BMC Infectious Diseases

Technical advance

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An improved microtiter assay for evaluating anti-HIV-1 neutralizing antibodies from sera or plasma

Chiyu Zhang[1,2], Yunyun Chen[1] and Kunlong Ben*[1,3]

Address: [1]Laboratory for Molecular and Cell Immunology, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China, [2]The School of Medical Technology, Jiangsu University, Zhenjiang, Jiangsu 212001, China and [3]Kunming Chinawave Biotechnology Company Ltd., Kunming, Yunnan 650106, China

Email: Chiyu Zhang - zhangcy1999@hotmail.com; Yunyun Chen - chenyy02@mails.tsinghua.edu.cn; Kunlong Ben* - kben@vip.sina.com

* Corresponding author

Published: 23 December 2003

BMC Infectious Diseases 2003, **3**:30

Received: 05 October 2003

Accepted: 23 December 2003

This article is available from: http://www.biomedcentral.com/1471-2334/3/30

Abstract

Background: The anti-HIV-1 neutralizing antibody assay is widely used in AIDS vaccine research and other experimental and clinical studies. The vital dye staining method applied in the detection of anti-HIV-1 neutralizing antibody has been used in many laboratories. However, the unknown factor(s) in sera or plasma affected cell growth and caused protection when the tested sera or plasma was continuously maintained in cell culture. In addition, the poor solubility of neutral red in medium (such as RPMI-1640) also limited the use of this assay.

Methods: In this study, human T cell line C8166 was used as host cells, and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) instead of neutral red was used as vital dye. In order to avoid the effect of the unknown factor(s), the tested sera or plasma was removed by a washout procedure after initial 3–6 h culture in the assay.

Result: This new assay eliminated the effect of the tested sera or plasma on cell growth, improved the reliability of detection of anti-HIV-1 neutralizing antibody, and showed excellent agreement with the p24 antigen method.

Conclusion: The results suggest that the improved assay is relatively simple, highly duplicable, cost-effective, and well reliable for evaluating anti-HIV-1 neutralizing antibodies from sera or plasma.

Background

The increasing spread of HIV-1 pandemic around world, especially in developing countries, is severely threatening human health. Neutralizing antibody (NAb) [1,2] and cytotoxic T lymphocyte (CTL) [3,4] are the major weapons of host defense system against viruses. NAb plays an important role in preventing the spread of cell-free virus *in vivo* [1,2]. Previous studies had shown that passive immunization of non-human primates with high titers of NAbs could protect non-human primates from SHIV challenge [5,6]. Currently, the study interest is focusing on the development of envelop-based vaccines that might elicit high titers of broadly, long-lasting NAbs [1,7]. The assay for evaluating NAb is a key tool, and is widely used in AIDS vaccine research and other experimental and clinical studies.

For detection of anti-HIV-1 neutralizing antibody, there are many methods used today, for example, vital dye staining [8], p24 antigen determination [9], polymerase chain reaction [10], flow cytometry [11], reverse transcriptase determination, luciferase activity assay, and so on. Among them, p24 antigen determination is widely used in many laboratories, but high cost limits its application. The vital dye staining can be used for neutralization of laboratory T cell line adapted (TCLA) HIV-1 strains, rather than primary HIV-1 isolates [8]. Because of its low cost, it is widely used in developing countries. In present study, we reported an improved, simple, highly duplicable, cost-effective, and well reliable microtiter neutralization assay for evaluating HIV-1 neutralizing antibody from sera or plasma.

Methods

Cells and virus culture

An HTLV-1-transformed human T lymphocyte cell line, C8166, was used as the host cell for the microtiter neutralization assay. High-titer HIV-1 IIIB stock were obtained by H9/ HIV-1 IIIB cultures. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus stock was determined by endpoint titration on C8166 cells [12]. Clones of C8166 and H9/ HIV-1 IIIB cell lines were donated from Medical Research Council (MRC), AIDS Reagent Project. All cultures were grown and maintained in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum.

