutrophil, have indicated that other second messengers, including guanine-nucleotide-binding proteins, protein kinase C, phosphoinositides, Ca²⁺ and arachidonic acid, may have a role in the regulation of the respiratory burst [6–12]. Further studies have suggested that tyrosine kinase may also have a role in activation of the respiratory burst in neutrophils [9,13–15]. Consistent with this suggestion, we have recently demonstrated a temporal relationship between the expression of two *src*-family tyrosine kinases, *hck* and *lyn*, and priming of the macrophage respiratory burst with lipopolysaccharide (LPS) and interferon- γ (IFN- γ) [16].

In this report we examine the role of tyrosine phosphorylation in the triggering of the macrophage respiratory burst. We demonstrate that zymosan, an agent that triggers the respiratory burst, stimulates tyrosine kinase activity in BMM and that the respiratory-burst priming agents, tumour necrosis factor α (TNF α), IFN- γ and LPS, enhance the ability of zymosan to induce tyrosine phosphorylation. Our results demonstrate a functional association between tyrosine phosphorylation and activation of the respiratory burst by zymosan. We suggest that triggering of the respiratory burst is mediated by the activation of a tyrosine kinase(s).

MATERIALS AND METHODS

Reagents

The following were obtained: aprotinin, horse heart cytochrome *c* type III, phenylmethanesulphonyl fluoride, phosphotyrosine, sodium orthovanadate, deoxycholate, Nonidet P40 and zymosan, from Sigma Chemical Company, St. Louis, MO, U.S.A.; RPMI-1640, from Flow Laboratories, McLean, VA, U.S.A.; fetal calf serum, from Commonwealth Serum Laboratories, Parkville, Victoria, Australia; affinity-purified mouse monoclonal anti-phosphotyrosine antibody (PY20) conjugated to horseradish peroxidase, from ICN Biochemicals, Sydney, Australia; enhanced chemiluminescence (ECL) Western-blotting-detection reagents and Hyperfilm-ECL from Amersham Corp. Sydney, Australia; BSA and chicken ovalbumin from Calbiochem, Sydney, Australia; LPS purified from *Escherichia coli* 0111:B4 by the Westphal method from Difco, Detroit, MI, U.S.A.; recombinant mouse IFN- γ from Nippon La Roche, Tokyo, Japan. ST638 (α -cyano-3-ethoxy-4-hydroxy-5-phenylmethylcinnamide) was generously provided by Dr. Tadayoshi Shiraishi, Biomedical Research Laboratories, Kanegafuchi Chemical Industry Co. Ltd., Takasago, Hyogo, Japan. Herbimycin A was supplied by Dr. Yoshito Kaziro, DNAX

Abbreviations used: BMM, bone-marrow-derived macrophage; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; TNF α , tumour necrosis factor α ; ST638, α -cyano-3-ethoxy-4-hydroxy-5-phenylmethylcinnamide.

Research Institute, CA, U.S.A. Recombinant mouse TNF α was a gift from Dr. G. F. Adolf, Boehringer Ingelheim, Vienna, Austria.

Cells

CBA mice (6–12 weeks old) were obtained from the Walter and Eliza Hall Institute (Parkville, Australia). Mouse BMM were grown from precursor cells obtained from mouse bone marrow as described previously [17]. Cells were seeded into either Linbro 24-well plates at 5×10^4 cells/ml or Linbro six-well plates at 2×10^5 cells/2 ml in RPMI culture medium containing 20% (v/v) L-cell-conditioned medium (as a source of the macrophage growth factor, colony-stimulating factor-1) and 10% (v/v) fetal calf serum and grown to confluence. After being washed with phosphate-buffered saline (PBS; 0.14 M-NaCl, 8.1 mM- Na_2HPO_4 , 1.4 mM- KH_2PO_4 , 2.7 mM-KCl, pH 7.3), the remaining adherent BMM are a relatively pure and homogeneous population of macrophages with more than 95% of the cells binding colony-stimulating factor-1 [18].

