BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 17–22 (1998)
ARTICLE NO. RC988370

Free Radical Stimulation of Tyrosine Kinase and Phosphatase Activity in Human Peripheral Blood Mononuclear Cells

Gordon M. Lowe,[1] Charlotte E. Hulley, Emma S. Rhodes, Andrew J. Young,* and Rodney F. Bilton
*School of Biomolecular Sciences and *School of Biological and Earth Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, United Kingdom*

Received November 17, 1998

Human lymphocytes were challenged with reactive oxygen species (ROS) generated by xanthine/xanthine oxidase leading to an increase in tyrosine phosphorylation, together with an increase in tyrosine phosphatase activity. In the presence of 50 μM vanadate and xanthine/xanthine oxidase, tyrosine phosphatase activity was inhibited and a marked increase in tyrosine phosphorylation was observed. The addition of catalase abolished the increase in tyrosine phosphorylation while the addition of superoxide dismutase had no effect. This suggests that vanadate together with hydrogen peroxide derived from xanthine/xanthine oxidase activity, interact to produce an agent that is an effective inhibitor of tyrosine phosphatase activity. When human lymphocytes were challenged with xanthine/xanthine oxidase in the presence of 50 μM $CuCl_2$, an increase in both tyrosine phosphatase and kinase activity was observed. Cupric ions inhibited xanthine oxidase activity by 84%; neither superoxide or hydroxyl radicals could be detected, but traces of hydrogen peroxide were detected in the medium. We conclude that unbound metals can interact with ROS and readily influence signalling mechanisms in human lymphocytes. © 1998 Academic Press

Key Words: **tyrosine kinases; tyrosine phosphatases; lymphocytes; xanthine oxidase; vanadate hydroperoxides.**

Free radicals have been shown to directly damage DNA and act as tumour promoters [1-4]. It is thought that free radicals put the cell under redox stress and may also participate in cell signalling. Initial signalling events include tyrosine phosphorylation, activation of protein kinase C (PKC), and the low molecular weight G-protein $p21^{ras}$, while NF-κB has been demonstrated to mediate signalling by reactive oxygen species (ROS) and reactive nitrogen [5-10].

Tyrosine phosphorylation is an important step in B and T cell activation (11,12). When lymphocytes are exposed to ROS at sites of inflammation, it is believed that the superoxide anion and hydrogen peroxide act as immunosuppressants [13,14]. Free radicals may also act to release bound metals from storage proteins [15-17]. The free metals may then interact with ROS to produce agents that can influence signalling in lymphocytic cells. This has been demonstrated when lymphocytic cell lines were subjected to ionising radiation in the presence of vanadate [5]. In this case the metal was oxidised in the presence of hydrogen peroxide to form vanadate hydroperoxides, which can cross the cell membrane and inhibit tyrosine phosphatase activity. In this study we have demonstrated tyrosine kinase and phosphatase activation in human lymphocytes, when the cells were challenged with exogeous hydrogen peroxide and superoxide. Superoxide is generated *in vivo* by several mechanisms including the activation of neutrophils and by the action of xanthine/xanthine oxidase. The superoxide ion can be dismutated to form hydrogen peroxide, and both these substances in the presence of metal ions can generate other oxy-radicals by Fenton and Haber-Weiss type reactions [2].

MATERIALS AND METHODS

Cell separation. Blood was collected by venipuncture from healthy volunteers, and was mixed with the anticoagulant 3.8% (w/v) trisodium citrate (9 vols blood : 1 vol citrate). 1.2 ml of Optiprep (60%, v/v) was added for every 10 ml of blood (unpublished data). After mixing the suspension was divided into 10 ml aliquots and centrifuged in at 1250 ×g for 30 mins at room temperature. The top 2ml of the upper layer was removed and washed in PBS at 450 ×g for 10 mins. This pelleted the mononuclear fraction, with only minimal platelet contamination. The washed cells were placed onto sterile plastic dishes for 20 mins at room temperature to remove the mono-

[1] Corresponding author. E-mail: G.M.Lowe@livjm.ac.uk.

Abbreviations used: PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species; ROI, reactive oxygen intermediates; SOD, superoxide dismutase.

