Research Article

Comparison of Spinoculation and Coculture Techniques for Facilitating HIV-1 Infection of Healthy PBMCs Using Patient-Derived PBMCs and Plasma

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ABSTRACT

The research evaluates two *in-vitro* approaches for HIV-1 culture from stored samples, *viz.* infection of donor PBMC with patient plasma by spinoculation and co-culture of patient PBMC with donor PBMC. The evaluation of virus production depended on detection of p24 antigen in culture supernatant and infection of TZM-bl reporter cells. Most of the samples infected using spinoculation method showed p24 absorbance levels exceeding the threshold value on day 7. The levels peaked by days 9/10 and decreased by day 13. The TZM-bl assay gave similar results. The PBMC co-culture methodology resulted in a steady increase in p24 antigen levels starting from day 5 and continued to increase gradually the levels of peak achieved by day 9 and decreased by day 13 over time. The results of our study indicate that spinoculation enables virus production from stored plasma, yet co-culture of PBMCs appears to be a more robust approach for virus amplification.

Keywords: Spinoculation, PBMC co-culture, HIV-1, TZM bl infectivity assay, p24 antigen detection, ELISA

1. INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection remains a vital worldwide health problem, affecting approximately 38 million individuals globally [1]. The major target of the virus is the CD4+ T cell, which gets gradually and progressively depleted during infection, resulting in immune system failure and AIDS in the absence of proper treatment. Examination of HIV-1 replication inside target cells stands as an essential component for screening tests for effective drug molecules and vaccine candidates [2-5]. Most studies use peripheral blood mononuclear cell (PBMC) co-culture as the standard *in vitro* method for virus production, since these are the cells that first experience HIV-1 attack within the human body [6-12]. However, some studies also employ spinoculation of donor PBMCs with plasma of HIV-1-positive patients for patient-specific virus production. The spinoculation process utilizes centrifugation to expose target cells to the virus for improved virus binding and infection. While the simplicity of operation makes this method advantageous for researchers, numerous studies have failed to establish sustained HIV-1 viral replication using this approach [13-17]. The co-culture method on the other hand allows PBMCs to interact directly with each other just as HIV-1 does in nature [18-19]. Available scientific evidence confirms co-culture as a more reliable method for improving sustained viral replication thus making it an excellent tool for research aimed at understanding HIV-1 pathogenesis and studies aimed at therapeutic assessment [20-25]. However, there are several challenges when the research study is restricted to the use of frozen samples for virus production. Plasma samples are easy to prepare and store, isolation and cryopreservation of PBMCs is a labour-intensive and costly process. If not stored appropriately, the viability and recovery of PBMCs can drop

drastically, making them unfit for future use. Though earlier studies have compared the efficiency of spinoculation and PBMC co-culture for raising viral culture from freshly collected samples, the present research evaluated the use of spinoculation with stored patient plasma versus co-culture of stored patient PBMC with healthy donor PBMC methods for virus production. The results demonstrate that PBMC co-culture appears to be better than spinoculation with plasma in terms of sustained HIV-1 replication and improved virus production, even in the case of stored samples.

2. Materials and Method

Study Design

The present study was nested into an ongoing cross-sectional observational research study that was aimed at assessing different parameters in HIV-1 patients enrolled at the ART (Antiretroviral Therapy) Centre located within the Rajiv Gandhi Government General Hospital, Chennai. Purposive sampling allowed us to handpick study participants who met essential requirements fitted to this investigation's research goals. Newly diagnosed HIV-1 infected individuals registered at the ART Clinic formed the basis for sample acquisition. Blood collection took place throughout a one year period at the ART clinic from September of 2023 to September of 2024. We chose this extended time period to achieve ample subject numbers together with seasonal contrasts assuring a complete patient demographic and minimizing statistical distortions in short research periods. The study employed a cross-sectional design to capture immediate data about variables thereby making it a suitable research procedure for this population.

Ethics Statement

The study obtained ethical approval of The Institutional Ethics Committee of The Tamil Nadu Dr. M.G.R. Medical University located in Chennai. A permission to collect blood samples from HIV-1 patients at the ART Centre was granted by the Tamil Nadu State AIDS Control Society (TANSACS).