Maintenance of LPS-free conditions

All tissue culture ware, if not of a sterile disposable nature, was thoroughly washed and heated to 180 °C for 3 h to destroy any contaminating LPS. Media and buffers were filtered through Zetapor membranes (AMF Cuno, Meriden, CT, U.S.A.) and reagents were routinely prepared in pyrogen-free water (Delta West Limited, Bentley, Western Australia). The maintenance of LPS-free conditions was regularly monitored using the *Limulus* lysate assay (Commonwealth Serum Laboratories) in which the minimal detectable levels of the assay was 10 pg/ml.

Respiratory burst

The respiratory burst of adherent BMM was assessed by the stimulus-induced reduction of cytochrome *c* [2]. After exposure to priming agents, the cells were washed once with PBS and incubated in 1 ml of Krebs–Ringer phosphate buffer (121 mM-NaCl, 5 mM-KCl, 1.3 mM- CaCl_2 , 1.2 mM- MgSO_4 , 3.1 mM- NaH_2PO_4 , 12.5 mM- Na_2HPO_4 and 11 mM-dextrose, pH 7.3) containing 80 μM -cytochrome *c* and 1 mg of boiled zymosan/ml for 90 min at 37 °C in 5% CO_2 . The cytochrome *c* reaction medium was then collected, clarified by centrifugation (13000 rev./min; Eppendorf Microfuge), and the absorbance monitored at 550 nm. After removal of the cytochrome *c* reaction medium, adherent cells were washed with PBS and the total protein in each culture well was determined by the Lowry method with BSA as standard [19]. Respiratory-burst activity was expressed as nmol of cytochrome *c* reduced/mg of cell protein and the results were presented as means $\pm$ S.E.M. of at least triplicate determinations. The superoxide dependence of the stimulus-induced cytochrome *c* reduction was confirmed by the inclusion of superoxide dismutase (30 μg /ml) in selected controls.

Phosphotyrosine detection

Cells were washed twice in ice-cold PBS and lysed in 100 mM-NaCl containing 10 mM-Tris/HCl, pH 7.2, 2 mM-EDTA, 0.5% (w/v) deoxycholate, 1% (v/v) Nonidet P40, 10 mM- MgCl_2 , 1 mM-phenylmethanesulphonyl fluoride, 10 μg of aprotinin/ml and 100 μM -sodium orthovanadate, for 15 min on ice. The protein content of each lysate was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.), with BSA as a standard. Lysates containing 50 μg of protein were mixed with an equal volume of Laemmli sample buffer [20], and heated for 5 min at 95 °C. After SDS/PAGE (10% gel) and electrophoretic transfer, the nitrocellulose filters were blocked by incubation in Tris-buffered saline [10 mM-

Tris/HCl, pH 7.4, 0.9% (w/v) NaCl] containing 5% (w/v) BSA, 1% (w/v) ovalbumin and 100 μM -sodium orthovanadate for 1 h at 37 °C. Phosphotyrosine-containing proteins were detected by incubation with an affinity-purified anti-phosphotyrosine antibody (PY20) (100 ng/ml) conjugated to horseradish peroxidase for 3 h at 22 °C. The filters were washed in Tris-buffered saline and visualized using an enhanced chemiluminescence detection system (ECL), performed as recommended by the manufacturer. The anti-phosphotyrosine antibody, PY20, does not recognize phosphoserine- or phosphothreonine-containing proteins [21].

^{125}I -Zymosan uptake

Zymosan was iodinated to a specific radioactivity of 5×10^7 c.p.m./mg as previously described [22]. The uptake of zymosan by control and inhibitor-treated BMM was assessed by incubating adherent cells in 0.5 ml of Krebs–Ringer phosphate buffer with 50 μg of ^{125}I -zymosan/ml in the presence or absence of a 20-fold excess of unlabelled zymosan for 10 min at 37 °C. The cells were then washed three times with PBS to remove free zymosan and the cell-associated radioactivity was determined by dissolving the cells in 0.2 ml of 0.2 M-NaOH and counting samples in a γ -radiation counter (Packard). Specific binding was calculated as total binding minus binding in the presence of excess unlabelled zymosan.

