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Superoxide enhances the spread of HIV-1 infection by cell-to-cell transmission

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Oxidative stress is thought to be involved in the progression of human immunodeficiency virus type 1 (HIV-1)-induced disease. We examined the effect of superoxide (O_2^-) on HIV-1 spread in cultured human $CD4^+$ cell lines. The O_2^- significantly enhanced cell-to-cell transmission of HIV-1, although its effect on HIV-1 replication was not evident, presumably due to its cytostatic activity The effect was notable on the HIV-1 transmission from macrophages to T lymphocytes by endogenous, macrophage-generated O_2^-. This amplification was specifically reduced to the steady-state level by antioxidants, and further to the basal level by anti-CD4 antibodies, indicating the specificity of O_2^- for enhancing HIV-1 spread by cell-to-cell transmission.

HIV-1; Superoxide; Syncytium formation; Antioxidant; AIDS

1. INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immune deficiency syndrome (AIDS) [1]. AIDS is characterized by depletion of the CD4 subset of T lymphocytes, which results in selective immunodeficiency [2]. CD4 present on the surface of helper T lymphocytes and mononuclear phagocytes serves as the receptor for HIV-1 infection through interaction with the HIV-1 envelope glycoprotein, gp120 [3]. It is thought that continued viral infection of $CD4^+$ T cells may be responsible for the progressive loss of immune function. There are two major ways that HIV-1 infection can spread to $CD4^+$ cells. HIV-1 produced from an infected cell may infect neighboring, uninfected cells. HIV-1 infection could be transmitted from cell-to-cell without being exposed to the extracellular environment, as evidenced by syncytia formation in in vitro co-culture system. Either way, the specific interaction between CD4 and gp120 is responsible for HIV-1 infection [4].

Recently, we showed significant amplification of superoxide (O_2^-) generation in cells of mononuclear phagocytic lineage by HIV-1 infection, which seemed to be due to HIV-1-provided cytosolic factor(s) [5]. This finding suggests a role of O_2^- in HIV-1-induced disease progression to AIDS. In fact, several reports have suggested the involvement of oxidative stress in the disease progression. An indirect argument in favor of the role of oxidative stress in the disease progression is the consumption of glutathione, a major intracellular antioxidant, or the elevated excretion of malondialdehyde into the urine, reflecting increased levels of lipid peroxidation in HIV-1-infected patients [6,7]. Thus, the precise role of O_2^- seems to be important for our understanding of the pathogenesis of AIDS. Recently, it has been suggested that HIV-1 replication is amplified by H_2O_2 in the Jurkat cell system in which transcriptional activation of NF-κB was observed [8]. In this study, we examined the direct effect of O_2^- on the replication and transmission of HIV-1 in cultured human $CD4^+$ cell lines. The results showed that O_2^- could affect HIV-1 spread by cell-to-cell transmission, although we did not identify a significant effect of O_2^- on HIV-1 replication.

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Abbreviations HIV-1, human immunodeficiency virus type 1; O_2^-, superoxide; AIDS, acquired immune deficiency syndrome; IF, immunofluorescence; RT, reverse transcriptase; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; MOI, multiplicity of infection; ceruloplasmin.

2. MATERIALS AND METHODS

2.1. *Cells and virus*

The promonocytic cell line U937 [9] and the T cell line MOLT-4 clone No. 8 (MOLT-#8) [10] were cultured in complete medium (RPMI-1640 supplemented with 5% fetal bovine serum). The LAI strain [1] was used as an HIV-1 inoculum. Persistently infected MOLT-#8 (MOLT-#8/LAI) was obtained by serial passage of the cells after infection with the LAI strain as described previously [11].

2.2. *Detection of HIV-1 expression*

To detect the HIV-1 antigen expression, indirect immunofluorescence (IF) was performed against acetone-fixed cells as previously described [12]. The production of HIV-1 particles was measured by reverse transcriptase (RT) assay as previously described [13].

