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Amplification of superoxide anion generation in phagocytic cells by HIV-1 infection

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Amplification of superoxide (O_2^-) generation by HIV-1 infection was examined in two human myeloid-monocytic cell lines. The level of O_2^- generation in HL-60 after infection became significantly higher than that of the steady-state. A similar phenomenon was also shown in U937, but only after acquisition of O_2^- generation ability by differentiation to macrophages. By means of the NADPH oxidase-coupled response in infected cells, we reconstituted the O_2^--generating machinery in cell-free system. The results suggested that cytosolic factor(s) exerted by infection might be responsible for the amplification of O_2^- generation. Thus, HIV-1 infection could elevate the level of oxidative stress in macrophages which might play an important role in disease progression.

HIV-1; Superoxide; Differentiation; Cytosolic factor; AIDS

1. INTRODUCTION

The preferential ability to generate superoxide anions (O_2^-) from phagocytic cells including neutrophils, monocytes and macrophages, is known as the host defense mechanism against microbial invasion [1]. Although the involvement of an oxidative response in viral infection remains to be elucidated, indirect evidence suggests the occurrence of O_2^--derived oxidative stress in human immunodeficiency virus type 1 (HIV-1)-positive individuals (see [2] for review).

O_2^- is metabolically generated from molecular oxygen through one electron transport by the action of the respiratory burst enzyme, NADPH oxidase, which is usually dormant in quiescent cells and activated by appropriate stimuli, such as phagocytosis [3]. The enzyme consists of membrane-associated, FAD-containing NADPH reductase [4], cytochrome b_{558} [5], and cytosolic components [6]. In fact, the catalytic activity of the NADPH oxidase in the membrane fraction for O_2^- generation can be significantly elevated by reconstitution with the cytosolic fraction [7,8].

To elucidate the involvement of O_2^- in the progression of HIV-1-induced disease, we examined the amplification of O_2^- generation in cells of mononuclear phagocytic lineage infected with HIV-1. The results showed significant amplification of O_2^- generation, which seemed to be due to HIV-1-provided cytosolic factor(s).

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Abbreviations: O_2^-, superoxide; HIV-1, human immunodeficiency virus type 1; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; RT, reverse transcriptase.

2. MATERIALS AND METHODS

2.1. *Cells and virus*

The human promyelocytic and promonocytic cell lines HL-60 [9] and U937 [10], respectively, were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum (Boehringer Mannheim, Germany) in a CO_2 incubator. Cells were maintained by passage into fresh medium every 3 or 4 days at a cell density of 5×10^5/ml. For U937 differentiation, the cells were suspended at a density of 5×10^5/ml in medium containing 20 ng/ml of phorbol 12-myristate 13-acetate (PMA). After incubation for 2 h, the cells were resuspended in fresh medium and incubated at a cell density of 5×10^5/ml to adhere onto the dishes for 3 days before HIV-1 infection.

The LAI strain of HIV-1 [11] was maintained in the persistently infected MOLT-4 clone No. 8 [12]. Virus infection was proceeded in a similar manner to that described previously [13]. After infection at a multiplicity of infection of 1 and adsorption for 2 h, the cells were given fresh medium and cultured for several days until the O_2^- generation was assayed.

2.2. *O_2^- assay*

O_2^- was generated from HL-60 or U937 cells by priming for 15 min with a low dose of PMA (30 pg for 2×10^6 cells). The O_2^- generated was photometrically assayed using superoxide dismutase (SOD) inhibitable ferricytochrome *c* reduction as described previously [14].

2.3. *Reverse transcriptase (RT) assay*

RT activity in conditioned medium was assayed using poly(rA)-oligo(dT) and [α-^{32}P]dTTP as described previously [15].

2.4. *Lineweaver–Burk analysis*

The relative affinity value of the oxidase to NADPH was determined by initial velocity kinetics [16] using a membrane fraction. Briefly, HL-60 cells uninfected or infected with HIV-1 and cultured for the appropriate period were primed with a low dose of PMA (30 pg for 2×10^6 cells) for 15 min. They were then disrupted in PBS containing 340 mM sucrose, 1 mM EGTA, and 1 mM phenylmeth-

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ylsulfonyl fluoride by sonication (Insonator 201M, Kubota Co. Lt., Tokyo, Japan) for three 15-s cycles. After centrifugation at 1,000 × *g* for 15 min, the supernatant was again centrifuged at 100,000 × *g* for 20 min. The final precipitate was washed, suspended in PBS then assayed for O_2^- generation as described above. Lineweaver–Burk plots were constructed according to the method of Tsunawaki and Nathan [17].

