# A Micro-Suspension-Test for Evaluation of Disinfectants against Human Immunodeficiency Virus

Yukio SHIMAKOSHI*,**, Kouichi SANO**, Takashi NAKANO**, Tsumukata NAKAMURA**, Saburo OHSHIBA*, Ken-ichi KATSU* and Masuyo NAKAI**

*Second Department of Internal Medicine, **Department of Microbiology, Osaka Medical College, Osaka, Japan

(Received: December 5, 1994)
(Accepted: February 2, 1995)

Key words: chemical disinfectant, persistently HIV-1-infected cells, infectivity assay, reverse transcriptase activity, giant cell formation

## Abstract

We devised a micro-suspension-test to evaluate disinfectants against human immunodeficiency virus type 1 (HIV-1) and confirmed its reliability. Suspensions of persistently HIV-1-infected Molt-4 cells were used as targets of disinfectants and residual infectivity was measured by an infectivity assay: after cocultivation with uninfected Molt-4 cells reverse transcriptase activity (RTA) in the supernatant and giant cell formation (GCF) were monitored. Our new infectivity assay consists of a short-term assay, that is RTA and GCF monitoring on the second day of co-culture, and a long-term assay, that is RTA monitoring up to the 28th day of co-culure. The sensitivity of the short-term assay was $1 \times 10^3$ infected cells and that of the long-term assay was $1 \times 10^1$ infected cells. All the chemical disinfectants examined in this study showed dose- and time-dependent inactivation of HIV-1. By 5-minute contact with ethanol, glutaraldehyde, formalin, sodium hypochlorite and povidone-iodine, HIV-1 was effectively inactivated at concentrations of 20, 0.01, 5, 0.05 and 0.1%, respectively. Since the micro-suspension-test is easy and sensitive, we recommend it as a method for evaluating disinfectants against HIV-1.

## Introduction

There is no universally accepted method for determining the *in vitro* efficacy of disinfectants against viruses. Efficacy has been monitored by an enzyme activity assay[1,2], an antigen assay[3], a morphological assay[4] and an infectivity assay[5]. The enzyme, antigen and morphological assays are important methods for evaluating the efficacy of disinfectants against nonculturable pathogens and to investigate the mechanism of disinfection. The most appropriate method of evaluation of disinfectants against culturable pathogens such as human immunodeficiency virus (HIV) is an infectivity assay, since disinfection is defined as the abolition of infectivity.

The essential factors of HIV transmission are cell-free HIV particles and HIV-infected cells. In the early studies of disinfection against HIV, cell-free virus particles were targeted in suspension (suspension test)[2,6,7,8,9]. Termination of the disinfection period was achieved by simple neutralization of the disinfectant[6], by dilution of a mixture of virus particles and disinfectant[7,8], or by ultracentrifugation after dilution or neutralization of the disinfectant[9]. However, the diluted dis-

Correspondence to: Yukiko SHIMAKOSHI, M.D.
Department of Microbiology, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki-shi, Osaka, 569 Japan

infectant or the neutralizer may influence the assay system, and virus particles can react with a low concentration of the disinfectant or the neutralizer during ultracentrifugation. In these cases, the efficacy of a disinfectant can be overestimated. Sattar and Springthorpe[10] pointed out the possibility of underestimation of infectious HIV particles in human specimens since it was difficult to determine the number of cell-associated viruses. Moreover, Dimitrov et al.[11] showed that the infectivity of HIV during cell-to-cell transmission is $10^2$ to $10^3$ times greater than that of cell-free virus. A new method of evaluation of disinfectants against viruses is therefore required, and the efficacy of disinfectants against HIV needs to be reevaluated.

In this article we describe the micro-suspension-test in which suspended HIV type 1 (HIV-1)-infected cells are used as the target of disinfection and residual infectivity is monitored by a new infectivity assay.

## Materials and Methods

Disinfectants

Glutaraldehyde (25 v/v% solution: TAAB Laboratories Equipment Ltd.), formalin (formaldehyde 37 v/v% solution: Nacalai Tesque Inc.), sodium hypochlorite (10 v/v% solution: Daiso Ltd.), ethanol (99.9 v/v% solution: Japan Alcohol Hanbai Ltd.), and povidone-iodine (available iodine 11.4 w/w% powder: Sigma) were used as disinfectants. Povidone-iodine was dissolved in PBS and the original solution was made at 10 w/v% (available iodine 1.14%). These disinfectants were used after being diluted to designated concentrations (v/v%) by PBS.