2.3. *Differentiation of U937 cells*

U937 cells (2.5×10^6 cells/ml) were treated with various concentra-

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tions of phorbol 12-myristate 13-acetate (PMA; 5–100 ng/ml) for 2 h at 37°C as described previously [5]. The cells were then washed, adjusted to 5×10^5 cells/ml, then incubated in a CO_2-incubator at 37°C for 10 days.

2.4. *Generation of O_2^-*

O_2^- was generated by two methods. O_2^- was enzymatically generated using various doses of xanthine oxidase and 2.5 mM hypoxanthine as a substrate. O_2^- was also generated from differentiated U937. The U937 cells were differentiated by treatment with a high dose of PMA followed by incubation for 10 days as described above, then primed for O_2^- generation for 15 min with a low dose of PMA (a final concentration of 30 pg/ml) as described previously [5].

2.5. *O_2^- assay*

O_2^- generation from cells was photometrically assayed using superoxide dismutase (SOD) inhibitable ferricytochrome *c* reduction as previously described [14].

2.6. *Effect of O_2^- on HIV-1 replication*

MOLT-#8 cells (2.5×10^6 cells/ml) were acutely infected with LAI at a multiplicity of infection (MOI) of 1 for 2 h at 37°C The cells were washed then incubated with enzymatically generated O_2^- for 30 min. The cells were washed again and incubated for up to 10 days. Then, the cells and culture fluids were subjected to IF and RT assay, respectively, for the determination of the HIV-1-specific antigen expression.

2.7. *Effect of O_2^- on HIV-1-induced syncytia formation*

The effect of O_2^- on HIV-1-induced syncytia formation was examined in three cell systems essentially as described previously [15]. Firstly, MOLT-#8/LAI was mixed with uninfected MOLT-#8 at a ratio of 1:9, then treated with enzymatically generated O_2^- for 30 min. After the cells were washed and co-cultured for 24 h, the cyncytia number formed was calculated from the average of triplicate assays. Secondly, U937 cells differentiated by treatment with a high dose of PMA as described above, were primed for 15 min with a low dose of PMA for O_2^- generation. The U937 cells were washed then co-cultured at a 1:1 ratio with MOLT-#8 which has been infected with HIV-1 at an MOI of 1 for 2 h. After incubation for 5 days, the cyncytia number was similarly calculated. Thirdly, U937 cells pretreated with PMA at a final concentration of 100 ng/ml at 37°C for 2 h were washed and then infected with HIV-1 at an MOI of 1. After adsorption for 2 h, the cells were again washed and cultured for 10 days for differentiation. These HIV-1-infected, differentiated U937 cells were primed for 15 min with a low dose of PMA for O_2^- generation. The U937 cells were washed again and then co-cultured with MOLT-#8 at a ratio of 1:1 for 5 days. The cyncytia number was similarly calculated.

2.8. *Blocking assays*

Two antioxidants, SOD and ceruloplasmin (CP), were used to determine the specificity of the O_2^- effect on HIV-1 induced syncytia formation. These antioxidants were mixed with the cells just before O_2^- generation in each cell system.

2.9. *Reagents*

Reagents were obtained commercially as follows: xanthine oxidase, hypoxanthine, and SOD from Wako Pure Chemical Industries (Osaka, Japan); PMA from Sigma (St. Louis, USA); CP from The Green Cross Corp. (Osaka, Japan); anti-Leu3A from Becton Dickinson Immunocytometry Systems (Mountain View, USA); FITC-conjugated rabbit anti-human IgG from Dakopatts A/S (Copenhagen, Denmark), and [α-^{32}P]dTTP (800 Ci/mmol) from DuPont-New England Nuclear (Boston, USA).