Plasma and sera

HIV-1 positive plasma YN02 and YN06 were obtained from HIV-1 infected asymptomatic individuals, and DH05, DH11, DH12 and DH16 were collected from HIV-1 infected injection drug users. Sera S1 and S2 were obtained from two rabbits immunized with a vaccine composed of HIV-1 non-gag multi-antigen-epitope peptides, and serum S6 was obtained from normal rabbit as negative control. All plasma and sera were heat-inactivated at 56°C for 45 min prior to assay.

MTT Assay for detecting the effect of unknown factors in plasma on cell growth

Six twofold serial dilutions of HIV-1 positive plasma were made in duplicate in a total 100 μl of growth medium per well in 96-well U-bottom plate. The serial dilutions ranged from 1:4 to 1:128. Then 4×10^4 dispersed C8166 cells in the logarithmic phase in 100 μl of growth medium were added into each well. Three experimental plates were prepared. The first plate was washed with growth medium twice after initial 6 h culture, the second plate was washed after overnight culture, and the third plate was continuously cultured without any cell washing process. Cell proliferation was measured by MTT (Sigma, Louis, Mo.) assay as previously described [13]. Briefly, after being cultured for 6 days, half of the C8166 cell suspension was discarded and 20 μl MTT solution (5 mg/ml in PBS) per well was added. After another 4–5 h incubation, 100 μl solution containing 20% SDS plus 50% DMF was added to melt formazan crystal. The OD values were read at 590 nm wavelength. The cell proliferation indices were calculated by the OD value in plasma or sera wells (cells plus plasma or sera) divided by the OD value in cell control wells (cells only), and then multiplied by 100.

Microtiter assay for HIV neutralizing antibody

Cell-free HIV-1IIIB and serial dilutions of the test plasma or sera were incubated in duplicate or triplicate for 1 h at 37°C, 5% CO_2 incubator before the addition of C8166 cells (4×10^4/well). Virus inoculum was approximately 8,000 TCID50 per well. To determine the optimal time point for washout procedure, four 96-well U bottom plates were used for each experiment of plasma YN02, YN06 and DH11-3. Of them, three plates were washed with growth medium twice after initial 3 h, 6 h and overnight (>12 h) culture respectively, and another was cultured without washout procedure. For plasma DH05-1, DH12-2, DH12-3 and DH16-1, two separate experimental plates for each plasma sample were prepared. One plate was washed with medium twice after initial 6 h culture, while another plate was not washed. After 6-day culture cytolysis occurred in approximately 80% of C8166 cells in virus control wells, MTT assay was performed as described above. Protection rate (%) was determined by the following formula:

$$\frac{\text{Mean OD}_{(\text{cells + plasma + virus})} - \text{Mean OD}_{(\text{cells + virus})}}{\text{Mean OD}_{(\text{cells})} - \text{Mean OD}_{(\text{cells + virus})}} \times 100$$

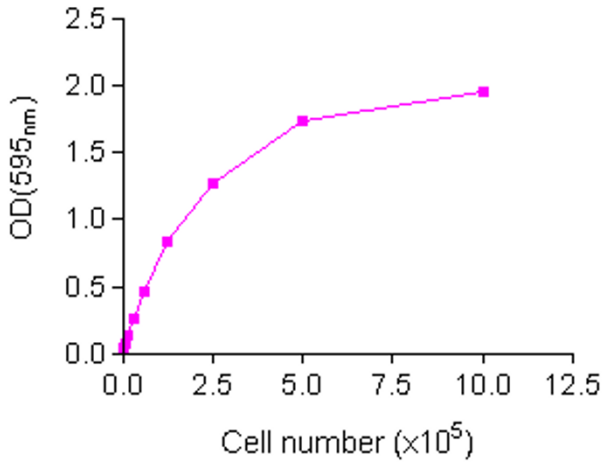

Figure 1
The relationship between the number of C8166 cells and MTT staining.

