ELSEVIER

Antiviral Research 35 (1997) 123–129

Antiviral Research

Short communication

Evaluation of anti-AIDS drugs in conventional mice implanted with a permeable membrane device containing human T cells infected with HIV

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Received 4 November 1996; accepted 4 March 1997

Abstract

We now report the confirmation of the work of Hollingshead et al. (1995) on development of a cell based hollow fiber (HF) system for evaluating potential anti-AIDS drugs in vivo using conventional mice rather than SCID mice. CD4 +, CEM-SS cells infected with HIV/1, strain RF, at a multiplicity of infection of 0.1 were placed into HFs. The fibers were implanted into the peritoneal cavity of outbred Swiss mice. Using this model, the antiviral activity of azidothymidine (AZT) at doses of ~150, 75 and 37.5 mg/kg/day was evaluated by administering AZT to the mice in drinking water. Upon fiber removal on day 6, AZT treatment was shown to significantly increase CEM cell viability over the untreated, virus control group and significantly reduced the levels of HIV p24 and HIV RT activity.

Keywords: Hollow fibers; AIDS drug evaluation; HIV animal model

The use of selective membranes has emerged as an alternative for cell transplantation. This approach is based on the concept of immune isolation. Transplanted cells are separated from the host by a semipermeable membrane that allows only molecules (oxygen, nutrients, etc.) of defined size to pass through the membrane. Polyacrylonitrile-polyvinylchloride (PAN/PVC) hollow fibers

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0166-3542/97/$17.00
PII S0166-3542(97)00014-4

with a molecular weight cutoff of 70 000 Da have been extensively tested in allogeneic and xenogeneic cell transplantation and have demonstrated good tissue compatibility and stability (Shoichet and Rein, 1996). Because hollow fibers with a molecular weight cutoff of 70 000 Da exclude both effector lymphocytes and immunoglobulins, rejection of allografts, as well as xenografts, has been prevented (Maki et al., 1991, 1995, 1996). PAN/PVC hollow fibers seeded with rodent, canine, porcine or bovine pancreatic islets were implanted for over 150 days in the peritoneal cavity of rats and only minimal tissue reactivity was observed (Altman et al., 1988; Lanza et al., 1991, 1992).

The treatment of human immunodeficiency virus (HIV) infection depends upon therapeutic agents effective against the infectious process. The development of new antiviral agents is aided by demonstrating their efficacy in animal models. Hollingshead et al. (1995) reported the use of a cell based hollow fiber system to transplant HIV infected CEM-SS human lymphoid CD4 + cells into mice. The study demonstrated that treatment of the host mice with antiviral agents decreased virus replication in the hollow fiber. Hollingshead et al. (1995), used polyvinylidene fluoride hollow fibers with a molecular weight cutoff of 500 000 Da implanted into severe combined immunodeficient (SCID) mice. Membranes with a molecular weight cutoff as large as 500 000 Da are permeable to immunoglobulins and other immune factors (e.g. complement, etc.). Since the mice used in those studies were SCID's, immunoglobulins may not have played a role in interfering with HIV infection. Complement components, however, may pass through that membrane and cause some inactivation of HIV (Hosoi et al., 1990). Regardless, the higher molecular weight cutoff of their membrane may preclude the use of immunocompetent animals.

In this study, we report the transplantation of HIV-infected CEM-SS human lymphoid CD4 + cells into conventional, immunocompetent mice (outbred Swiss mice, CD-1 from Charles River). Because both effector lymphocytes and immunoglobulins are excluded using a lower molecular weight cutoff (70 000 Da) fiber, interference with HIV infection may not occur (Maki et al., 1991, 1995, 1996). This system allows the membrane implant to be removed after several days to assess the cell viability and viral replication while screening antiviral drugs using an inexpensive animal model. The membrane used in this report was formed by a phase inversion spinning process from a polymeric casting solution made of polyacrylonitrile-polyvinylchloride (PAN/PVC) copolymer, according to the process developed by Michaels (1971). The resulting tubular ultrafiltration membranes were tested for permselectivity with substances of varying molecular weights, including bovine serum albumin and immunoglobulin G. The membrane allowed passage of albumin, but rejected 99–100% of immunoglobulin G and proteins of greater molecular weights (Sullivan et al., 1991).

The hollow fibers were approximately 15 mm long with a 3 mm diameter and had a volume capacity of 80 μl. The hollow fibers (HFs) were obtained from W.R. Grace. The HFs were prepared with one end sealed, placed in cryovials containing water and sterilized by gamma irradiation. Before use the HFs were placed in RPMI 1640 medium without serum for one night. Cells and/or virus were placed in HFs in 80 μl volumes and the remaining end was heat sealed.