Cell

Molt-4 cells were used as uninfected cells. To produce persistently HIV-1-infected Molt-4 cells, we added 2000 TCID of LAV-$1_{BRU}$[12] per ml to uninfected Molt-4 cells and cultured them for over 6 months with replacement of three-quarters of the medium every 3 or 4 days. The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics (158 units of penicillin and 77 $\mu$g of streptomycin per ml).

The number of proviral DNA copies in the persistently HIV-1-infected cell line was determined by the quantitative PCR method. In brief, the cell pellets, in which the number of cells was serially tenfold reduced from $1 \times 10^5$ to 1, were lysed in 50 $\mu$l of 10 mM Tris-HCl (pH 8.3) containing 2.5 mM $MgCl_2$, 50 mM KCl, 0.5% Tween 20, 0.5% NP40 and 120 $\mu$g of proteinase K per ml. The lysates were incubated at 50°C for 1 hour, then mixed with 50 $\mu$l of 10 mM Tris-HCl (pH 8.3) containing 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.2 mmol of the four dNTPs, 50 pmol of each primer, and 2.5 U of Taq DNA polymerase (Pharmacia Biotech). The solutions were overlaid with mineral oil (Sigma), and 30 cycles of amplification were performed. The DNA of each sample was analyzed by 7% polyacrylamide gel electrophoresis, and the gel was stained with ethydium bromide. The cell dilution from which a distinct signal was detected was determined to be positive. The smallest number of cells that were positive was taken as the end-point. We used the primer pair M667/AA55[13] to amplify the 140 base-pairs in the R-U5 region of the HIV-1 long terminal repeat.

Construction and Characterization of Micro-Suspension-Test

A pellet of $1 \times 10^5$ HIV-1-infected Molt-4 cells was resuspended in 1 ml of disinfectant in a 1.5-ml microtube and allowed to react for a designated time at room temperature in a conventional biosafety cabinet. The cells and disinfectant were then separated by centrifugation (HF-120, Tomy Seiko Ltd.) at 2000 $\times$g for 1 minute. The cells were washed three times with the medium and resuspended in 1 ml of the medium. One hundred microliters of cell suspension were added to each well containing uninfected Molt-4 cells ($1 \times 10^4$ cells/100 $\mu$l/well) in a 96-well flat-bottomed microtiter plate and cultured at 37°C in 5% $CO_2$. The culture supernatant was harvested to measure reverse transcriptase

平成7年5月20日

activity (RTA)[14], and giant cell formation (GCF)[15] was observed under a microscope on the second day of co-culture (short-term assay). All cultures were continued for about 4 weeks with replacement of three-quarters of the medium every 3 or 4 days, and RTA in the culture supernatant was measured every 6 or 8 days (long-term assay). RTA and GCF were monitored in triplicate. For monitoring RTA, a mixture of supernatants from the three wells was used. In GCF monitoring, when giant cells were found in at least one of the three wells, residual infectivity was considered to be present. In the short-term assay, a positive result from either RTA or GCF monitoring was considered to be positive for residual infectivity.

To eveluate the micro-suspension-test, after 5 minutes of contact with the designated concentrations of ethanol, glutaraldehyde, formalin, sodium hypochlorite and povidone-iodine, and the designated time of contact with a critical concentration of glutaraldehyde, the cultures were examined for disinfection according to the protocol.

To determine the sensitivity of the short- and long-term assays, 100 $\mu$l of HIV-1-infected Molt-4 cells tenfold serially diluted from $1 \times 10^5$ to $1 \times 10^0$ were inoculated into a 96-well flat-bottomed microtiter plate. To each well, 100 $\mu$l of uninfected Molt-4 cells ($1 \times 10^4$ cells/100 $\mu$l/well) were added, and the suspensions were cultured at 37°C in 5% $CO_2$. On the second day of culture the number of giant cells was counted under a microscope and the culture supernatant was harvested to measure RTA (short-term assay). RTA in the culture supernatant was measured every 6 or 8 days up to the 28th day of culture (long-term assay).

## Results

The PCR study showed the virus load of persistently HIV-1-infected Molt-4 cells to be five copies of HIV-1 proviral DNA in $1 \times 10^3$ cells.