3. RESULTS AND DISCUSSION

It has been hypothesized that oxidative stress is involved in the progression of HIV-1-induced disease [6,7]. One explanation is the amplification of HIV-1

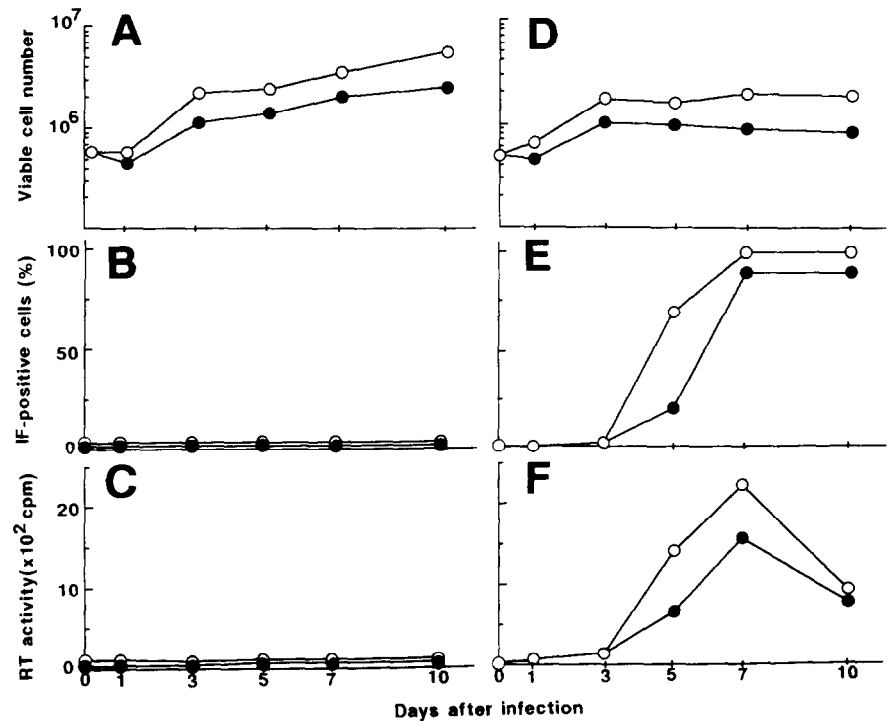

Fig. 1. Effect on O_2^- on HIV-1 replication. MOLT-#8 cells mock-infected (A–C) or acutely infected with HIV-1 (D–F) were untreated (○) or treated with O_2^- generated from 0.02 units/ml of xanthine oxidase and 2.5 mM hypoxanthine (●), and cultured for up to 10 days. (A and D) Viable cell number counted by Trypan blue exclusion. (B and E) HIV-1 antigen expression in infected cells visualized by IF. (C and F) RT activity in the fluid of infected cells.

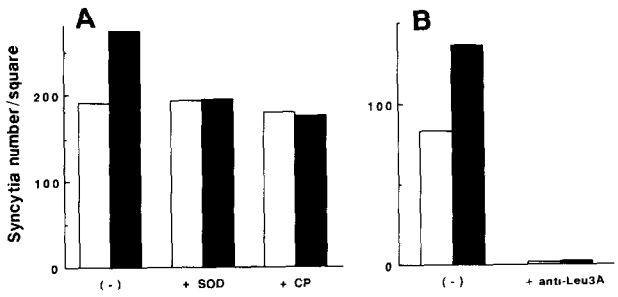

Fig 2. Effect of enzymatically generated O_2^- on syncytia formation. The syncytia number was counted in co-culture of uninfected MOLT-#8 with MOLT-#8/LAI in the absence (□) or presence of O_2^- which was generated from 0.02 units/ml of xanthine oxidase and 2.5 mM hypoxanthine (■). The cells were incubated without (−) or with antioxidant, SOD or CP, just before O_2^- generation (A) In an independent experiment, the cells were also untreated (−) or treated with anti-Leu3A, just before O_2^- generation (B).