2.5. *O_2^- generation in reconstituted cell-free system*

The NADPH oxidase system in cell-free preparations was assayed according to Steinback et al. [18] with slight modifications. Briefly, HL-60 and undifferentiated and differentiated U937 which were infected with HIV-1 and cultured for appropriate periods were suspended at a concentration of 5×10^7 cells/ml in relaxing buffer (2.7 mM KCl, 136 mM NaCl, 1.5 mM KH_2PO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, and 20 mM leupeptin). The membrane and cytosolic fractions were prepared as precipitate and supernatant, respectively, after centrifugation of the disrupted cells at 100,000 × *g* for 20 min as described above. O_2^- generation was assayed in 1 ml of reaction buffer containing 0.1 mM cytochrome *c*, 10 mM arachidonic acid, and cytosolic and membrane fractions equivalent to 1×10^6 cells in PBS. The reaction was started by adding NADPH to a final concentration of 1 mM.

2.6. *Chemical reagents*

Reagents were obtained commercially; cytochrome *c* (Type III), leupeptin, and PMA from Sigma (St. Louis, USA); arachidonic acid from Funakoshi Co., Ltd. (Tokyo, Japan); SOD and phenylmethylsulfonyl fluoride from Wako Chemicals (Osaka, Japan); NADPH from Oriental Yeast Co., Ltd. (Osaka, Japan); and [α-^{32}P]dTTP (800 Ci/mmol) from New England Nuclear (Boston, USA).

3. RESULTS

3.1. *Amplification of O_2^- generation in HL-60 and differentiated U937 by HIV-1 infection*

To determine the effect of HIV-1 infection on the respiratory burst response in HL-60, the O_2^--generating capacity was compared between HIV-1-infected and uninfected control cells. As shown in Fig. 1A, the O_2^- generation level in infected cells was significantly higher than the steady-state level of uninfected control cells for 3 weeks. In addition, the RT activity assay in the conditioned media of infected cells revealed an increase of HIV-1 production that was almost proportional to the O_2^- generation (Fig. 1D). In contrast to HL-60, neither HIV-1-infected nor uninfected U937 cells generated O_2^- (Fig. 1B). Then, the time course of the O_2^--generating capacity was examined in U937 after differentiation into adherent cells by PMA. The results showed that