0006-291X/98 $25.00

cytes. The remaining lymphocytes were collected and counted in an improved Neubaur counting chamber. Typically 1×10^7 cells were obtained for every 10 ml of blood collected. Giemsa staining showed that the cells were contaminated with less than 1% red cells, and more than 95% excluded Trypan blue dye.

Free radical generation. Xanthine oxidase catalyses the oxidation of xanthine to produce uric acid as the final product. During this reaction both superoxide ions and hydrogen peroxide are produced. Xanthine was prepared as a 1.6 mM solution in PBS and xanthine oxidase (Grade III from buttermilk) was used without further purification at 200 mU/ml in PBS.

The generation of superoxide was performed in a 1 ml volume, with 160 μM xanthine and 20mU of xanthine oxidase. The reaction was monitored either by the reduction of 12.5mg/ml cytochrome c at 550 nm [18] or the production of uric acid at 290 nm [19] using a Milton Roy MR3000 Diode Array spectrophotometer. The cytochrome c reduction by superoxide was inhibited by the addition of 50U of superoxide dismutase to the reaction mixture.

Free radical attack on cells. Lymphocytes (3×10^6) were mixed with 160 μM xanthine and 50 μM of either Na_3VO_4, $CuCl_2$, $Na_2Cr_2O_7$, or $FeSO_4$ and the volume was adjusted to 900 μl with PBS. Superoxide dismutase was used at 50 U/ml and catalase at 22 μg/ml. The reaction was performed at 37°C and initiated by the addition of 20 mU xanthine oxidase. The reaction was terminated by rapid cooling and the cells pelleted by centrifugation at 13000 $\times$g. Cell protein was then extracted to investigate tyrosine kinase activity.

Phosphotyrosine detection. Detection of phosphotyrosine was undertaken as previously described [20]. Briefly, proteins were extracted from the cells by the addition of 0.5 ml of cold Triton X-100 lysis buffer (1% v/v Triton X-100, 150 mM NaCl, 5 mM EDTA, 13 mM sodium pyrophosphate, 50 mM sodium fluoride, 1.1 mM sodium orthovanadate and 10mM Tris, pH 7.2). This buffer was supplemented with 9 μg/ml of aprotinin, leupeptin, pepstatin A, iodoacetamide and 1mM PMSF prior to use. The extracts were left on ice for 10 min, prior to centrifugation at 4°C in a minifuge at 13000 $\times$g for 10 min. 150 μl of lysate was mixed with 50 μl of double strength sample buffer and heated at 100°C for 5 mins.

The sample (100μl) was applied to a 5-15% (v/v) SDS polyacrylamide gel, alongside prestained and unstained molecular weight markers. The gel was run at 200 V for 4.5 h and blotted onto Immobilon P membrane overnight at 30V. The lane containing the unstained markers was excised and stained with 0.2% (w/v) Ponceau S (Merck, U.K.) in 3% (w/v) TCA.

The non-specific antigenic sites of the remaining proteins on the membrane were blocked using 3% (w/v) gelatin in T/N-TBS buffer (100 mM NaCl, 25mM Tris, pH 7.2 (pH adjusted using 4M HCl), 0.05%, v/v, Tween 20 and 0.05%v/v, IGE-PAL CA-630) at 37°C for 1 h. The membrane was probed using a 1 in 10000 dilution of antiphosphotyrosine PY-20HRP (ICN, High Wycombe, U.K.) in 0.5% (v/v) gelatin in T/N-TBS for 2h at room temperature. The blot was the washed extensively with T/N-TBS and tyrosine phosphorylated proteins were revealed using an ECL kit (Amersham International, U.K.).

Tyrosine phosphatase assay. Human lymphocytes were challenged with free radicals generated by xanthine/xanthine oxidase as described above. The cells were then pelleted and lysed with ice-cold 0.5 ml lysis buffer (1% (v/v) Triton X-100, 150 mM Tris, 5 mM EDTA, 13 mM sodium pyrophosphate, 1mg/ml BSA (fatty acid free) and 0.1% (v/v), 2-mercaptoethanol, 1 mM PMSF, pH 7.2). The cells were then placed on ice for 5 min and spun at 13000 $\times$g at 4°C for 10 min. The supernatant was then removed and stored at -20°C.