RESULTS

Effect of zymosan on BMM intracellular tyrosine phosphorylation and respiratory-burst activity

The level of tyrosine phosphorylation was assessed by Western-blot analysis using a mouse monoclonal anti-phosphotyrosine antibody (PY20) conjugated to horseradish peroxidase. Exposure of BMM to zymosan resulted in a time-dependent increase in tyrosine phosphorylation (Fig. 1). Zymosan-induced phosphorylation was detected on at least ten protein substrates, the most prominent having molecular masses of 53, 55, 71, 75, 91 and 94 kDa. Quantification of tyrosine phosphorylation by densitometry confirmed that zymosan increased tyrosine phosphorylation within 5 min and was maximal at 10 min with

Fig. 1. Time course of zymosan-triggered tyrosine phosphorylation

BMM were exposed to boiled zymosan (1 mg/ml) for 0–90 min at 37 °C. Cells were scraped into lysis buffer and the proteins separated by SDS/PAGE. After Western transfer, blots were incubated with a phosphotyrosine-specific antibody (PY20) conjugated to horseradish peroxidase. Tyrosine phosphorylation was detected by enhanced chemiluminescence. The Hyperfilm-ECL was exposed for 10 min. Remizole-labelled molecular-mass marker proteins were phosphorylase *b* (98 kDa), albumin (71 kDa), ovalbumin (47 kDa), and carbonic anhydrase (32 kDa).

Fig. 2. Comparison of the zymosan time course between tyrosine phosphorylation and respiratory-burst activity

BMM were exposed to boiled zymosan (1 mg/ml) for 0–90 min at 37 °C. Zymosan-induced tyrosine phosphorylation was quantified by densitometric analysis of the exposed Hyperfilm-ECL using a Molecular Dynamics densitometer (▲). The densitometric data are expressed as a percentage of the maximal response; 100% maximal zymosan response represents a 6.67-fold increase in total phosphorylation compared with the unstimulated control. Results are expressed as means $\pm$ S.E.M. ($n = 3$). The zymosan-triggered respiratory-burst activity was assessed by the reduction of cytochrome *c* (□). Results are expressed as means $\pm$ S.E.M. ($n = 3$).

Fig. 4. Comparison of the zymosan dose-response between tyrosine phosphorylation and respiratory-burst activity

BMM were exposed to boiled zymosan (0–1000 µg/ml) at 37 °C for 10 min. Zymosan-induced tyrosine phosphorylation was quantified by densitometric analysis of the exposed Hyperfilm-ECL as described in the legend to Fig. 2 (▲). The densitometric data are expressed as a percentage of the maximal response. Results are expressed as means $\pm$ S.E.M. ($n = 3$). The zymosan-triggered respiratory-burst activity was assessed by the reduction of cytochrome *c* (□). Results are expressed as means $\pm$ S.E.M. ($n = 3$).

Fig. 3. Dose-response of zymosan-triggered tyrosine phosphorylation

BMM were exposed to boiled zymosan (0–1000 µg/ml) at 37 °C for 10 min. Tyrosine phosphorylation was assessed by Western blot and visualized by enhanced chemiluminescence. The Hyperfilm-ECL was exposed for 10 min.

a 6.7 ± 2.5 -fold increase compared with the unstimulated control (mean $\pm$ S.E.M., $n = 7$). The level of tyrosine phosphorylation declined thereafter (Fig. 2). In parallel cultures, zymosan induced a linear increase in respiratory-burst activity over the 0–90 min period (Fig. 2). The increase in tyrosine phosphorylation after exposure to zymosan was dose-dependent (Fig. 3). Densitometric analysis indicates that tyrosine phosphorylation induced by zymosan was observed between 10 and 50 µg/ml and reached maximal levels by 500 µg/ml (Fig. 4). A similar dose-dependence was observed for the zymosan-induced respiratory burst (Fig. 4). Detection of phosphotyrosine-containing proteins was completely eliminated by the inclusion of 4.5 mM-phosphotyrosine, confirming the specificity of the anti-phosphotyrosine antibody (results not shown). Zymosan-induced tyrosine phosphorylation