replication as shown by the specific activation of DNA-binding and the appearance in the nucleus of the transcription factor NF-κB, in Jurkat cells exposed to H_2O_2 [8]. Here, we examined the effect of O_2^- on the replication rate of HIV-1 in the human $CD4^+$ T cell line, MOLT-8 (Fig. 1). The O_2^- was enzymatically generated using various doses of xanthine oxidase and 2.5 mM hypoxanthine. The results at doses of 0.02 units/ml of xanthine oxidase was 2.5 mM of hypoxanthine which generated 33 nmol/5 min of O_2^- were shown in Fig. 1, as representative, since there were no effect at lower doses of xanthine oxidase and cytostatic effect at this dose (Fig. 1A and D), whereas there was cytotoxic effect at higher doses. HIV-1-specific IF and RT assays showed rather a suppressed effect on the HIV-1 replication in infected MOLT-#8 at this dose (Fig. 1E and F). However, this effect seems to be due to the cytostatic effect of O_2^- against infected MOLT-#8 as well as uninfected MOLT-#8, as shown by their decreased cell growth rates in the presence of O_2^- (Fig. 1C and D). The growth of the infected cells was more suppressed compared with uninfected cells (Fig. 1C and D), presumably due to a cytopathic effect induced by HIV-1 replication. Next, we examined the effect of O_2^- on HIV-1-induced syncytia formation.

The effect of enzymatically generated O_2^- on HIV-1-induced syncytia formation was examined by means of a co-culture system of MOLT-#8/LAI and uninfected MOLT-#8 (Fig. 2). There was significant amplification of syncytia formation in the co-culture in the presence of O_2^-, compared with the steadystate level in the absence of O_2^-. The number of syncytia reached the maximal levels at around 24 h incubation. In Fig. 2, the