The cells used in these studies were CEM-SS cells (NIH AIDS Research and Reference Program), which were grown in RPMI 1640 plus 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml Streptomycin, 50 μg/ml gentamicin and 25 mM HEPES. The cells were passaged the day before the experiment. Cultures were maintained in disposable tissue culture labware in an incubator at 37°C in a humidified atmosphere of 5% CO_2 in air. Prior to performing the experiment the cells were counted and suspended at 1×10^5 cells per ml. In order to place cells in HFs, the HFs were placed in wells of 96 well microtiter plates, with the sealed end down.

The virus used in this study was HIV/1, strain RF (NIH AIDS Research and Reference Program). In most experiments, it was used at a multiplicity of infection (MOI) of 0.1 as previously observed in a CPE assay for determining 50% tissue culture infectious doses ($TCID_{50}$).

In order to determine cell viability, cells were mixed with trypan blue stain and counted on a hemocytometer or samples of 50 μl were analyzed using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This material is taken up and metabolized to an insoluble, colored formazan product by live cells. The optical density value obtained is a function of the amount of formazan product which is proportional to the number of viable cells. In the experiments, cells were exposed to MTT in an incubator for 6 h. At that time 30% SDS was added to solubilize the cells and the MTT formazan. The SDS was incubated with the cell suspension for 18 h. The optical density of the MTT formazan was read in a plate reader at 570 nm. The optical densities are used to calculate the percent of viable cells when infected and/or drug-treated cells are compared to untreated control cells.

In order to establish that the HFs were nontoxic to cells and that HIV infection could take place in them a preliminary in vitro experiment was performed. Uninfected cells and virus-infected cells were placed in 80 μl volumes in the HFs and heat sealed. The HFs were placed in cryovials and 1 ml of RPMI 1640 medium + 10% FBS was added to the vial. In the first study, the growth and degree of infection (cytopathic effect [CPE] development) was observed.

HF samples (three per group) of uninfected and infected cells were taken on days 4, 6 and 8 after loading the HFs. On day 4, the cell counts had increased from 100 000 to 410 000/ml with 96.9% being viable. On day 6, the cell number had increased to 1 503 000/ml with 97.4% being viable. On day 8, cell number had increased to 3 390 000/ml with 83.4% being viable. On day 8, the medium surrounding the HFs had become yellow indicating acidic changes in the medium due to cell growth and excretion. Extensive CPE was observed in infected CEM cells by day 4 with few cells surviving to be counted. On days 6 and 8 there were essentially no cells surviving. Since day 6 is the day on which essentially all cells were dead and is the day we use for our microtiter drug assays, we chose day 6 as the appropriate day for further studies.

In a second experiment, uninfected (control cells) and infected cells were placed into HFs and divided into groups of three fibers. Four groups were placed in medium that contained the following concentrations of AZT: 1, 0.1, 0.01 or 0.001 μg/ml. HF samples were collected on day 6 after infection and placement into fibers. Cell viability was measured by MTT assay as we normally do for our in vitro drug assays. In this experiment with AZT, the uninfected cell controls had a mean O.D. value of 1.464 and the virus controls had a mean value of 0.174 which indicated that virus had destroyed most cells and reduced the viability by 88% (Table 1) after 6 days. For fibers exposed to medium containing AZT, the O.D. values were greater than the virus controls. AZT at concentrations of 1 and 0.1 μg/ml produced essentially 100% protection. AZT at 0.01 μg/ml showed a reduction of cytopathic effect by 53.6%. This value is close to the 50% inhibitory dose that we usually observe in our in vitro drug assay.

For implantation of fibers into mice, the mice were anesthetized with an injection of ketamine-xylazine. Eye lubrication was applied and the surgical site was prepared. The hair was removed by clipping and a surgical preparation was performed using betadine scrub and betadine solution. Sterile instruments were used to make incisions of approximately 1 cm length in the ventral midline abdominal skin and musculature and then through the peritoneum. The HFs were inserted and the incisions in the peritoneum and skin were closed separately with PDL suture.

Table 1
Effect of AZT treatment on the viability of uninfected and HIV-infected CEM cells entrapped in hollow fibers and cultured in vitro

Group	Mean O.D.	Percent reduction of CPE
Cell controls	1.464	—
Virus controls	0.174	—
AZT 1.0 μg/ml	1.435	100.0
AZT 0.1 μg/ml	1.566	100.0
AZT 0.01 μg/ml	0.691	53.6
AZT 0.001 μg/ml	0.223	17.3

In order to determine if cell growth and virus infection could take place in HFs implanted intraperitoneally in mice, we performed a preliminary experiment. The cells of some fibers were inoculated with strain RF of HIV/1 at an MOI of 0.1 or 0.05. Samples were collected on day 6 to perform MTT assays and on days 4, 6 and 8 to observe CPE and measure p24 antigen. The p24 was measured using 5 μl aliquots of sample. These aliquots were diluted 1:100 and 200 μl were used for the assay. Measurement was performed using a commercially available ELISA kit (Coulter Diagnostics) with the manufacturer's use instructions being followed.