was not suppressed in BMM pretreated with cycloheximide (10 µg/ml) or actinomycin D (5 µg/ml) for 15 min at 37 °C, conditions that inhibit transcription and translation in BMM (S. P. Green, J. A. Hamilton & W. A. Phillips, unpublished work). Consequently, the increase in tyrosine phosphorylation is unlikely to be due to increased synthesis of constitutively phosphorylated proteins.

Amplification of zymosan-induced tyrosine phosphorylation and respiratory-burst activity by priming agents

The respiratory burst of BMM can be enhanced by prior exposure to various priming agents including TNF α , IFN- γ and LPS [2,3] for 24–48 h. We have exposed BMM to these agents for 48 h, triggered the respiratory burst with zymosan (1 mg/ml) for 10 min and analysed subsequent tyrosine phosphorylation (Fig. 5). Addition of TNF α (1 nM), IFN- γ (100 units/ml) and LPS (100 ng/ml) for 48 h had modest effects on the tyrosine phosphorylation of a number of substrates (Fig. 5). In the absence of a triggering stimulus, none of these priming agents were able to generate a respiratory burst. In contrast with the modest effects of the priming agents alone, the degree of zymosan-induced tyrosine phosphorylation was dramatically potentiated in cells pretreated with TNF α , IFN- γ and LPS (Fig. 5). Densitometric analysis indicates that TNF α , LPS and IFN- γ enhanced the zymosan-triggered tyrosine phosphorylation from 1.0 (unprimed) to $3.2 \pm 0.6^{**}$ ($n = 5$), $1.6 \pm 0.2^*$ ($n = 3$) and $1.8 \pm 0.3^*$ ($n = 3$) fold respectively. Under the same conditions TNF α , IFN- γ and LPS enhanced the zymosan-triggered respiratory burst from 78 ± 17 ($n = 13$) (zymosan alone) to $217 \pm 40^{**}$ ($n = 12$), 101 ± 25 ($n = 6$) and $161 \pm 24^{**}$ ($n = 12$) nmol/90 min per mg respectively (means $\pm$ S.E.M., $^*P < 0.1$; $^{**}P < 0.05$; paired *t* test).

Inhibition of tyrosine phosphorylation and respiratory-burst activity by herbimycin A and ST638

To examine further the apparent relationship between tyrosine phosphorylation and respiratory-burst activity, we investigated

Fig. 5. Effect of priming agents on zymosan-triggered tyrosine phosphorylation

BMM were cultured with medium alone (control), 1 nm-TNF α , 100 ng of LPS/ml or 100 units of IFN- γ /ml for 48 h at 37 °C before stimulation with 1 mg of zymosan/ml for 10 min. Tyrosine phosphorylation was assessed by Western blot and visualized by enhanced chemiluminescence. The Hyperfilm-ECL was exposed for 10 min.

Fig. 6. Inhibition of zymosan-triggered tyrosine phosphorylation by ST638 and herbimycin A

BMM were incubated with medium alone or medium containing 100 μ M-ST638 for 10 min or 5 μ g of herbimycin A/ml for 4 h at 37 °C before stimulation with zymosan (1 mg/ml) for 10 min. Tyrosine phosphorylation was assessed by Western blot and visualized by enhanced chemiluminescence. The Hyperfilm-ECL was exposed for 15 min. Lane A, medium alone; lane B, zymosan alone; lane C, herbimycin A followed by stimulation with zymosan; lane D, ST638 followed by stimulation with zymosan.