The number of giant cells and the RTA in the culture supernatant on the second day of co-culture showed a linear correlation with the number of infected cells inoculated in a well. The short-term assay detected at least $1 \times 10^3$ infected cells per well (Figs. 1 and 2). To confirm the above sensitivity,

Fig. 1 Kinetics of Giant Cell Formation
Persistently HIV-1-infected Molt-4 cell suspensions tenfold serially diluted from $1 \times 10^5$ to $1 \times 10^0$ cells were co-cultured with $1 \times 10^4$ uninfected Molt-4 cells. On the second day of co-culture, the giant cells were counted under a microscope. (---): cut off

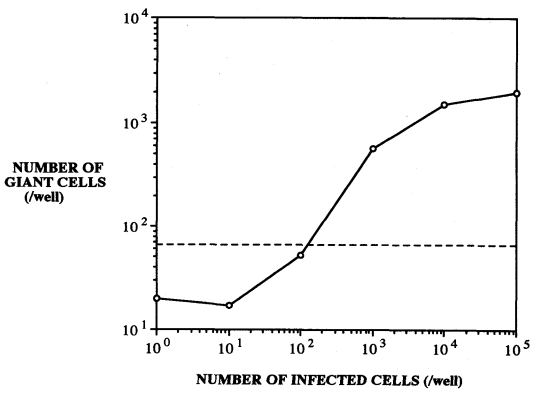


Fig. 2 Reverse Transcriptase Activity in Culture Supernatant
Persistently HIV-1-infected Molt-4 cells suspensions tenfold serially diluted from $1 \times 10^5$ to $1 \times 10^0$ cells were co-cultured with $1 \times 10^4$ uninfected Molt-4 cells for 48 hours. Reverse transcriptase activity in the culture supernatant was measured. (---): cut off

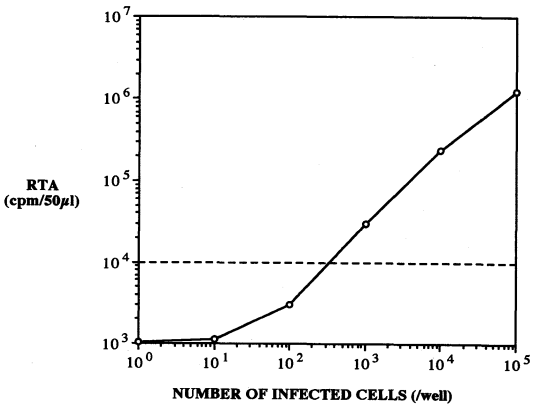

Table 1 Sensitivity of infectivity assay*

| Number of infected cells[a] | Short-term assay[b] | | Long-term assay[c] RTA[e] | Result[d] (day of culture[f]) |
|---|---|---|---|---|
| | GCF | RTA[e] | | |
| $1\times10^5$ | + | 1,236,986(+) | 1,236,986(+) | detectable(2) |
| $1\times10^4$ | + | 233,938(+) | 384,521(+) | detectable(2) |
| $1\times10^3$ | + | 29,957(+) | 568,319(+) | detectable(2) |
| $1\times10^2$ | − | 3,006(−) | 1,286,540(+) | detectable(7) |
| $1\times10^1$ | − | 1,118(−) | 808,058(+) | detectable(7) |
| $1\times10^0$ | − | 1,033(−) | 2,340(−) | not detectable |
| none(control) | − | 884(−) | 5,595(−) | |

*GCF : giant cell formation ; RTA : reverse transcriptase activity
[a]number of HIV-1-infected Molt-4 cells per well
[b]GCF and RTA monitorings on the second day of co-culture
[c]highest RTA during culture for 4 weeks (cpm/50 $\mu$l)
[d]final infectivity
[e]cut off : 10,000 cpm/50 $\mu$l[14]
[f]first day that infectivity could be detected