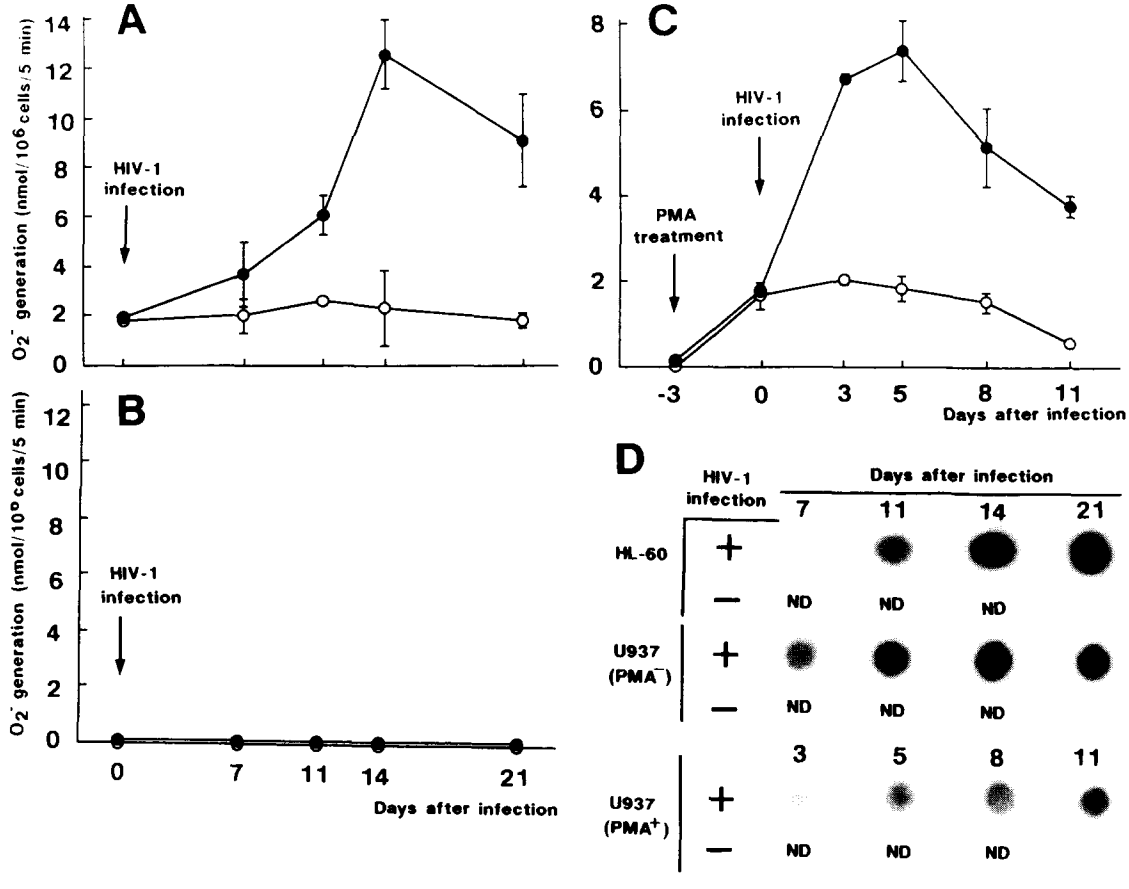

Fig. 1. Amplification of O_2^- generated from human cell lines of myeloid-monocytic lineage by HIV-1 infection. HL-60 (A), undifferentiated U937 (B) and differentiated U937 exposed to 20 ng/ml of PMA for 2 h, then cultures for 3 days (C) were uninfected (○) or infected with HIV-1 (●). The cells were cultured for the indicated days until assayed for O_2^- generation. Bars represent standard deviations. The RT activities in the conditioned media of HL-60, undifferentiated U937 (PMA$^-$) and differentiated U937 (PMA$^+$) were visualized on X-ray film (D). ND, not determined.

uninfected U937 generated O_2^- 3 days after exposure to PMA and thereafter continued at a significant level for over 5 days (Fig. 1C). Therefore, we also examined the effect of HIV-1 infection on the O_2^- generation in differentiated U937. The level of O_2^- generation from these differentiated U937 cells was similarly elevated by HIV-1 infection (Fig. 1C), as observed in HL-60 cells (Fig. 1A). The RT activity in the conditioned medium of HIV-1-infected, differentiated U937 was rather reduced compared with those in the conditioned medium of HIV-1-infected, undifferentiated U937 cells (Fig. 1D). This was consistent with a previous report in which PMA rendered the THP-1 monocytic line cells permissive to macrophage-tropic HIV-1_{Bal} but 10-fold less so to lymphocyte-tropic HIV-1_{LAI} [19]. Thus, the ability to generate O_2^- was significantly elevated by HIV-1 infection in HL-60 and differentiated U937 cells.

3.2. *The kinetics of NADPH oxidase in infected and uninfected cell membranes*

O_2^- is metabolically generated from molecular oxygen by membrane-associated NADPH oxidase [3]. To clarify the mechanism of the amplified O_2^- generation by HIV-1 infection, we examined whether the increased O_2^- generation is due to the activation of NADPH oxidase in infected cell membranes, in which the enzyme seems to be already coupled to a certain amount of cytosolic component(s). The relative affinity value of the enzyme to its substrate, NADPH, was compared between membrane fractions from infected and uninfected HL-60 cells by Lineweaver–Burk analyses. However, both preparations were similar as shown in Fig. 2, indicating the contribution of elevated level of cytosolic factor(s) to the amplified O_2^- generation by HIV-1 infection. Therefore, membrane fraction from uninfected cells was reconstituted with the cytosolic fractions from HIV-1-infected cells to study O_2^- generation in cell-free systems.

3.3. *Reconstitution of cell-free preparations for O_2^- generation*

The O_2^--generating machinery in cell-free systems was reconstituted using the plasma membrane fraction from uninfected cells and the cytosolic fraction from infected or uninfected cells as described in section 2 (Fig. 3). Under conditions in which the O_2^--generating capacity in the membrane fraction from uninfected HL-60 was basal, the O_2^- generation was augmented by adding the cytosolic fraction from infected cells, but not from uninfected cells (Fig. 3A). This result indicated that the amplification of O_2^- generation in infected HL-60 (Fig. 1A) is due to cytosolic factor(s) induced by HIV-1 infection.