Tyrosine phosphatase activity was monitored using an ELISA kit (Boeringher Mannheim, U.K.). Peptides with tyrosine phosphorylated residues were bound to strepavidin pre-coated microtitre wells. After washing the wells three times with PBS, 10 μl of distilled H_2O, 25 μl of lysis buffer and 25 μl of cell lysis supernatant were then added to the peptides. The plate was incubated at 37°C for 20 min, and then the samples were then processed according to manufacturer's instructions.

TABLE 1

Effects of Metal Ions upon Cytochrome c Reduction and Uric Acid Production in a Xanthine/Xanthine Oxidase System

Treatment	Superoxide (A550 nm/min)	Uric acid (A290 nm/min)
X/XO	0.073	0.048
X/XO + Fe^{2+}	0.054	0.043
X/XO + Cu^{2+}	N/D	0.008
X/XO + V^{5+}	0.077	0.046
X/XO + SOD	0.007	0.045

Note. Superoxide was monitored by the reduction of cytochrome c. Xanthine (X), xanthine oxidase (XO) were incubated at 25 °C in the presence of 12.5 mg/ml cytochrome c. The reduction of cytochrome c was monitored by the increase in absorbance at 550 nm. Uric acid production was monitored at 290 nm (see materials and methods). All metals were added at 50 μM. Results are expressed as a mean of three separate experiments. (N/D-none detected).

RESULTS

Free Radical Generation by Xanthine Oxidase

Superoxide production was monitored by the reduction of cytochrome c at 550 nm. Generation of superoxide continued at a constant rate (0.074 ΔA550nm/min) over 5 min. The reduction of cytochrome c was inhibited by 96 % with the inclusion of 50U of SOD (Table 1).

The inclusion of vanadate and chromate (results not shown) did not result in a significant decrease in the activity of xanthine oxidase. However ferrous ions inhibited the reduction of cytochrome c by 26%, and Cu^{2+} reduced superoxide production by 96%. When xanthine oxidase activity was monitored by the production of uric acid, then ferrous iron reduced activity by 11% and Cu^{2+} reduced it by 84% (Table 1). This suggests that both ferrous and cupric ions have superoxide scavenging properties, and act to dismutate superoxide to hydrogen peroxide. Xanthine/xanthine oxidase alone and in the presence of SOD produced hydrogen peroxide at 30 μM, after a 5 min period. When xanthine/xanthine oxidase was reacted in the presence of Cu^{2+} the concentration of hydrogen peroxide was reduced to 3μM.

Furthermore, the tyrosine kinase inhibitors tyrphostin B46 and A27 at a concentration of 20μM did not inhibit xanthine oxidase activity (data not shown).

Tyrosine Phosphoproteins

Xanthine/xanthine oxidase. When peripheral blood mononuclear cells (PMBC) were challenged with xanthine/xanthine oxidase, a minor increase in tyrosine phosphorylation was observed 1 min following stimulation. This was associated with an increase in tyrosine

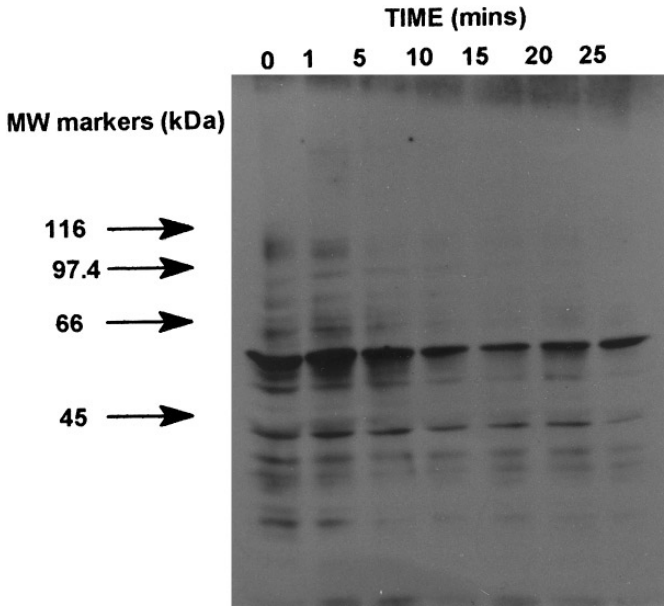

FIG. 1. Human peripheral blood mononuclear cells, were isolated, and challenged with xanthine/xanthine oxidase over a 25 min period. The cells were lysed using Triton X-100 (see materials and methods), and the proteins were separated using SDS-PAGE electrophoresis. The proteins were transferred to Immobilon P and probed using antiphosphotyrosine PY-20.

phosphatase activity (Fig. 1, and Table 2). which was characterised by a decrease in tyrosine phosphorylation. This decrease in phosphorylation may also be explained by protein leakage from damaged cells, however significant release of lactate dehydrogenase was not observed until 30 min following stimulation of the cells (results not shown).