the effects of two tyrosine kinase inhibitors, herbimycin A and ST638. Exposure of BMM to either herbimycin A (5 μ g/ml) for 4 h or ST638 (100 μ M) for 30 min partially suppressed the zymosan-induced tyrosine phosphorylation (Fig. 6, lanes C and D). Quantification of tyrosine phosphorylation by densitometry revealed an inhibition of $48 \pm 10\%$ (n = 3) and $46 \pm 11\%$ (n = 4) (mean $\pm$ S.E.M.) using herbimycin A and ST638 respectively. The zymosan-triggered respiratory burst was similarly inhibited by $35 \pm 4\%$ (n = 3) and $30 \pm 3\%$ (n = 4) (mean $\pm$ S.E.M.) using 5 μ g of herbimycin A/ml and 100 μ M-ST638 respectively (** P < 0.05; paired t test). In addition, the respiratory burst of primed BMM was also suppressed by treatment

Fig. 7. Enhancement of zymosan-triggered tyrosine phosphorylation and respiratory-burst activity by vanadate

(a) BMM were cultured in medium alone or with 100 μ M-vanadate at 37 °C for 3 h before stimulation with 1 mg of zymosan/ml for 10 min (where indicated). (b) BMM were incubated with medium alone or vanadate, without further stimulation. Tyrosine phosphorylation was detected by enhanced chemiluminescence. The Hyperfilms-ECL for (a) and (b) were exposed for 10 min and 20 min respectively.

with herbimycin A and ST638. Incubation with 5 μ g of herbimycin A/ml for 4 h inhibited the zymosan-triggered respiratory burst in TNF α and LPS-primed BMM by $30 \pm 6\%$ and $10 \pm 5\%$ respectively (means $\pm$ S.E.M., n = 3). Similarly, treatment with 100 μ M-ST638 for 30 min inhibited the zymosan-triggered respiratory burst in TNF α - and LPS-primed BMM by $22 \pm 10\%$ and $31 \pm 9\%$ respectively (mean $\pm$ S.E.M., n = 5, * P < 0.1; ** P < 0.05; paired t test). To confirm the specificity of ST638 for tyrosine kinases, we have shown that this inhibitor alone did not modulate the levels or subcellular distribution of protein kinase C, or cell viability as assessed by cell adherence, Trypan Blue exclusion and total cellular protein measurements (S. P. Green, J. A. Hamilton & W. A. Phillips, unpublished work). Importantly, equivalent concentrations of the dimethyl sulphoxide carrier had no effect on zymosan-triggered tyrosine phosphorylation or respiratory-burst activity (results not shown).

Effect of herbimycin A and ST638 on 125 I-zymosan uptake

Since ST638 has been reported to interfere with FMLP binding in neutrophils [13], it was important to determine whether the tyrosine kinase inhibitors used in this study had similar effects on zymosan uptake in BMM. Incubation of BMM with 5 μ g of herbimycin A/ml for 4 h or 100 μ M-ST638 for 30 min had no effect on 125 I-zymosan uptake. The amount of cell-associated 125 I-zymosan after a 10 min incubation was $106 \pm 6\%$ and $96 \pm 11\%$ (mean $\pm$ S.E.M., n = 3) compared with the untreated control (100%) for ST638- or herbimycin A-treated BMM respectively. A 100% uptake of 125 I-zymosan represents 346 c.p.m./ μ g of soluble protein (BioRad protein assay).

Enhancement of tyrosine phosphorylation and the respiratory burst by vanadate

Since the observed level of tyrosine phosphorylation is likely to be the result of a balance between tyrosine kinase activity and the opposing endogenous phosphotyrosine phosphatase activity, we have investigated the effects of a phosphotyrosine phosphatase inhibitor, vanadate. Pretreatment of BMM with 100 μ M-vanadate for 3 h greatly facilitated zymosan-triggered tyrosine phosphorylation (Fig. 7a). Interestingly, pretreatment of BMM with 100 μ M-vanadate also enhanced the zymosan-triggered respiratory-burst

activity from 54 ± 3 (control) to 254 ± 31 nmol/90 min per mg (vanadate-treated) (mean $\pm$ S.E.M., $n = 3$, $P < 0.001$, paired t test). Vanadate alone did not induce tyrosine phosphorylation (Fig. 7b) or respiratory-burst activity. The priming effects of $\text{TNF}\alpha$, LPS and $\text{IFN-}\gamma$ on both the zymosan-triggered tyrosine phosphorylation and respiratory burst were also greatly amplified in vanadate-treated cells (results not shown).