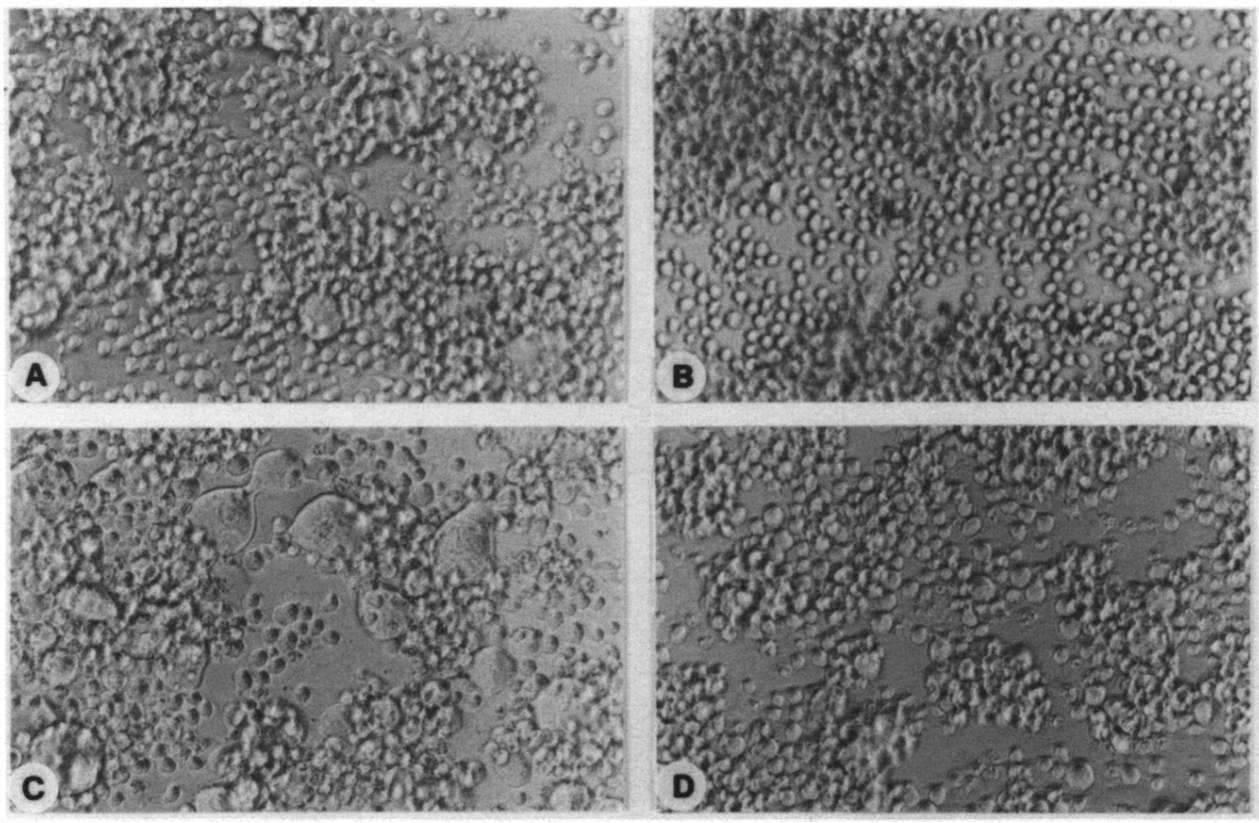

Fig. 3. Profiles of amplified syncytia formation by O_2^- and its specific reduction by anti-Leu3A. The cell system was the same as that described in the legend to Fig. 2. The co-cultured cells were untreated (A and C) or treated with anti-Leu3A (B and D) before exposure to (C and D) or without (A and B) O_2^-.

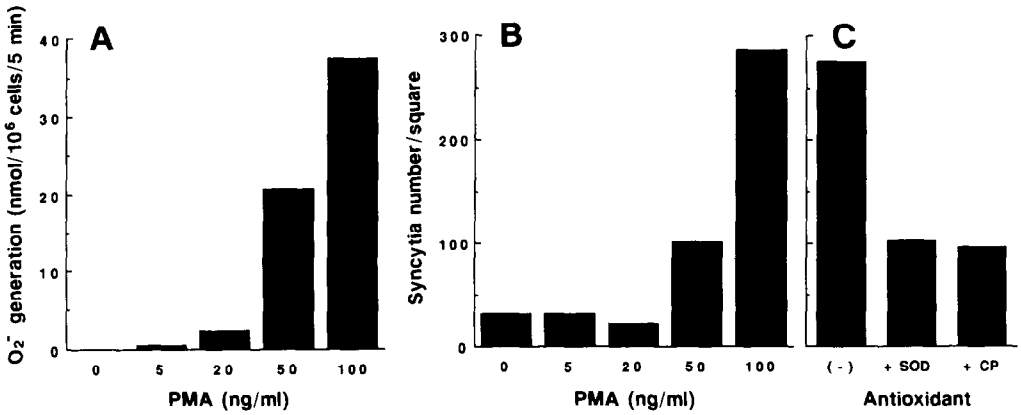

Fig. 4. Effect of exogenous O_2^- from U937 on syncytia formation. O_2^- generation ability was examined in U937 cells by incubation with PMA at final concentrations of 0 to 100 ng/ml (A) MOLT-#8 acutely infected with HIV-1 was mixed with the same differentiated U937 as an O_2^- source, and the cyncytia number was counted (B). In an independent experiment, the cells were untreated (−) or treated with SOD or CP during O_2^- generation (C).

results using 0.02 units/ml of xanthine oxidase and 2.5 mM hypoxanthine are shown as described in Fig. 1, since the amplification by O_2^- was dose-dependent and was maximal at these doses of xanthine oxidase and hypoxanthine. To assess the availability of O_2^- in our system, we assayed the blocking action of the antioxidants, CP and SOD. Both reduced the amplified syncytia formation in O_2^--treated cells to the steady-state level found in untreated cells (Fig. 2A). These results indicate the specificity of O_2^- for the effect on the HIV-1-induced syncytia formation. We then examined whether the amplified syncytia formed by O_2^- was mediated by CD4–gp120 interaction, using an anti-Leu3A monoclonal antibody (anti-CD4), which recognizes the region reactive with gp120 [16]. Under the condition using 1 μg/ml of the monoclonal antibody which completely blocked the steady-state syncytia formation in untreated cells to the basal level as shown previously [15], the HIV-1-induced syncytia formation amplified by O_2^--treatment was also completely blocked to the basal level (Fig. 2B). This result showed that the amplified syncytia formation by O_2^- was also mediated by CD4. Representative profiles are shown in Fig. 3.