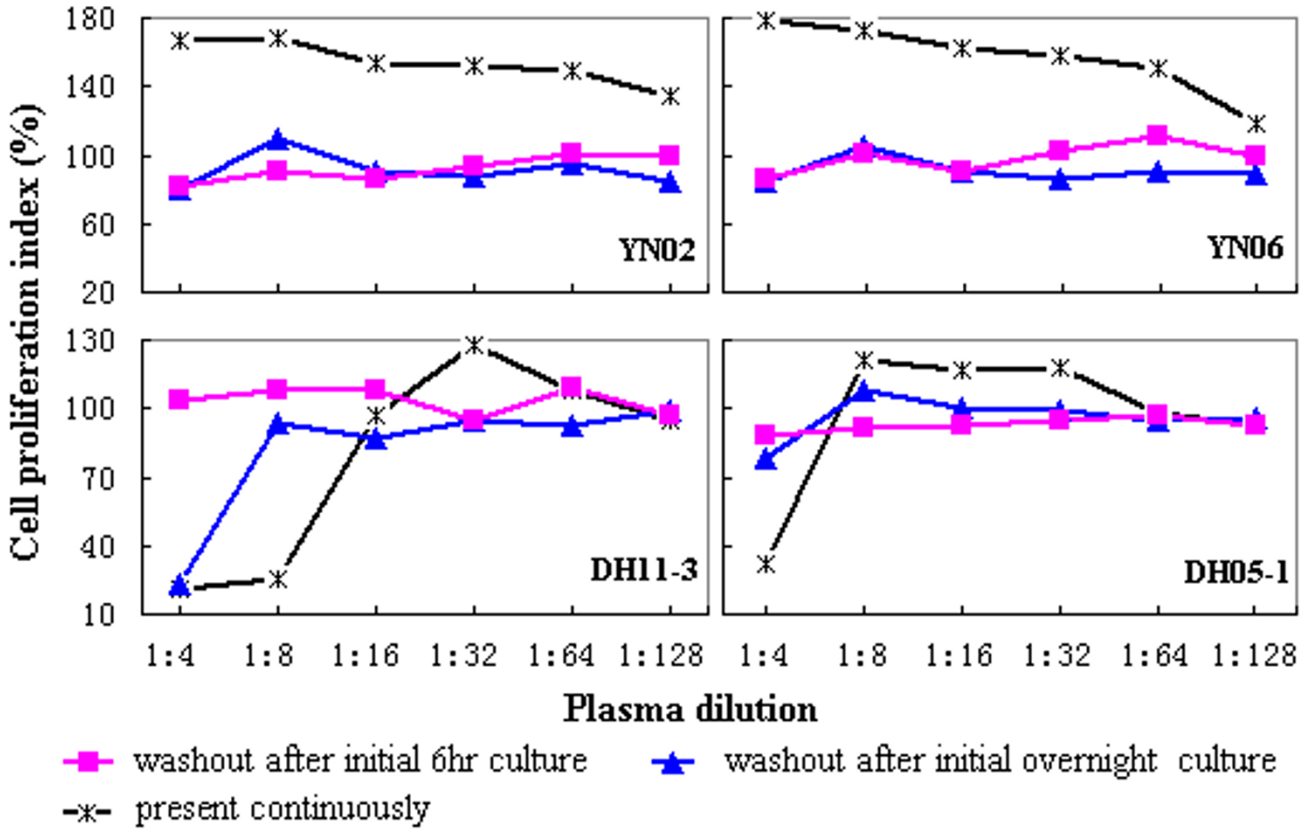

Figure 2
The effect of plasma on C8166 cell growth. Tested plasma were maintained in the continuous culture or were removed by washout procedure after initial 6 h and overnight culture respectively.