More notable results were obtained in cell-free systems using preparations from U937 cells. No significant amplification of the O_2^- generation level occurred in the cell-free systems using the membrane fraction from undifferentiated U937, reconstituted with any cytosolic fraction (Fig. 3B). Thus, the membrane fraction from

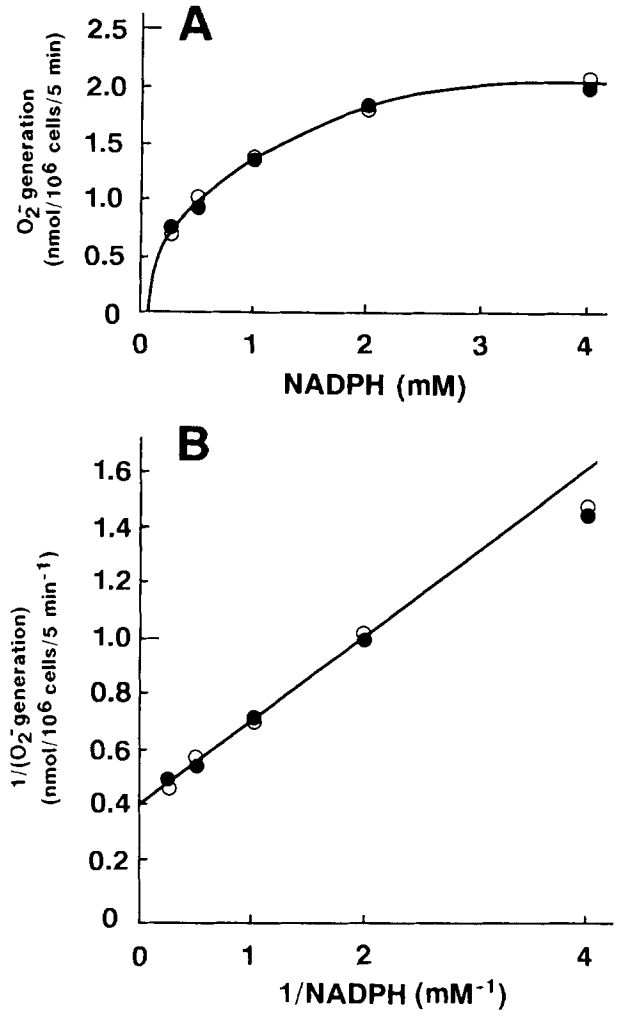

Fig. 2. Lineweaver–Burk analysis of NADPH oxidase in the membrane fraction from HL-60 cells. The uninfected (○) or HIV-1-infected HL-60 cells (●) were harvested at day 11 after infection when increased RT activity was seen in the conditioned medium. The membrane fractions (equivalent to 1×10^6 cells) prepared from these cells were assayed for O_2^- generation by SOD inhibitable ferricytochrome *c* reduction after adding various concentrations of NADPH (A). Lineweaver–Burk plots of the NADPH oxidase activity obtained in (A) are shown in (B).

undifferentiated cells seemed to be immature with respect to O_2^- generation, as observed in Fig. 1B. However, the membrane fraction from differentiated cells increased the level of O_2^- generation by reconstitution with the cytosolic fractions from uninfected, differentiated U937 (Fig. 3C). This result may reflect the acquisition of O_2^- generation ability in uninfected U937 cells only after their differentiation (Fig. 1C). Under these conditions, notable amplification was observed in the cell-free systems using the membrane fraction from differentiated U937 which was reconstituted with the cytosolic fractions from infected U937, irrespective of the differentiation (Fig. 3C). Thus, the HIV-1-induced amplifica-