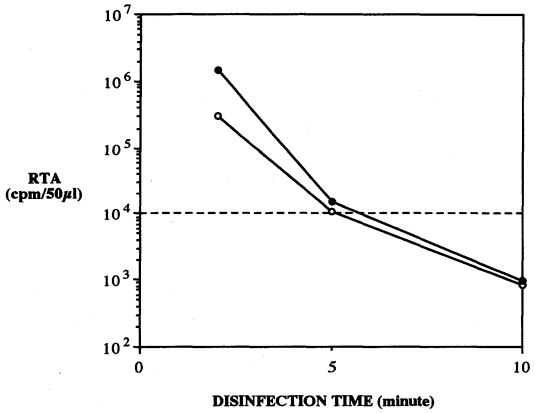


Fig. 3 Time-Dependent Effect of a Disinfectant (0.005% glutaraldehyde)
Persistently HIV-1-infected Molt-4 cells ($1 \times 10^5$) were exposed to 0.005% glutaraldehyde for 2, 5 or 10 minutes in the micro-suspension-test. Reverse transcriptase activity was measured on the 7th day (open circles) and the 13th day (closed circles) of co-culture. (---): cut off

we continued to culture the cells for about 4 weeks. No HIV-1 was detected in the wells with less than $1 \times 10^1$ infected cells (Table 1).

All of the chemical disinfectants examined in this study showed dose-dependent inactivation of HIV-1 (Table 2). The minimal effective concentrations of the disinfectants, except for formalin, showed identical results in short-term and long-term assay. The minimal effective concentrations for 5 minutes of contact with formalin in the short-term assay and long-term assay were 0.5% and 5%, respectively. The minimal effective concentrations of ethanol, glutaraldehyde, sodium hypochlorite and povidone-iodine, were 20, 0.01, 0.05 and 0.1%, respectively.

Our study of the critical concentration of 0.005% glutaraldehyde showed time-dependent inactivation of HIV-1 (Fig. 3).

## Discussion

To prevent the obstructive cytotoxicity in the assay system the removal of a disinfectant from an infectious target is one of the most important procedures in the study of disinfectants against viruses. In our study of disinfectants against persistently HIV-1-infected cells, we were able to separate them by centrifugation at 2000 ×g for 1 minute (data not shown). Aranda-Anzaldo et al.[16]

平成7年5月20日

Table 2 Comparison of short-term assay and long-term assay in evaluation of effective concentration after 5-minute contact time*

| Disinfectant & concentration (v/v%) | Short-term assay[a] | | Long-term assay[b] | Result[c] |
|---|---|---|---|---|
| | GCF | RTA[d] | RTA[d] | (days of culture[e]) |
| Ethanol | | | | |
| 70 | − | 1,836(−) | 1,836(−) | effective |
| 50 | − | 2,266(−) | 2,634(−) | effective |
| 20 | − | 745(−) | 1,825(−) | effective |
| 5 | + | 30,060(+) | 93,639(+) | ineffective (2) |
| 1 | + | 26,040(+) | 74,663(+) | ineffective (2) |
| Glutaraldehyde | | | | |
| 2 | − | 3,006(−) | 3,006(−) | effective |
| 1 | − | 3,980(−) | 3,980(−) | effective |
| 0.1 | − | 3,085(−) | 3,085(−) | effective |
| 0.01 | − | 5,045(−) | 5,045(−) | effective |
| 0.001 | + | 5,850(−) | 99,071(+) | ineffective (7) |
| Formalin | | | | |
| 5 | − | 2,358(−) | 2,358(−) | effective |
| 0.5 | − | 3,990(−) | 31,858(+) | ineffective(28) |
| 0.05 | + | 3,817(−) | 417,533(+) | ineffective(14) |
| 0.005 | + | 18,960(+) | 78,211(+) | ineffective (2) |
| 0.0005 | + | 44,787(+) | 44,787(+) | ineffective (2) |
| Sodium hypochlorite | | | | |
| 0.1 | − | 3,264(−) | 3,264(−) | effective |
| 0.05 | − | 4,210(−) | 4,210(−) | effective |
| 0.01 | + | 89,149(+) | 89,149(+) | ineffective (2) |
| 0.001 | + | 83,412(+) | 337,286(+) | ineffective (2) |
| 0.0001 | + | 112,210(+) | 610,379(+) | ineffective (2) |
| Povidone iodine | | | | |
| 10 | − | 764(−) | 1,142(−) | effective |
| 7.5 | − | 1,615(−) | 2,745(−) | effective |
| 1 | − | 661(−) | 1,644(−) | effective |
| 0.1 | − | 1,833(−) | 1,833(−) | effective |
| 0.01 | + | 11,482(+) | 2,202,697(+) | ineffective (2) |
| None(control) | + | 108,866(+) | 774,970(+) | |

*GCF : giant cell formation ; RTA : reverse transcriptase activity

[a]GCF and RTA monitorings on the second day of co-culture

[b]highest RTA during culture for 4 weeks (cpm/50 $\mu$l)

[c]final efficacy of the disinfectant

[d]cut off : 10,000 cpm/50 $\mu$l[14]

[e]first day that residual infectivity could be detected

stated that the residual cytotoxicity of the chemical may mask or mimic the true virucidal activity. We measured the residual cytotoxicity of the disinfectants after washing by using uninfected Molt-4 cells as the target of the disinfectant. On the second and seventh day of co-culture with disinfected and undisinfected Molt-4 cells, we counted the viable cells under a microscope. No residual cytotoxicity was found at the concentration that we used (data not shown).