Study Participants

A total of forty participants were included in this sub-study. All participants were in the age group of 18 to 55 years and had viral loads above 1000 copies/ml. The study excluded both pregnant patients and terminally ill patients.

Sample Collection and Sample Processing

Six millilitres of whole blood were collected from the HIV-1 patients through venipuncture into K_2 EDTA anticoagulant-coated vacutainers after obtaining informed consent in writing. Blood samples were shipped to the molecular virology laboratory in transportation containers containing ice packs to maintain the cold chain. In the laboratory plasma was separated by centrifugation and stored as 1 ml aliquots at -80°C. PBMCs were isolated from the cell pellet using density gradient centrifugation with Ficoll-hypaque and stored at -150°C.

Infection of healthy donor PBMCs with HIV-1-infected patient plasma using spinoculation method

Blood samples from healthy donors and buffy coats from VHS hospital in Chennai were obtained and used to obtain PBMCs using Ficoll-Hypaque density gradient centrifugation. Cells were washed with complete RPMI medium containing 10% FBS and resuspended in medium containing complete RPMI and 10% foetal bovine serum (FBS) with 100 units of penicillin and 0.1 mg/mL streptomycin. Five million PBMCs were seeded in 25ml flasks and the cells were stimulated with 5 µg/ml phytohemagglutinin (PHA) for 48 hours at 37^0C. Frozen HIV-1 positive plasma was thawed at 37°C and 400 µL was added to the activated donor PBMCs. The ratio of plasma volume to cell concentration was adjusted based on infection efficiency obtained in pilot studies. Infection was allowed to occur by slow centrifugation at 2000 rpm for 2 hours. After infection, the supernatant was removed and 500 µL of fresh culture medium was added. The cells were transferred to a 12 well plate and another 500 µL of complete RPMI medium 5µL of Interleukin-2 was added to each well. The infected cells were grown in the incubator (37°C and 5% CO_2) for 5–14 days. Small aliquots of culture supernatants were collected on days 5, 7,

9, 11 and 13 for measuring virus production using p24 antigen detection ELISA and TZM-bl viral infectivity assay.

Co-culture of patient PBMC with activated healthy donor PBMC

Healthy, HIV negative donor PBMCs were obtained as described above and stimulated with 5 µg/mL phytohemagglutinin (PHA) for 48 hours. The PBMCs washed once, resuspended in complete RPMI medium containing 10% FBS, and counted. Frozen patient PBMCs were revived from storage, thawed at 37^0C, washed and resuspended in complete RPMI medium containing 10% FBS. Equal numbers of patient and donor PBMC were mixed (2-5 x 10^6 cells) and cultured in a T25 flask for 5-14 days in 5ml RPMI medium containing IL-2 and Polybrene (5 µg/mL). One ml of culture supernatant was collected from the flask on days 5, 7, 9, 11 and 13 and replaced with an equal volume of fresh RPMI. The collected supernatants were tested for virus production using p24 ELISA and TZM-bl infectivity assay.

P^{24} antigen detection

The p24 ELISA measures the amount of HIV-1 capsid protein as a marker of viral replication (26–30). We used the commercially available 4th Generation HIV Ag & Ab kit from J. Mitra & Co. for the detection of HIV-1 p24 antigen in cell culture supernatants. Cell culture supernatants were harvested at the collection times mentioned above and tested using the manufacturer's protocol. Briefly, 100µL of the test samples and positive controls, as well as negative controls and calibration standards, were added to pre-coated microtiter wells. Antigen-antibody binding was allowed to occur at 37°C for 60 minutes. After incubation, the wells were washed, and 100µL of enzyme conjugate solution was added to the wells. After 30 minutes incubation the wells were washed again and 100µL of substrate solution was added to plates and incubated at room temperature in dark for 10 minutes [26-30]. A stop solution was added for ending the reaction and the absorbance was read at 450nm using a microplate reader. Samples that gave absorbance values greater than the threshold were considered positive for HIV-1 p24 antigen. This assay allowed qualitative measurement of viral protein expression in cell culture supernatants to determine the success of HIV-1 infection.