Vanadate. When lymphocytes were challenged with xanthine/xanthine oxidase in the presence of vanadate, a marked increase in tyrosine phosphorylation was observed (Fig. 2). This increase in phosphorylation was observed at 20 min following the challenge, especially in the 142, 109, 101, 93, 73, 68, 60 and 56 kDa proteins.

Copper. An increase in tyrosine phosphorylation was observed in the presence of copper, although Cu^{2+} inhibits superoxide production by xanthine/xanthine oxidase. This occurred after 5 min following challenge of the cells and persisted for a further 10 min. Proteins that had increased tyrosine phosphorylation include those of 66, 60, 56, 53 and 37.1 kDa (Fig. 3A).

Treatment with catalase reduced both vanadate- and copper- activation of tyrosine phosphorylation (Fig. 3A and 3B), while SOD only had a minor inhibitory effect on the activation of tyrosine kinases. The tyrosine kinase inhibitors, tyrphostin B46 and A27 and genistein, were effective in reducing tyrosine phosphorylation of several substrates (Fig. 4). The Erbstatin analogue was less effective than genistein, although both inhibited a common substrate at 33 kDa. These results demonstrate that the increase in tyrosine phosphorylation was due to an increase in tyrosine kinase activity. Interestingly, when PBMC were stimulated by xanthine/xanthine oxidase in the presence of Cu^{2+} the phosphorylation of a 56 kDa substrate was inhibited both by catalase and tyrphostin B46. Therefore tyrphostin may be used subsequently in further studies to investigate the role of the 56 kDa protein. The inclusion of the hydroxyl radical scavengers, thiourea and mannitol, reduced the level of tyrosine phosphorylation when Cu^{2+} was included in the xanthine/xanthine oxidase system, suggesting that hydroxyl radicals are implicated in the initiation of tyrosine kinase activation. However the presence of any hydroxyl radicals in this study (using salicylic acid as a trapping agent [21]) could not be observed (results not shown).

Tyrosine phosphatases. Treatment of the cells with vanadate in the presence of xanthine/xanthine oxidase resulted in a significant reduction in tyrosine phosphatase activity in the cell. However in the presence of xanthine/xanthine oxidase alone or additionally with Cu^{2+} a significant increase in tyrosine phosphatase activity resulted (Table 2).

TABLE 2

The Effect of Oxidant Production on the Activation of Tyrosine Phosphatases in PBMCs

Treatment	Activity (% of control)
Control	100
Xanthine/xanthine oxidase (5 min)	143 ± 6.1
Xanthine/xanthine oxidase (10 min)	153 ± 4.6
Xanthine/xanthine oxidase + Cu^{2+} (5 min)	128 ± 4.9
Xanthine/xanthine oxidase + vanadate (25 min)	9.8 ± 2.6

Note. Human PBMCs were challenged with either xanthine/xanthine oxidase or were challenged with xanthine/xanthine oxidase in the presence of 50μM copper chloride or vanadate. The cells were lysed using a Triton X-100 lysis buffer (see Materials and Methods). The extract was incubated with tyrosine phosphorylated peptides and the degree of tyrosine phosphatase activity was assessed using an ELISA technique. The results are expressed as mean % activity ± SEM compared to the control (Human PMBC incubated, with the relevant metal at 50 μM, with no addition of xanthine oxidase). The means are a composite of three separate experiments. Treatment with xanthine/xanthine oxidase in the presence of vanadate for 25 min produced a marked inhibition of tyrosine phosphatase activity in the PMBC, whereas incubation of PMBCs with vanadate alone under the same conditions did not affect tyrosine phosphatase activity in the cell. This would suggest and that vanadate can interact with ROS to form a potent tyrosine phosphatase inhibitor. The treatment with copper for 5 min was to determine if the increase in tyrosine phosphorylation at 5 min was accompanied by an increase or decrease in tyrosine phosphatase activity. Xanthine/xanthine oxidase treatment alone for 5 and 10 min was to confirm that the observed decrease in tyrosine phosphorylation was due to an increase in tyrosine phosphatase activity in the cell (see fig. 1).