P24 antigen determination for anti-HIV-1 neutralization assay

The p24 antigen determination used for neutralization assay was performed according to Mascola's method with minor modification [9]. In present assay, human T cell line C8166 was used as host cells instead of peripheral blood mononuclear cells (PBMC), and HIV-1IIIB as virus inoculum. Briefly, the neutralization assay of sera S1, S2, plasma YN06 and negative control serum S6 at four three-fold serial dilutions was performed using approximate 5,000 $TCID_{50}$ HIV-1IIIB virus inoculum for each well. After 6 h culture, the U-bottom plate was washed with growth medium thrice to completely remove both the free virus inoculum and all the residual anti-p24 antibodies from the serum or plasma. Two days later, 10 μl culture supernatant was collected from each well for quantifying the p24 antigen production. The quantification of p24 antigen was carried out using RETRO-TEK HIV-1 p24 ELISA (ZeptoMetrix Corporation, New York) according to the manufacturer's instructions. Percent protection was determined by calculating the reduction in p24 production relative to the virus control [9]. Another four days later, MTT assay was performed as described above.

Results and discussion

MTT as the vital dye

MTT [14] and neutral red (NR) [15] are well known vital dyes used in the cell viability assay. In previous microtiter neutralization assay, NR was used as the vital dye [8]. In our experience, however, NR appears to have poor solubility in growth medium or RPMI-1640 medium. On the other hand, when NR is used, the microtiter neutralization assay needs a series of complex experimental processes including coating plate with poly-L-lysine (PLL), adhering cells to PLL-coated plate, washout procedure, extracting NR from cells, and so on [8]. In contrast to NR, MTT has a good solubility, and MTT assay has a relatively simple experimental process [13,14]. Therefore, we used MTT as the vital dye in our microtiter neutralization assay. Fig. 1 showed an excellent linear relationship (r = 0.9307;