The determination of the minimal effective concentration of a disinfectant and the duration of contact is important in the study of disinfection. Our system can determine both. The long-term assay detected infectivity after treatment with 0.5% formalin (10 times more concentrated than in the short-term assay). We showed that the long-term assay was approximately $10^2$ times more sensitive

than the short-term assay. The probable reason for the higher sensitivity of the long-term assay is the number of infectious HIV particles in the supernatant. During the longer period of culture infectious virus is amplified in both the uninfected cells and the surviving infected cells. Since cell-to-cell transmission occurs earlier than virus-to-cell transmission[17], the short-term assay may reflect only the cell-to-cell transmission. These results show that the short-term assay is useful in testing the efficacy of a disinfectant; however, the long-term assay is needed for definitive determination of its efficacy.

Spire et al.[2] reported the minimal effective concentration of a disinfectant against cell-free HIV-1, monitored by RTA, as an indicator of viral inactivation. They determined the minimal effective concentrations by 5-minute treatment of ethanol, glutaraldehyde, formalin and sodium hypochlorite to be 19, 0.0125, 0.1 and 0.1%, respectively. Our values were 20, 0.01, 5 and 0.05%, respectively. We believe that they underestimated the minimal effective concentration of formalin, probably because cell-free virus is less resistant than the virus-infected cell[11]. In our method the infectious target was persistently HIV-1-infected Molt-4 cells containing five copies of HIV-1 proviral DNA in $1 \times 10^3$ cells which kept producing HIV-1. Both the cell itself and the virus particles carried in the cell act as targets of the disinfectant. In the practical use of disinfectants against HIV, infected cells are targets, and in the laboratory it is easier to handle a cell than a virus. Therefore, infected cells should be used in the evaluation of disinfectants.

To determine the effect of a disinfectant during a short period of disinfection, we shortened the time of washing of the disinfectant from the infectious target. The other situation in which the contact time is minimized is the carrier test[18]. In the carrier test, the infectious target is fixed by drying on a solid material, allowed brief contact with a disinfectant and rinsed by dipping into a liquid. The easy rinsing is a convenient way to shorten washing time and to avoid unnecessary contact between the disinfectant and the target. It is, however, not clear whether dried infectious material is safe during the assay and whether infectious targets are shed from the solid phase during the disinfecting and washing procedures.

With our method, since an infected cell is the target of the disinfectant and also the carrier of virus particles, some disinfectants with strong cytotoxicity due to very low osmotic pressure or strong acidity cannot be examined. Low osmotic pressure of low pH destroys cells, so the targeted cells cannot be recovered by centrifugation. Since our micro-suspension-test is easy and sensitive and can be carried out in a conventional biosafety cabinet, we can use this method to evaluate disinfectants against HIV-1.

## Acknowledgment

Part of this work was supported by a grant for AIDS research from the Osaka Association for Public Health.

## References

1) Nagumo, T., Takeuchi,Y. & Hoshino, H.: Inhibition of heat inactivation of reverse transcriptase of human immunodeficiency virus type 1 by seropositive sera. Jpn. J. Cancer Res., 83: 594—600, 1992.
2) Spire, B., Barré-Sinoussi, F., Montagnier, L. & Chermann, J.C.: Inactivation of lymphadenopathy associated virus by cheminal disinfectants. Lancet, ii: 899—901, 1984.
3) Fauvel, M. & Ozanne, G.: Immunofluorescence assay for human immunodeficiency virus antibody: investigation of cell fixation for virus inactivation and antigenic preservation. J. Clin. Microbiol., 27(8): 1810—1813, 1989.
4) Rodgers, F.G., Hufton, P., Kurzawska, E., Molloy, C. & Morgan, S.: Morphological response of human rotavirus to ultra-violet radiation, heat and disinfectants. J. Med. Microbiol., 20(1): 123—130, 1985.
5) Malkovsky, M., Newell, A. & Dalgleish, A.G.: Inactivation of HIV by nonoxynol-9. Lancet, i: 645, 1988.
6) Bloomfield, S.F., Smith-Burchnell, C.A. & Dalgleish, A.G.: Evaluation of hypochlorite-releasing disinfectants