DISCUSSION

We have investigated the role of tyrosine phosphorylation in the activation of the respiratory burst of BMM. Using a specific affinity-purified anti-phosphotyrosine antibody that does not recognize phosphoserine or phosphothreonine [21], we have measured the net amount of tyrosine phosphorylation that occurs in intact cells by Western-blot analysis. This approach allows us to observe the net tyrosine phosphorylation of individual endogenous proteins under physiological conditions, thus eliminating many of the potential difficulties in interpretation associated with studying the phosphorylation of artificial substrates in disrupted cell preparations. The detection of two tyrosine-phosphorylated proteins with apparent molecular masses of 53 and 55 kDa in unstimulated control cells suggests that these proteins may be constitutively phosphorylated on tyrosine. Zymosan, a stimulus used to trigger the respiratory burst, rapidly induced tyrosine phosphorylation on a number of protein substrates (Figs. 1 and 3). Detection of phosphotyrosine-containing proteins was completely eliminated by the inclusion of 4.5 mM-phosphotyrosine, confirming the specificity of the anti-phosphotyrosine antibody.

Since the level of tyrosine phosphorylation *in vivo* is determined by the balance between the activities of tyrosine kinase(s) and the opposing phosphotyrosine phosphatase(s), the ability of zymosan to increase levels of tyrosine phosphorylation may be the result of either a stimulation of a tyrosine kinase(s) or an inhibition of a phosphotyrosine phosphatase(s). Incubation of BMM with the tyrosine kinase inhibitors, ST638 [23] and herbimycin A, suppressed the zymosan-triggered tyrosine phosphorylation (Fig. 6). Concomitantly, herbimycin A and ST638 also suppressed the zymosan-triggered respiratory burst by a similar extent. Both herbimycin A and ST638 were also found to suppress the zymosan-triggered respiratory burst in primed BMM. Our data are consistent with the previously reported inhibition of the zymosan-stimulated respiratory burst in neutrophils by ST638 [14,15].

It is important to note that, although the inhibition of the respiratory burst by ST638 and herbimycin A was only modest, this inhibition correlated well with the level of inhibition of tyrosine phosphorylation. It is not clear why these inhibitors failed to inhibit tyrosine kinase activity completely. It may be that we were unable to achieve an appropriate intracellular concentration under our experimental conditions. Alternatively, it may reflect a poor specificity of these inhibitors for the particular tyrosine kinase(s) stimulated by zymosan. Indeed, ST638 has been reported to be a poor inhibitor of cytosolic tyrosine kinase activity [13].

In order to examine the involvement of phosphotyrosine phosphatase(s) on the level of zymosan-triggered tyrosine phosphorylation, we have used vanadate, a phosphotyrosine phosphatase inhibitor [24], to block endogenous phosphotyrosine phosphatase activity. Pre-exposure of BMM to vanadate dramatically enhanced the level of tyrosine phosphorylation observed when the cells were subsequently stimulated with zymosan (Fig. 7a). Vanadate also enhanced the zymosan-triggered respiratory-burst activity, providing further support for an association between tyrosine phosphorylation and

triggering of the respiratory burst, and demonstrates a potential role for phosphotyrosine phosphatases in regulation of the respiratory burst. Interestingly, vanadate alone did not elevate tyrosine phosphorylation (Fig. 7b). Since the ability of vanadate to enhance tyrosine phosphorylation in response to zymosan and phorbol myristate acetate (unpublished work) indicates that vanadate is able to inhibit intracellular phosphotyrosine phosphatases, the inability of vanadate alone to induce tyrosine phosphorylation would suggest that simply decreasing phosphotyrosine phosphatase activity is insufficient to enhance tyrosine phosphorylation in the absence of a stimulus for tyrosine kinase(s). It therefore follows that the phosphorylation observed in response to zymosan is the result of the activation of a tyrosine kinase(s) rather than inhibition of phosphotyrosine phosphatase activity. Consistent with its inability to induce tyrosine phosphorylation, vanadate alone did not trigger a respiratory burst in intact BMM. In contrast, others have reported triggering of the respiratory burst with vanadate, but this was demonstrated using permeabilized neutrophils [25] or HL60 cells [26].