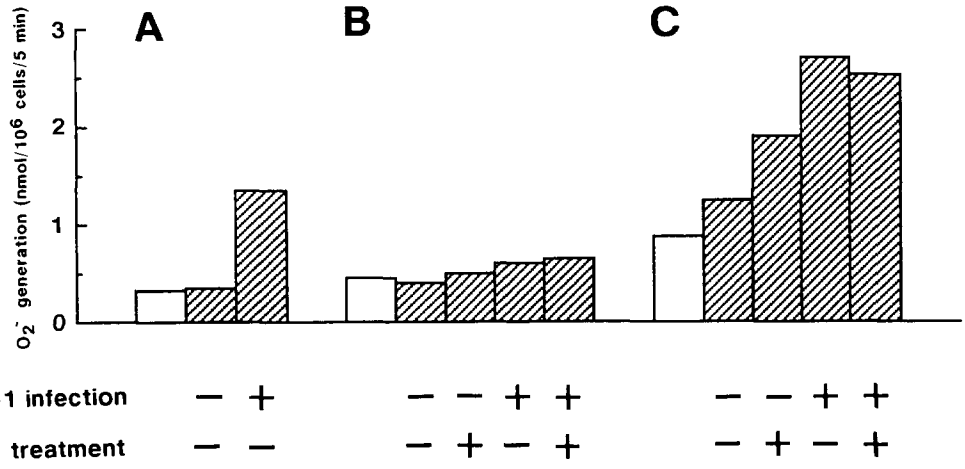

Fig. 3. Amplification of O_2^- generation in cell-free systems by reconstitution with cytosolic fractions from HIV-1-infected cells. The membrane fractions from HL-60 (A), undifferentiated U937 (B), and differentiated U937 (C) were reconstituted with several cytosolic fractions prepared from HIV-1-uninfected or -infected, and PMA-undifferentiated or -differentiated U937 as indicated at the bottom of the figure (▨). To prepare cytosolic fractions, the HIV-1-infected HL-60 and U937 cells were harvested at days 11 and 9 after infection respectively, when increased RT activities were seen in their conditioned media. The O_2^- level in the system without any cytosolic fraction is shown as a control (□). In the cell-free systems, the fractions were equivalent to 1×10^6 cells.

tion of O_2^- generation in U937 cells (Fig. 1C) could also be due to cytosolic factor(s) induced by HIV-1 infection.

4. DISCUSSION

In this study, we revealed the amplification of O_2^- generated from human cell lines of myeloid-monocytic lineage by HIV-1 infection (Fig. 1). In HL-60 cells, the level of O_2^- generation was significantly amplified by HIV-1 infection (Fig. 1A). On the other hand, neither uninfected nor infected U937 cells generated O_2^- (Fig. 1B). However, O_2^- generation was facilitated by exposing the cells to PMA for differentiation to macrophages and also amplified by HIV-1 infection in the differentiated U937 cells (Fig. 1C).

Several viruses prime active oxygen metabolites in neutrophils [20] and macrophages [21], although the mechanism(s) responsible for the amplified activation by the infection remains unresolved. Here, we investigated the mechanism of HIV-1-induced amplification of O_2^- generation in cell-free systems. Tsunawaki and Nathan [17] described the kinetic analysis of O_2^- generation in macrophage lysates. They showed that activated macrophages secreted increased amounts of O_2^- which seemed to be mainly due to an increased affinity of the oxidase to NADPH in the cell membrane fraction. However, activation of NADPH oxidase was not observed in our system using HIV-1-infected HL-60 cells (Fig. 2), suggesting an alternative mechanism of activation. One explanation for this mechanism is the assembly of membranous cytochrome b_{558} with the cytosolic factors, $p47^{phox}$, $p67^{phox}$, and small GTP-binding proteins such as $p21^{ras}$, as recently observed by their involvement in activating the O_2^--generation system in neutrophils [22,23]. In fact, O_2^- augmentation by HIV-1 infection seemed to be due to the increase of cytosolic factor(s) in infected cells (Fig. 3), although the factor(s) in our system have not yet been characterized. The factor(s) could be HIV-1-specific or -induced protein(s) in infected cells. However, the cytosolic fraction from infected, but undifferentiated U937 cells also amplified O_2^- generation to a rate similar to that in infected, differentiated U937 when the membrane fraction from differentiated U937 cells was reconstituted. Therefore, it is suggested that the factor(s) may be a host cellular protein(s) which is activated by HIV-1 infection, but not by HIV-1-specific protein(s), since the amount of HIV-1-specific proteins could be much higher in undifferentiated, than in differentiated U937, as evidenced by the RT assay (Fig. 1D).

Evidences, such as decreased levels of acid-soluble thiols in plasma and leukocytes [24] and depression of the glutathione levels in bronchoalveolar lavage fluid from HIV-1-infected individuals [25], indicate that oxidative stress plays an important role in the progression of acquired immune deficiency syndrome. The amplification of O_2^- generation in phagocytic cells by HIV-1 infection as shown here could contribute to such oxidative stress as well as the O_2^- generated by opportunistic infections.

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