TZM-bl infectivity assay

The TZM-bl infectivity assay correctly identifies and quantitates the number of infectious, replication-competent HIV-1 particles [31-35]. Briefly, TZM-bl cells were plated at a density of 10,000–15,000 cells per well in a 96 well plate containing 100µL of DMEM supplemented with 10% FBS and incubated at 37°C in a 5% CO_2 incubator overnight to promote adherence of cells. On the following day, HIV containing cell culture supernatants were serially diluted (1:100) in DMEM supplemented with 10% FBS, and 100 µL of each dilution was added to duplicate wells. The experimental setup included a positive control (known infectious virus) and a negative control (uninfected cell culture supernatant). The plate was incubated for 48 hours at 37°C in a 5% CO_2 incubator. After incubation, the supernatant was removed, 100µL of lysis buffer was added to the wells and the plate was incubated at ambient temperature for 5 to 10 minutes. Luciferase substrate was mixed with an equal amount of cell lysate and luminescence was quantified using a luminometer. Viral infectivity was measured as relative light units (RLU) in a luminometer, after normalizing the background signal obtained from the negative control wells.

3. Results

We employed a dual approach of measuring virus production using p24 antigen ELISA and viral infectivity using the TZM-bl assay to enable precise measurement of HIV-1 replication dynamics and efficiency of infection. Tables 1 and 2 provide the results of p24 antigen levels and viral infectivity of viruses produced using the spinoculation method for culturing activated donor PBMC with frozen patient plasma.

High levels of HIV-1 infection were observed in most of the samples as evidenced by high p24 antigen levels in day 7 and 13 culture supernatants (absorbance values exceeding the cut-off value of 0.459). On Day 7, all samples except two, HIVDRCHN-17 and HIVDRCHN-20 gave peak p24 values. Sample HIVDRCHN-05 gave the highest value of 1.497. On Day 13, though most samples still produced p24 antigen,

the levels were significantly lower than that of Day 7 indicating a decline in virus production with time. However, in two samples, HIVDRCHN-07 and HIVDRCHN-14, p24 levels remained stable indicating that infection was sustained over time.

Table 1: p24 antigen levels measured in day 7 and day 13 supernatants of spinoculation cultures of healthy donor PBMCs and HIV-1 positive patients' plasma

Sample No.	Absorbance value of Day 7 supernatant	Absorbance value of Day 13 supernatant
HIVDRCHN-01	1.134	0.76
HIVDRCHN-02	1.294	0.861
HIVDRCHN-03	1.402	0.99
HIVDRCHN-04	1.348	0.273
HIVDRCHN-05	1.497	1.226
HIVDRCHN-06	1.355	1.181
HIVDRCHN-07	1.264	1.302
HIVDRCHN-08	1.169	1.106
HIVDRCHN-09	0.636	0.397
HIVDRCHN-10	0.864	0.822
HIVDRCHN-11	0.746	0.258
HIVDRCHN-12	1.342	1.127
HIVDRCHN-13	1.358	1.095
HIVDRCHN-14	1.383	1.168
HIVDRCHN-15	1.295	1.189
HIVDRCHN-16	1.397	1.151
HIVDRCHN-17	0.216	0.967
HIVDRCHN-18	0.693	0.455
HIVDRCHN-19	0.758	0.675
HIVDRCHN-20	0.599	0.332

Table 2: Assessment of viral infectivity of spinoculation supernatants using TZM-bl cells

Sample ID	RLU of Day 7 supernatant	RLU of Day 13 supernatant
HIVDRCHN-01	421	251
HIVDRCHN-02	987	273
HIVDRCHN-03	398	283
HIVDRCHN-04	334	263
HIVDRCHN-05	601	269
HIVDRCHN-06	322	221
HIVDRCHN-07	294	243
HIVDRCHN-08	459	341
HIVDRCHN-09	297	371
HIVDRCHN-10	407	399
HIVDRCHN-11	369	390
HIVDRCHN-12	352	352
HIVDRCHN-13	364	344
HIVDRCHN-14	343	319
HIVDRCHN-15	367	333
HIVDRCHN-16	268	214
HIVDRCHN-17	284	300
HIVDRCHN-18	326	365
HIVDRCHN-19	381	303
HIVDRCHN-20	377	348
POSITIVE CONTROL 1	36016	31922
POSITIVE CONTROL 2	15701	8100
NEGATIVE CONTROL	536	482

The results of this experiment confirmed effective infection of donor PBMC by cell-free virus present in stored patients' plasma and effective virus production using the spinoculation approach.