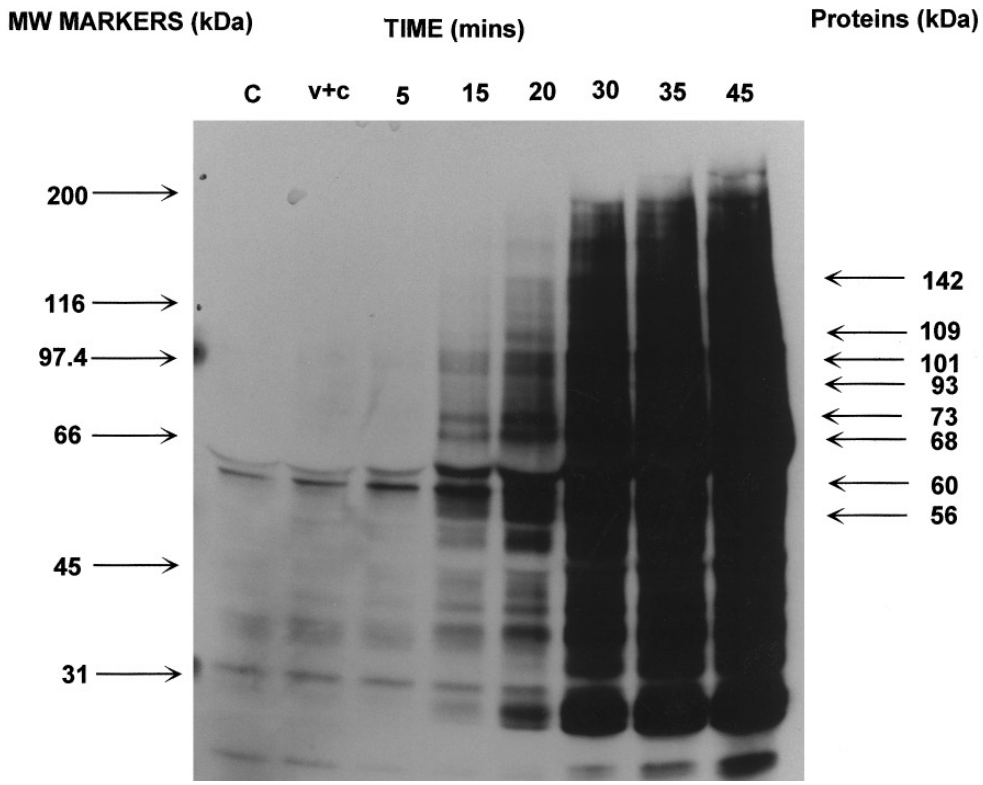

FIG. 2. Human peripheral blood mononuclear cells were challenged with xanthine/xanthine oxidase in the presence of 50 μM sodium orthovanadate. The control PBMC were incubated in the presence of xanthine and vanadate for 45 min. Following lysis and separation of the proteins by SDS-PAGE, The proteins were transferred to an Immobilon P membrane, and probed using antiphosphotyrosine PY-20-HRP. Control PBMC alone (Lane C) and PBMC incubated along with 50 μM vanadate (Lane V+C) for 45 min at 37°C, no increase in tyrosine phosphorylation was observed in these cells. A marked increase in tyrosine phosphorylation was observed after cells were challenged with xanthine/xanthine oxidase in the presence of vanadate, for 20 min at 37°C.

DISCUSSION

Hydrogen peroxide and ROI such as the superoxide anion are produced in pathological states such as inflammation. In addition it has been suggested that metal ions may be released from storage proteins by the action of free radicals [15-17]. The free metals may participate in Fenton type reactions to generate further ROS such as the hydroxyl radical.