Although LPS, $\text{TNF}\alpha$ and $\text{IFN-}\gamma$ have been shown to trigger rapid tyrosine phosphorylation in a number of cell types [27–29], these priming agents alone do not trigger the macrophage respiratory burst. Instead, incubation with these priming agents for longer periods (12–48 h) primes the macrophage for an enhanced respiratory burst after stimulation with a triggering agent. Similarly, the ability of zymosan to induce tyrosine phosphorylation was also enhanced in primed BMM (Fig. 5). Interestingly, this priming effect on both tyrosine phosphorylation and the respiratory burst was not observed when BMM were incubated with the priming agents for shorter periods (1–6 h) (results not shown).

The mechanism underlying the increase in zymosan-triggered tyrosine phosphorylation in primed BMM is not clear, but may be due to an increase in the level and/or specific activity of endogenous tyrosine kinases. Consistent with this suggestion, we have recently demonstrated that LPS and $\text{IFN-}\gamma$ increase transcription and translation of *hck* and *lyn* tyrosine kinases in BMM [16]. A good correlation between the expression of *hck* and *lyn* and the BMM respiratory burst was found, suggesting a possible role for these two kinases in the activation of the respiratory burst. The induction of *hck* by LPS has also been reported in human monocyte-derived macrophages [30]. Alternatively, the ability of vanadate, a phosphotyrosine phosphatase inhibitor, to enhance the respiratory burst of activated macrophages raises the possibility that putative physiological priming agents, such as LPS and cytokines [2,8,31–33], may also suppress phosphotyrosine phosphatase activity.

The data presented in this report demonstrate that zymosan triggers tyrosine phosphorylation in BMM. The level of this phosphorylation correlates well with the activity of the respiratory burst. However, the physiological relationship between tyrosine phosphorylation and the activation of the respiratory burst is not clear. The accumulation of tyrosine-phosphorylated proteins appears to precede the respiratory burst and declines while the rate of the respiratory burst is maintained (Fig. 2), suggesting that, although tyrosine phosphorylation may be required to initiate the respiratory burst, the maintenance of tyrosine phosphorylation does not appear to be necessary for continued activity.

This work was supported by the Eileen Urquhart Memorial Scholarship, Arthritis Foundation of Australia (S.P.G.) and a Program Grant from the National Health and Medical Research Council of Australia. We thank Dr. Tadayoshi Shiraishi for kindly providing ST638, Dr. Yoshito Kaziro for supplying us with herbimycin A and Dr. G. R. Adolf for generously donating the $\text{TNF}\alpha$ used in this study. We

also thank Drs. Ashley Dunn, Steve Ralph and Stella Clark for helpful discussions.