Since the p24 ELISA does not provide direct proof of viral infectivity or competence of viral replication, we used the TZM-bl viral infectivity assay to further confirm the findings of the p24 assay. The results of the TZM-bl assay revealed that the infectivity of the culture supernatants was clearly lower than that of the positive controls both on Day 7 and Day 13, indicating the absence or presence of very few infectious viral particles produced using this approach. Only two samples - HIVDRCHN-02 and HIVDRCHN-05, had detectable levels of infectious viruses on Day 7, but even in these samples the titres declined significantly on Day 13. The findings of our study showed that the spinoculation method of infecting activated donor PBMC with frozen patient plasma under the conditions of this study had limited utility in robust virus production. Our findings also emphasize the need to simultaneously assess viral infectivity along with expression of viral protein to accurately determine the efficacy of infection and virus production.

Table 3: Absorbance values of p24 antigen ELISA of supernatants of patient and donor PBMC co-culture tested on Days 7 and 13

Sample ID	Absorbance value of Day 7 supernatant	Absorbance value of Day 13 supernatant
HIVDR-01	1.394	1.081
HIVDR-02	1.291	0.623
HIVDR-03	0.883	0.494
HIVDR-04	0.632	0.52
HIVDR-05	0.464	0.533
HIVDR-06	0.641	1.334
HIVDR-07	1.14	0.845
HIVDR-08	1.113	1.187
HIVDR-09	1.02	0.945
HIVDR-10	0.63	0.634
HIVCHN-11	0.616	0.665
HIVCHN-12	0.493	0.658
HIVCHN-13	0.689	1.124
HIVCHN-14	1.017	0.436
HIVCHN-15	0.803	0.309
HIVCHN-16	0.31	0.403
HIVCHN-17	1.129	0.829
HIVCHN-18	0.766	0.524
HIVCHN-19	0.97	0.7
HIVCHN-20	0.86	0.757

The results of the p24 antigen ELISA and viral infectivity of culture supernatants of co-cultured activated healthy donor PBMC and frozen patient PBMC are presented in Tables 3 and 4.

As can be seen from Table 3, the co-culture approach resulted in positive cultures for HIV-1 p24 antigen in most cases as indicated by absorbance values >0.290 on both days 7 and 13, indicating successful establishment of infection and viral protein production. On Day 7, few samples (HIVDR-01, HIVDR-02, HIVDR-07 and HIVDR-08) had very high p24 levels, some had moderate levels, while two samples (HIVDR-05 and HIVCHN-12) had low levels. p24 antigen levels declined in few samples, but remained stable or increased slightly up to Day 13. Two samples (HIVDR-06 and HIVCHN-13) showed peak p24 production on Day 13, indicating slow but steady increase in viral replication. These results seem to indicate that the PBMC co-culture approach ensures stable viral production between days 7 and 13, and potentially longer is feeder cells are provided during this period.

Table 4: Results of the TZM-bl assay of Day 7 and Day 13 supernatants of frozen patient PBMC with activated donor PBMC

Sample ID	RLU of Day 7 supernatant	RLU of Day 13 supernatant
HIVDR-01	49442	26570
HIVDR-02	116791	69331
HIVDR-03	8530	6335
HIVDR-04	260072	82892
HIVDR-05	1103	1064
HIVDR-06	15969	62233
HIVDR-07	5862	11091
HIVDR-08	9672	5278
HIVDR-09	15474	2599
HIVDR-10	721	525
HIVCHN-11	1854	1340
HIVCHN-12	7868	4862
HIVCHN-13	1629	2002
HIVCHN-14	21897	27717
HIVCHN-15	5822	5060
HIVCHN-16	9756	6426
HIVCHN-17	8120	5060
HIVCHN-18	4690	5180
HIVCHN-19	216791	2426
HIVCHN-20	15467	9683
POSITIVE CONTROL 1	35372	42043
POSITIVE CONTROL 2	5836	4872
NEGATIVE CONTROL	315	451