We focused upon the availability of macrophage-generating O_2^-. When U937 cells were differentiated into adherent cells by PMA at final concentrations of 5–100 ng/ml, the cells generated O_2^- in a dose-dependent manner (Fig. 4A). Again, this O_2^- was also effective in enhancing HIV-1-induced syncytia formation in acutely HIV-1-infected MOLT-#8. The number of syncytia reaching the maximal levels after 5 days is shown in Fig. 4B. The period necessary for the maximum syncytia formation was longer than the 24 h required in co-cultured MOLT-#8 with persistently infected cells (Fig. 2), since MOLT-#8 was acutely infected with cell-free HIV-1. The number of syncytia proportionally increased with the concentration of the PMA-induced O_2^- in U937. Similar to that shown as in Fig. 2A, antioxidants reduced the amplified syncytia formation (Fig. 4C).

Finally, we examined the possible amplification of HIV-1 transmission from macrophages to T lymphocytes by the O_2^- endogenously generated by the infected macrophages (Fig. 5). MOLT-#8 was co-cultured with the PMA-differentiated, HIV-1-infected U937 which were unprimed or primed with a low dose of PMA for O_2^- generation. The results showed that the efficiency of amplifying syncytium formation in the co-culture between infected U937 and uninfected MOLT-#8 (Fig. 5) by endogenous O_2^- was much higher than that in the co-culture between infected and uninfected MOLT-#8 by exogenous O_2^- from xanthine oxidase/hypoxanthine (Fig. 2A) or that in differentiated U937 (Fig. 4B). Similarly, antioxidants reduced the syncytia formation am-

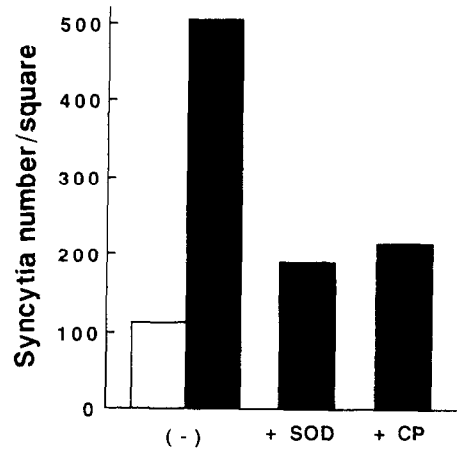

Fig. 5. Effect of endogenous O_2^- from infected U937 on syncytia formation. The cyncytia number was counted in co-culture of uninfected MOLT-#8 with differentiated U937 cells. U937 cells were pretreated with 100 ng/ml of PMA and then infected with HIV-1, and then unprimed (□) or primed (■) with a low dose of PMA. The co-culture with the primed U937 cells were untreated (−) or treated with SOD or CP during O_2^- generation.

plified by endogenous O_2^- (Fig. 5). Although U937 was infected 10 days prior to the co-culture, the maximum increase in syncytia formation was observed after the culture for 5 days, indicating the difficulty of the transmission from macrophage to T lymphocyte.

In this report, we described that HIV-1 spread is amplified during cell-to-cell transmission by O_2^- (Figs. 2–5). Generally, it is believed that the spread of HIV-1 predominantly occurs by cell-to-cell transmission. Therefore, the amplified generation of O_2^- not only by HIV-1 infection [5] but also by opportunistic infections is noteworthy for efficient HIV-1 transmission. The effect on amplified transmission was observed in acutely (Fig. 4B) or persistently (Fig. 2A) infected cells and uninfected $CD4^+$ cells. Especially, the efficiency of the amplification was notable on the transmission of HIV-1 from U937 to MOLT-#8 using endogenously generated O_2^- from the U937 (Fig. 5). This might be due to amplified generation of O_2^- from U937 by HIV-1 infection as reported previously [5]. Macrophage-tropic clones are important for the persistence of HIV-1 infection especially during the early asymptomatic phase and moreover, a qualitative shift to more T-cell-tropic clones occurs along with progression of HIV-1 infection [17]. It is of particular interest to clarify the involvement of endogenous O_2^- from macrophage in the shift from a macrophage-tropic to a T-cell-tropic virus population. Thus, our findings support the hypothesis that oxidative stress is involved in the progression of AIDS and may provide an alternative approach to therapy using antioxidant drugs.

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