平成7年5月20日

against the human immunodeficiency virus (HIV). J. Hosp. Infect., 15(3): 273—278, 1990.
7) Martin, L.S., McDougal J.S. & Loskoski, S.L.: Disinfection and inactivation of the human T lymphotropic virus type III/lymphadenopathy-associated virus. J. Infect. Dis., 152(2): 400—403, 1985.
8) Resnick, L., Veren, K., Salahuddin, Z., Tondreau, S. & Markham, P.D.: Stability and inactivation of HTLV-III/LAV under clinical and laboratory environments. JAMA, 255(14): 1887—1891, 1986.
9) Tjøtta, E., Hungnes, O. & Grinde, B.: Survival of HIV-1 activity after disinfection, temperature and pH changes, or drying. J. Med. Virol., 35(4): 223—227, 1991.
10) Sattar, S.A. & Springthorpe, V.S.: Survival and disinfectant inactivation of the human immunodeficiency virus: a critical review. Rev. Infect. Dis., 13(3): 430—447, 1991.
11) Dimitrov, D.S., Willey R.L., Sato, H., Chang, L-J., Blumenthal, R. & Martin, M.A.: Quantitation of human immunodeficiency virus type 1 infection kinetics. J. Virol., 67(4): 2182—2190, 1993.
12) Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L.: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science, 220: 868—871, 1983.
13) Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A. & Chen, I.S.Y.: HIV-1 entry into quiescent primary lymphocytes: Molecular analysis reveals a labile, latent viral structure. Cell, 61: 213—222, 1990.
14) Lee, M.H., Sano, K., Morales, F.E. & Imagawa, D.T.: Sensitive reverse transcriptase assay to detect and quantitate human immunodeficiency virus. J. Clin. Microbiol., 25(9): 1717—1721, 1987.
15) Tochikura, T.S., Nakashima, H., Tanabe, A. & Yamamoto, N.: Human immunodeficiency virus (HIV)-induced cell fusion: quantification and its application for the simple and rapid screening of anti-HIV substances *in vitro*. Virology, 164: 542—546, 1988.
16) Aranda-Anzaldo, A., Viza, D. & Busnel, R.G.: Chemical inactivation of human immunodeficiency virus in vitro. J. Virol. Methods, 37: 71—82, 1992.
17) Sato, H., Orenstein, J., Dimitrov, D. & Martin, M.: Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. Virology, 186: 712—724, 1992.
18) Hanson, P.J., Gor, D., Jeffries, D.J. & Collins, J.V.: Chemical inactivation of HIV on surfaces. BMJ, 298: 862—864, 1989.

# Micro-Suspension-Test ;
## ヒト免疫不全ウイルスに対する消毒薬の効果判定方法

大阪医科大学，*第2内科学教室，**微生物学教室
島越由紀子*** 佐野　浩一** 中野　隆史** 中村　積方**
大柴　三郎* 勝　健一* 中井　益代**

**要　旨**

我々は，ヒト免疫不全ウイルス(HIV-1)に対する消毒薬の効果判定方法であるmicro-suspension-testを開発し，その信頼性を検討した．浮遊状態のHIV-1持続感染細胞を消毒薬と作用させ，非感染細胞と混合培養し，巨細胞形成(GCF)と上清中の逆転写酵素活性(RTA)によって，残存する感染性を調べた．培養2日目にGCFとRTAを検討するshort-term assayと，培養28日目までRTAを検討するlong-term assayとで構成されており，感度はそれぞれ$1\times10^3$，$1\times10^1$感染細胞であった．エタノール，グルタールアルデヒド，ホルマリン，次亜塩素酸ナトリウム，ポビドンヨードの5分間処理での最低有効濃度は，それぞれ20v/v%，0.01v/v%，5v/v%，0.05v/v，0.1v/v%であり，濃度依存性，時間依存性の消毒効果をみることができた．micro-suspension-testは，簡便で，感度もよいため，HIV-1に対する消毒薬の効果判定として有効であると考えた．