In order to determine the replication-competence of the viruses produced upon co-culture, TZM-bl cells were infected with Day 7 and Day 13 culture supernatants and the titre of infectious virus particles present were quantified by measuring RLUs in a luminometer (Table 4). It was observed that the samples that gave the highest values on Day 7 were HIVDR-04 (260,072) and HIVDR-02 (116,791) representing the presence of very high titres of infectious virus in these cultures. Samples HIVDR-01 (49,442) and HIVDR-06 (15,969) had values closer to that of the positive controls used indicating that these samples were also highly infectious samples. The infectivity of the other samples was relatively lower. In the case of Day 13 culture supernatants, the infectivity was relatively stable or slightly decreased in most of the samples. However, in a few samples, peak levels of infectivity were observed in Day 13 supernatants.

Thus, we demonstrated through this study that the PBMC co-culture method was superior to infection of donor PBMC with patient plasma for the production of high titres of infectious viruses that may be required for other purposes. We also showed that peak virus production happened around Day 7 for most samples and declined over time, whereas in some cases virus production continued until Day 13 with the peak virus production occurring in the second week after infection, suggesting that the PBMC co-culture approach allows sustained viral replication for extended periods of time. Finally, the study provides evidence to support the use of multiple assays, such as the p24 antigen ELISA and TZM-bl infectivity assay, to measure accurately measure viral replication and infectivity dynamics.

4. Discussion

The present study investigated the optimal methodology for production of viruses from stored plasma and PBMC of HIV-1 infected persons. Using two different techniques of HIV-1 infection, namely, infection of activated donor PBMC with frozen plasma using the spinoculation method and co-culture of frozen patient PBMC with HIV-positive patient PBMC in a ratio of 1:1, this study demonstrated that the PBMC co-culture approach was a superior method for virus production. This was in spite of the decline in viable recovery that was noticed in the cryopreserved PBMCs. For infection of donor PBMC with patient plasma we used the spinoculation method, which used slow centrifugation to increase infection of target cells

[13–17]. This method also allowed infection of donor PBMC as indicated by detection of p24 antigen in cell cultures by Day 7, but in most cases the cultures became negative for p24 by Day 13, indicating rapid viral clearance. Repeat testing of the spinoculation culture supernatants using the TZM-bl assay also showed low levels of viral infectivity indicating that spinoculation may not be an effective way of achieving durable viral replication as suggested by some [36].

More sustained viral replication and increased production of infectious viral particles was observed in the PBMC co-culture method. It is well known that long-term storage of PBMCs can affect the viability and recovery of cells. However, in spite of these limitations, our study clearly indicates that PBMC co-culture of patient PBMC with activated healthy donor PBMCs resulted in robust viral production via direct interaction between the infected and uninfected cells. The direct cell-cell contact actually facilitates viral dissemination and leads to sustained viral production [18-19]. We found many samples that had elevated viral titres up to Day 13 of culture. Thus, our findings corroborate with prior reports that PBMC co-culture techniques is an effective means for enhancing long term viral replication in PBMCs [18, 37]. Though spinoculation also resulted in efficient initiation of infection, we noticed a rapid decline in virus production, particularly infectious viruses that were replication competent. Thus, the efficiency of the spinoculation method for infection of healthy donor PBMC with patients' plasma appears to be very limited [38- 39]. The comparison between the two methods provides clear evidence to suggest that co-culture of patient PBMC with activated healthy donor PBMC is a more robust method for studying HIV-1 replication kinetics and dynamics of antiretroviral treatment efficacy, as well as for virus production for other experimental studies. Our study also provides proof to show that p24 antigen detection in culture supernatants, though simple, does not provide an accurate measure of the number of infectious viral particles, which is crucial to know for the purpose of several downstream applications. Hence, other approaches such as the TZM-bl assay appear to be better methods to ascertain the number of replication competent viruses [40]. The ability of the co-culture approach to sustain viral replication for longer periods of time is another reason to suggest that PBMC co-culture is an optimal method for virus production from patient samples.

5. Conclusion

To summarize, the results of our study clearly demonstrate that the PBMC co-culture method is a far more effective method for sustained viral replication and virus production and would therefore be a more reliable model to study HIV-1 infection, anti-HIV compound screening and other such studies. Optimizing the infection protocol will enhance the effectiveness of this method to facilitate high levels of sustained viral replication to support various kinds of HIV research.

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