Such effects of ROS were simulated *in vitro* by exposing lymphocytes to free radicals produced by xanthine/xanthine oxidase in the absence or presence of metal ions. Xanthine/xanthine oxidase produces both superoxide and hydrogen peroxide. In response, human lymphocytes exhibited, a small increase in tyrosine kinase activity after 1 min exposure. However, at the same time there is also an increase in tyrosine phosphatase activity. This increase in phosphatase activity may be a protective mechanism to ensure that detrimental signalling pathways are not activated in the cell.

When lymphocytes were challenged with xanthine/xanthine oxidase alone for 1 min there was a slight increase in tyrosine phosphorylation (Fig. 1). The increase in tyrosine kinase activity in PMBCs may be explained by tyrosine phosphatases positively controlling tyrosine kinase activity. An example of this can be seen with CD45 (present in human lymphocytes), which dephosphorylates at residue tyr-505 at the C-terminus of $p56^{lyk}$, resulting in the activation of this kinase [22,23].

When lymphocytes were challenged in the presence of Fe^{2+}, or Cr^{6+}, similar patterns of tyrosine phosphorylation were observed to that seen when xanthine/xanthine oxidase was used alone.

When vanadate was included in the system, there was a marked increase in tyrosine phosphorylation, along with an inhibition of tyrosine phosphatase activity. Subsequent studies using catalase and SOD (Fig. 3b) indicate that vanadate is reacting with the hydrogen peroxide formed by the action of xanthine/xanthine oxidase. This leads to the formation of vanadate hydroperoxides, which can readily cross the membrane and inhibit tyrosine phosphatases, a process demonstrated in several cell types [24,25].

The inclusion of Cu^{2+} resulted in a 94% inhibition in the production of superoxide by xanthine/xanthine oxidase. The enzyme itself was inhibited by 84%, when its activity was monitored by the production of uric