REFERENCES

- Morel, F., Doussiere, J. & Vignais, P. V. (1991) *Eur. J. Biochem.* **201**, 523–546
- Phillips, W. A. & Hamilton, J. A. (1989) *J. Immunol.* **142**, 2445–2449
- Warren, J. S., Kunkel, S. L., Cunningham, T. W., Johnson, K. J. & Ward, P. A. (1988) *Am. J. Pathol.* **130**, 489–495
- Veis, N. & Hamilton, J. A. (1991) *J. Cell. Physiol.* **147**, 298–305
- Hamilton, J. A., Veis, N., Bordun, A.-M., Vairo, G., Gonda, T. J. & Phillips, W. A. (1989) *J. Cell. Physiol.* **141**, 618–626
- Richter, J., Anderson, T. & Olsson, I. (1989) *J. Immunol.* **142**, 3199–3205
- McColl, S. R., Kreis, C., DiPersio, J. F., Borgeat, P. & Naccache, P. H. (1989) *Blood* **73**, 588–591
- Gomez-Cambronero, J., Yamazaki, M., Metwally, F., Molski, T. F., Bonak, V. A., Huang, C. K., Becker, E. L. & Sha'afi, R. I. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3569–3573
- Nasmith, P. E., Mills, G. B. & Grinstein, S. (1989) *Biochem. J.* **257**, 893–897
- Heidel, J. R., Taylor, S. M., Silflow, R. M., Laegreid, W. W. & Leid, R. W. (1991) *Inflammation* **15**, 31–42
- Pittet, D., Krause, K. H., Wollheim, C. B., Bruzzzone, R. & Lew, D. P. (1987) *J. Biol. Chem.* **262**, 10072–10076
- Sako, T., Tauber, A. I., Jeng, A. Y., Yuspa, S. H. & Blumberg, P. M. (1988) *Cancer Res.* **48**, 4646–4650
- Berkow, R. L., Dodson, R. W. & Kraft, A. S. (1989) *Biochim. Biophys. Acta* **997**, 292–301
- Gomez-Cambronero, J., Huang, C. K., Bonak, V. A., Wang, E., Casnellie, J. E., Shiraishi, T. & Sha'afi, R. I. (1989) *Biochem. Biophys. Res. Commun.* **162**, 1478–1485
- McColl, S. R., DiPersio, J. F., Caon, A. C., Ho, P. & Naccache, P. H. (1991) *Blood* **78**, 1842–1852
- Boulet, I., Ralph, S., Stanley, E., Lock, P., Dunn, A. R., Green, S. P. & Phillips, W. A. (1992) *Oncogene* **7**, 703–710
- Vairo, G. & Hamilton, J. A. (1985) *Biochem. Biophys. Res. Commun.* **132**, 430–437
- Tushinski, R. J., Oliver, I. T., Guilbert, L. J., Tynan, P. W., Warner, J. R. & Stanley, E. R. (1982) *Cell* **28**, 71–81
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Glenney, J. R. Jr., Zokas, L. & Kamps, M. P. (1988) *J. Immunol. Methods* **109**, 277–285
- Phillips, W. A., Croatto, M., Veiss, N. & Hamilton, J. A. (1992) *J. Cell. Physiol.* **152**, 64–70
- Shiraishi, T., Owada, M. K., Tatsuka, M., Fuse, Y., Watanabe, K. & Kakunaga, T. (1990) *Jpn. J. Cancer Res.* **81**, 645–652
- Swarup, G., Speeg, K. V. Jr., Cohen, S. & Garbers, D. L. (1982) *J. Biol. Chem.* **257**, 7298–7301
- Grinstein, S., Furuya, W., Lu, D. J. & Mills, G. B. (1990) *J. Biol. Chem.* **265**, 318–327
- Trudel, S., Downey, G. P., Grinstein, S. & Paquet, M. R. (1990) *Biochem. J.* **269**, 127–131
- Weinstein, S. L., Gold, M. R. & DeFranco, A. L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4148–4152
- Evans, J. P. M., Mire-Sluis, A. R., Hoffbrand, A. V. & Wickremasinghe, R. G. (1990) *Blood* **75**, 88–95
- Kohn, M., Nishizawa, N., Tsujimoto, M. & Nomoto, H. (1990) *Biochem. J.* **267**, 91–98
- Ziegler, S. F., Wilson, C. B. & Perlmutter, R. M. (1988) *J. Exp. Med.* **168**, 1801–1810
- Berkow, R. L. & Dodson, M. R. (1988) *J. Leuk. Biol.* **44**, 345–352
- Davila, D. R., Edwards, C. K., III, Arkins, S., Simon, J. & Kelley, K. W. (1990) *FASEB J.* **4**, 2906–2911
- Phillips, W. A., Croatto, M. & Hamilton, J. A. (1990) *Immunology* **70**, 498–503

Received 21 January 1992/20 May 1992; accepted 17 June 1992