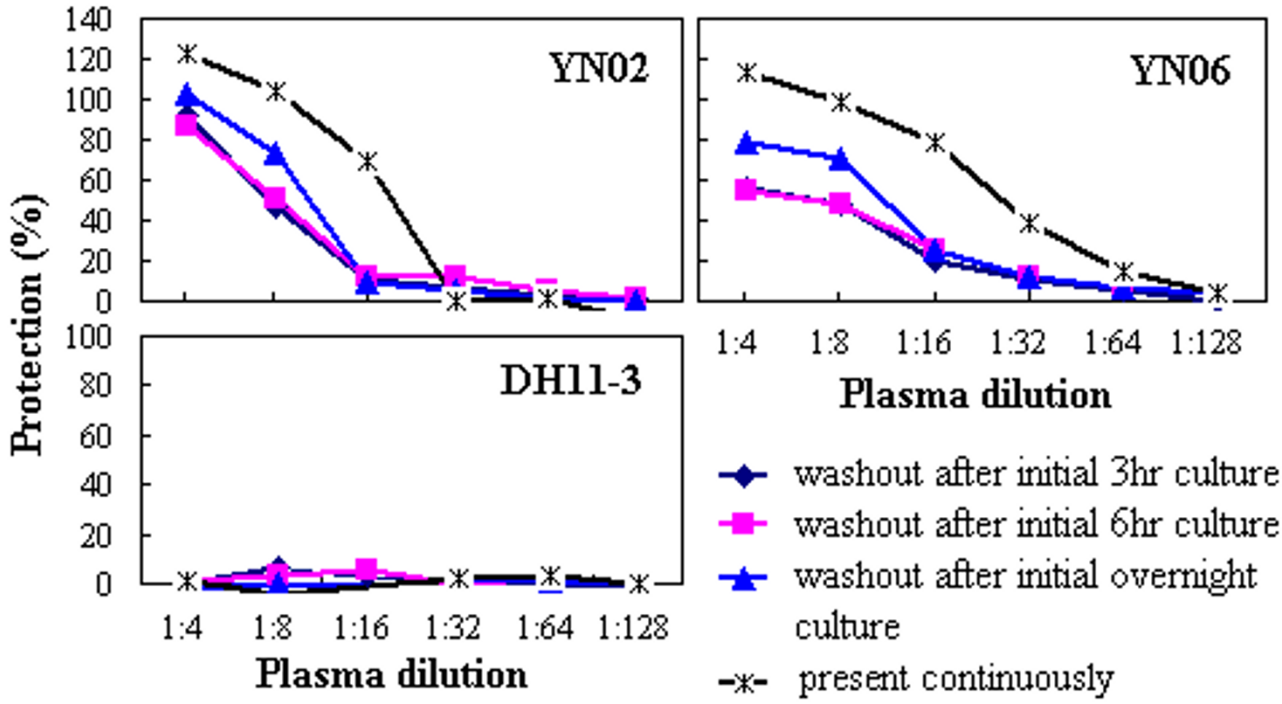

Figure 3
Determination of the optimal time point for washout procedure in the improved microtiter neutralization assay. Tested plasma were maintained in the continuous culture or were removed by washout procedure after initial 3 h, 6 h and overnight culture respectively.

$P < 0.0001$) between the numbers of the C8166 cells in the range of 1.95×10^3 to 5×10^5 per well and MTT staining.

The unknown factors in tested plasma or sera affected cell growth

Previously, tested human plasma or sera were maintained in the whole cell culture of microtiter neutralization assay. We found that the unknown factors in tested human plasma YN02 and YN06 stimulated the growth of host cells and resulted in overestimate of HIV-1 neutralization when the tested plasma were maintained in whole assay (Fig. 2). In contrast, the unknown factors in some other clinical plasma (such as DH05-1 and DH11-3) surprisingly inhibited cell growth dramatically at high concentration (Fig. 2). In order to eliminate these effects, we washed host cells with medium after initial 6 h or overnight culture. The results showed that washout procedure effectively decreased or eliminated these adverse effects of the factors in tested plasma (Fig. 2), suggesting that some unknown factors in these clinical plasma might be responsible for the stimulation or inhibition of cell growth.

The unknown factors in tested plasma caused inaccurate anti-HIV-1 protection using previous microtiter neutralization assay

The microtiter neutralization assay was established on the base of the quantification of virus-induced extensive cytolysis of host cells [8], the unknown factors in clinical plasma affects cell growth, and therefore lead to inaccurate determination of HIV-1 neutralization. To testify the hypothesis, HIV-1 neutralization by these clinical human plasma was determined using previous microtiter neutralization assay. When tested plasma was maintained in whole assay, the plasma at dilution of 1:4 (such as YN02 and YN06) stimulated cell growth, their protection rates distinctly were beyond 100% (Fig. 3). On the contrary, the plasma DH05-1 at dilution of 1:4 inhibited cell growth, appeared to have no protection, which was much lower than at higher dilutions (Fig. 4A). The above overestimation or underestimation of anti-HIV-1 protections at high plasma concentration suggested that previous method was unable to accurately evaluate anti-HIV-1 neutralization of clinical human plasma, and the elimination of these plasma effects is necessary for microtiter neutralization assay.

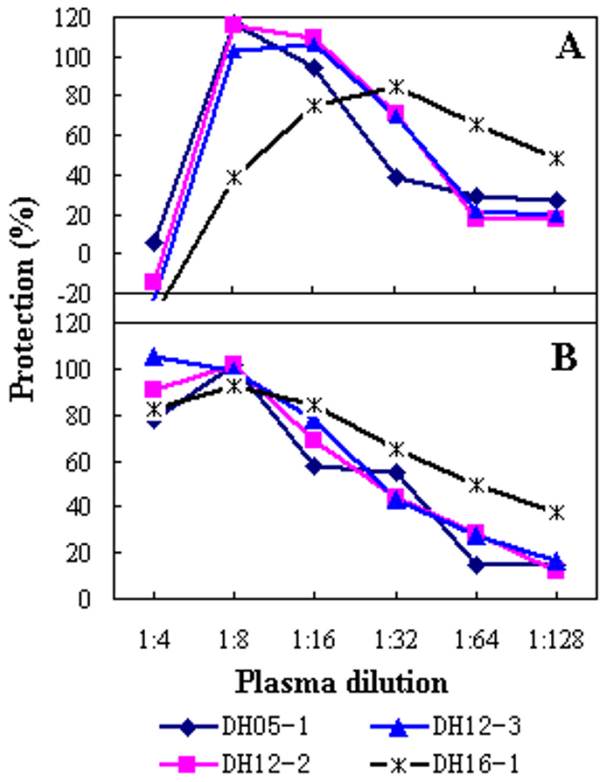

Figure 4
The anti-HIV-1 neutralizing activities of various patient plasma were measured by the previous microtiter neutralization assay (**A**) and by the improved microtiter neutralization assay (**B**).