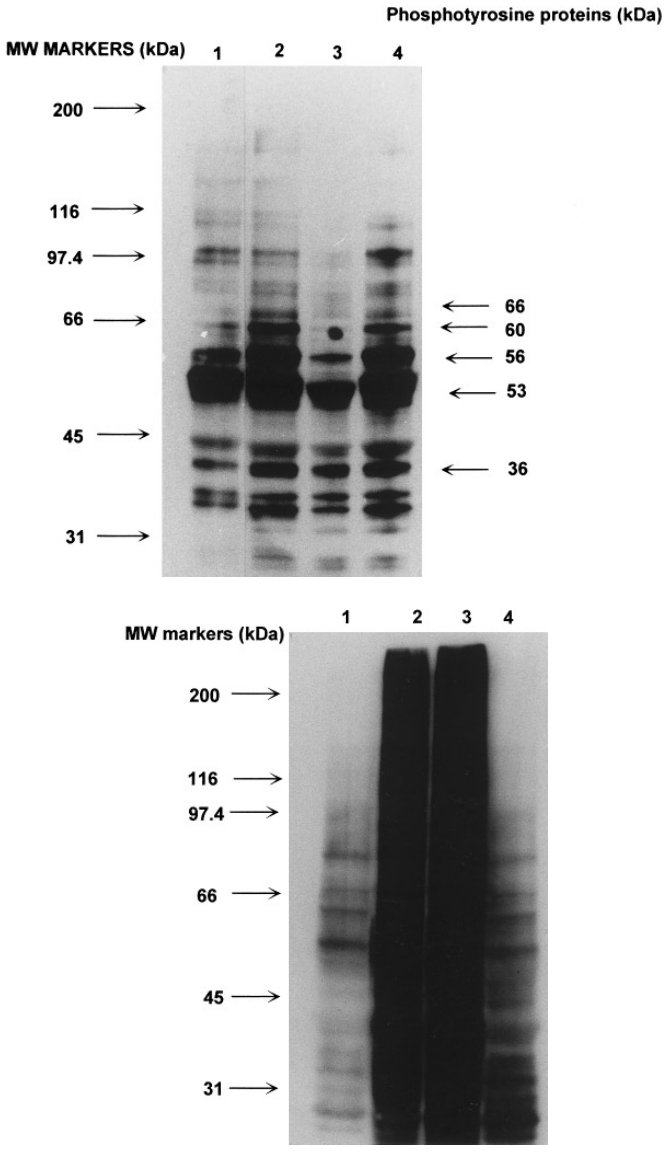

FIG. 3. (A) Human peripheral blood mononuclear cells were challenged with xanthine/xanthine oxidase in the presence of 50μM $CuCl_2$ for 5 min (lane 2). The cells were also challenged with the above reagents in the presence of catalase (lane 3) and SOD (lane 4) for 5 min. Control (lane 1) contained xanthine and $CuCl_2$, and were incubated under identical conditions to that of the test samples. (B) PBMCs were challenged with xanthine/xanthine oxidase in the presence of 50 μM sodium orthovanadate for 30 min (lane 2). The effect of co-incubation with SOD (50U/ml), and catalase 22 μg/ml, can be seen in lanes 3 and 4, respectively. Note that catalase abolishes the marked increase in tyrosine kinase activity.

acid. This indicates that free Cu^{2+} in solution is interacting with superoxide in an unknown manner. One possible mode of action is that copper can act to dismutate superoxide to hydrogen peroxide. Despite the decrease in xanthine oxidase activity, an increase in tyrosine kinase activity was observed 5 min following stimulation of the cells. This activation was inhibited by the inclusion of catalase and the hydroxyl radical scavengers, thiourea and mannitol. The production of hydrogen peroxide was confirmed in this system using fluorometry, and therefore it can be assumed that either hydrogen peroxide or hydroxyl radicals are involved in the activation of tyrosine kinases.

Hydrogen peroxide alone has been shown to activate $p56^{lck}$ in T-cells [26]. However this and similar studies were performed using hydrogen peroxide in the millimolar range [25-27]. In this study the concentration of hydrogen peroxide produced by xanthine/xanthine oxidase never exceeded 50 μM, and when copper was included levels of hydrogen peroxide never exceeded 3 μM. This indicates that hydrogen peroxide alone was not the causative agent for the increase in tyrosine kinase stimulated by xanthine/xanthine oxidase in the presence of 50 μM $CuCl_2$. Another likely candidate is the hydroxyl radical, however we could not detect the presence of hydroxyl radicals in aqueous solution, but the addition of both thiourea and mannitol served to reduce the levels of tyrosine phosphorylation. A mechanism involving hydrogen peroxide or hydroxyl radicals is therefore likely to be responsible for the increase in tyrosine kinase activity. Similar results were found by Lynch [28] when human LDLs were challenged with xanthine/xanthine oxidase and copper. Although no superoxide or hydroxyl radicals were directly detected, oxidative damage to LDL protein was observed.

When free radicals (generated by the action of xanthine/xanthine oxidase) interact with isolated PBMC, an increase in tyrosine phosphatase activity was observed (Table 2). However when this reaction is performed in the presence of vanadate or copper, an increase in tyrosine kinase activity is observed. This increase in tyrosine

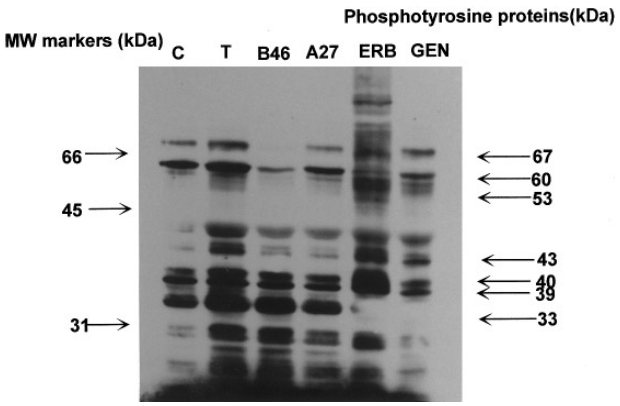

FIG. 4. Human peripheral mononuclear cells were incubated with tyrosine kinase inhibitors,(20 μM of tyrphostin B46, A27, erbstatin analogue and genistein) at 37°C for 30 min. All cells were washed and with the exception of the control (lane C) challenged with xanthine/xanthine oxidase in the presence of 50 μM $CuCl_2$. Following lysis and separation of the proteins by SDS-PAGE, The proteins were transferred to an Immobilon P membrane, and probed using antiphosphotyrosine PY-20. (laneT-cells challenged without the addition of tyrosine kinase inhibitor.)

kinase activity may have implications for the reactivity of lymphocytes in inflamed areas in the body.