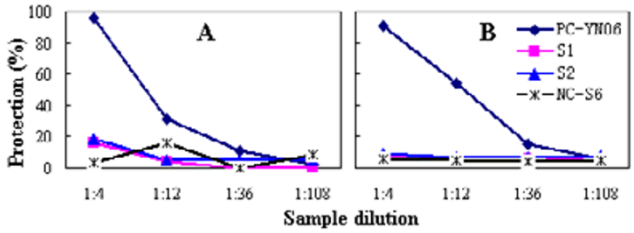

Figure 5
The comparison of the anti-HIV-1 neutralizing activities of some samples detected by the modified improved neutralization assay (**A**) with those by p24 neutralization assay (**B**).

Optimal time point for removing the unknown factors

In order to eliminate the effect of the factors on HIV-1 neutralization assay, we removed the factors from cell culture system by adding a washout procedure at 3 h, 6 h and overnight after culture. We found that washout after both initial 3 h and 6 h culture resulted in consistent protection rates at various dilutions of tested plasma (Fig. 3: YN02 and YN06), suggesting that washout after initial 3 to 6 h culture could completely eliminate the effect of the unknown factors in plasma on anti-HIV-1 neutralization assay.

Anti-HIV-1 neutralization of plasma YN05-1, DH12-2, DH12-3 and DH16-1, which inhibited distinctly cell growth at high concentration, was determined using the improved microtiter neutralization assay. The results showed that the underestimation of HIV-1 protection rates obtained by previous method were corrected (Fig. 4B). These results suggested the improved assay was able to eliminate both the effect of the unknown factors in plasma (or sera) on anti-HIV-1 neutralization and prolong time exposure cells to virus and plasma (or sera).

The comparison between the improved microtiter neutralization assay and p24 antigen neutralization assay

P24 antigen assay was widely used for evaluating HIV-1 NAb by detecting the reduction of p24 antigen production caused by NAbs [9]. To detect the reliability of the improved microtiter neutralization assay, both assays were performed with the same host cells, cell densities and virus inoculum. The results showed an excellent agreement of the protection rate in both assays (Fig. 5), suggesting the improved microtiter neutralization assay is as sensitive and reliable as p24 antigen determination in evaluating HIV-1 NAbs from sera or plasma.

Conclusion

Currently, HIV-1 neutralization assay is a key tool, and is widely used in AIDS vaccine research and other experimental and clinical studies. Because of cost considerations, the vital dye staining method, such as previous microtiter neutralization assay [8], is widely accepted in many laboratories. However, previous assay neglected the effect of the unknown factors in some tested plasma or sera on cell growth, and might lead to inaccurate anti-HIV-1 neutralization. Here, we modified the previous microtiter neutralization assay by using MTT as vital dye and by removing the factors in tested plasma or sera. In comparison with the other methods, the improved method increased the reliability of anti-HIV-1 neutralization assay for plasma or sera, and is relatively simple, highly duplicable, cost-effective for evaluating anti-HIV-1 neutralizing antibody from sera or plasma.

Competing interests

None declared.

Authors' contributions

Zhang CY participated in the design of the study, carried out the most of all experiments and drafted the manuscript. Chen YY participated in the experiments for detecting the effect of unknown factors in plasma on cell growth and the microtiter assay for HIV neutralizing antibody. Ben K conceived the study, and checked and approved the draft. All authors read and approved the final manuscript.

Acknowledgements

Authors are grateful to Dr. Chen YH for providing sera S1, S2 and S6. Authors also thank Xia XS and Zhang GH for technical assistance. This work was supported by the State 863-Program (2001AA215181).

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2334/3/30/prepub