ACKNOWLEDGMENTS

We thank the World Cancer Research Fund for financing this project. We also thank Dr. S. W. Edwards for his technical assistance.

REFERENCES

1. Ames, B. N., Shigenaga, M. K., and Gold, L. S. (1993) *Environ. Health Perspect.* **101,** 35–44.
2. Halliwell, B., and Gutteridge, J. M. C. (1989) Free Radicals in Biology and Medicine, 2nd ed., Oxford Univ. Press, New York.
3. Adelson, R., Saul, R. L., and Ames, B. N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2706–2710.
4. Muelmatter, D., Larsson, R., and Cerutti, P. (1988) *Carcinogenesis* **9,** 239–247.
5. Uckun, F. M., Tuel-Ahlgren, L., Song, C. W., Waddick, K., Myers, D. E., Kirihara, J., Ledbetter, J. A., and Schieven, G. L. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9005–9009.
6. Schieven, G. L., Kirihara, J. M., Myers, D. E., Ledbetter, J. A., and Uckun, F. M. (1993) *Blood* **82,** 1212–1220.
7. Uckun, F. M., Schieven, G. L., Tuel-Ahlgren, L. M., Dibirdik, I., Myers, D. E., Ledbetter, J. A., and Song, C. W. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 252–256.
8. Lander, H. M., Levine, D. M., and Novogrodsky, A. (1993) *Biochem. J.* **291,** 281–287.
9. Anderson, M. T., Staal, F. J. T., Gitler, C., Herzenberg, L. A., and Herzenberg, L. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11527–11531.
10. Lander, H. M., Ogiste, J. S., Teng, K. K., and Novogrodsky, A. (1995) *J. Biol. Chem.* **270,** 21195–21198.
11. Lane, P. J. L., Ledbetter, J. A., McConnell, F. M., Draves, K., Deans, J., Schieven, G. L., and Clarke, E. A. (1991) *J. Immunol.* **146,** 715–722.
12. June, C. H., Fletcher, M. C., Ledbetter, J. A., Schieven, G. L., Siegel, J. N., Phillips, A. F., and Samelsson, L. E. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7722–7726.
13. El-Hag, A., Lipsky, P. E., Bennett, M., and Clark, R. A. (1986) *J. Immunol.* **136,** 3420–3426.
14. El-Hag, A., and Clark, R. A. (1987) *J. Immunol.* **139,** 2406–2413.
15. Swain, J. A., Darley-Usmar, V., and Gutteridge, J. M. C. (1994) *FEBS Lett.* **342,** 49–52.
16. Hartmann, H. J., Deters, D., and Weser, U. (1996) *Biometals* **9,** 345–349.
17. Gutteridge, J. M. C. (1995) *Clin. Chem.* **41,** 1819–1828.
18. Edwards, S. W., Nurcombe, H. L., and Hart, C. A. (1987) *Biochem. J.* **245,** 925–928.
19. Mondal, M. S., Behere, D. V., and Mitra, S. (1994) *Proc. Indian Acad. Sci.* **106,** 29–35.
20. Lowe, G. M., Slupsky, J. R., Galvani, D. W., and Edwards, S. W. (1996) *Biochem. Biophys. Res. Commun.* **220,** 484–490.
21. Halliwell, B. (1978) *FEBS Lett.* **92,** 321–326.
22. Amrein, K. E., and Sefton, B. M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4247–4251.
23. Ostergaard, H. L., and Trowbridge, I. S. (1990) *J. Exp. Med.* **172,** 347–350.
24. Seglen, P. O., and Gordon, P. B. (1981) *J. Biol. Chem.* **256,** 7699–7701.
25. Heffetz, D., Bushkin, I., Dror, R., and Zick, Y. (1990) *J. Biol. Chem.* **265,** 2896–2902.
26. Yurchak, L. K., Hardwick, J. S., Amrein, K., Pierno, K., and Sefton, B. M. (1996) *J. Biol. Chem.* **271,** 12549–12554.
27. Schieven, G. L., Kirihara, J. M., Burg, D. L., Geahlen, R. L., and Ledbetter, J. A. (1993) *J. Biol. Chem.* **268,** 16688–16692.
28. Lynch, S. M., and Frei, B. (1993) *J. Lipid Res.* **34,** 1745–1753.