On day 4 after implantation of the fiber uninfected cell counts had increased from 100 000 to 1 200 000 cells/ml. The day 6 and 8 counts were 2 660 000 and 2 470 000 respectively. For infected cells, when the high amount of HIV inoculum was used, extensive CPE was found by day 4 and essentially all cells showed CPE by day 6. The cells had disintegrated by day 8. This was not quite as fast an infection as we had seen in the in vitro experiment. The infection produced by the lower inoculum of virus progressed slower with less CPE, but the CPE appeared to be essentially 100% by day 8. Samples from fibers containing cells that had been infected with an MOI of 0.1 had p24 values of 475 and 1250 pg on days 4 and 8. The samples from fibers containing cells that had been infected with an MOI of 0.05 had p24 values of 109 and 755 pg on days 4 and 8. For the MTT assay performed on cells from fibers collected on day 6, the mean O.D. value for the cell controls was 1.238. The O.D.s of the high and low virus inocula were 0.333 and 0.862. From these findings, we concluded that the same MOI that we used in our in vitro studies was also appropriate for in vivo studies.

In order to evaluate the effect of drug treatment on uninfected cells or infected cells in HFs implanted in vivo, AZT was dissolved in the drinking water of the mice. The AZT oral treatment was begun 2 days prior to implantation of the HFs. The dosages were based on calculations of the amount of water that was consumed daily by the mice. The dosages were $\sim$150, 75 and 37.5 mg/kg per day. Treatment was begun before fiber implantation because of the long time involved in preparing fibers and implanting them. Due to this preparation time, the infection had been in progress for a period of 4–8 h before exposure to plasma in the peritoneal cavity. At the time of implantation 10% of the cells would have progressed into the virus replication cycle, but 90% of the cells would still be uninfected. The following groups were included in the study: Cell-free fibers, media controls (five mice); uninfected cells, animals untreated, cell controls (ten mice); uninfected cells, animals treated with $\sim$150 mg of AZT per kg/day (five mice); uninfected cells, animals treated with $\sim$75 mg of AZT per kg/day (five mice); uninfected cells, animals treated with $\sim$37.5 mg of AZT per kg/day (five mice); infected cells, animals untreated, virus control (ten mice); infected cells, animals treated with $\sim$150 mg of AZT per kg/day (ten mice); infected cells, animals treated with $\sim$75 mg of AZT per kg/day (ten mice); and infected cells, animals treated with $\sim$37.5 mg of AZT per kg/day (ten mice).

To measure cell viability in this experiment, we used the MTT assay and took the mean of all O.D. values. To measure virus infection, we observed CPE microscopically, performed p24 assays and also assayed reverse transcriptase (RT) levels. The methodology for measuring RT is as follows. A 5 μl aliquot of sample was diluted 1:10 and 25 μl was assayed. The RT reactions, which can be performed in microtiter plates or in 0.5 ml Eppendorf tubes, contain the following reagents: oligo dT, poly rA, Tris HCl (pH 7.4), DTT, $MgCl_2$, and EDTA. The reaction proceeds for 45–90 min at 37°C. Following the reaction, the total volume was spotted onto DE81 chromatography paper, washed five times with a large volume of 5% sodium phosphate buffer, twice with distilled water and twice with ethanol. Following the ethanol wash, the samples were air dried or dried under a heat lamp. The amount of incorporated radioactive thymidine was quantitated by liquid scintillation counting using a toluene-base fluor. The results are presented as disintegrations per minute (DPM).

Table 2
Effect of oral administration of AZT on the viability of uninfected cells or HIV-infected CEM cells entrapped in hollow fibers and implanted intraperitoneally in swiss mice

Group	Mean O.D.	S.E.M.[a]	P^b
Uninfected cells (cell control)	1.780	0.216	—
Uninfected cells, AZT-treated ~150 mg/kg per day	1.390	0.315	0.190
Uninfected cells, AZT-treated ~75 mg/kg per day	0.973	0.193	0.076
Uninfected cells, AZT-treated ~37.5 mg/kg per day	1.810	0.276	0.940
Infected cells, untreated (virus control)	0.262	0.046	—
Infected cells, AZT-treated ~150 mg/kg per day	0.970	0.134	<0.0001
Infected cells, AZT-treated ~75 mg/kg per day	0.465	0.084	0.023
Infected cells, AZT-treated ~37.5 mg/kg per day	0.404	0.076	0.063

[a] S.E.M. = standard error of the mean.
[b] Probability by Mann-Whitney *U*-test, uninfected cells were compared to the uninfected cell control and infected cells were compared to the infected, untreated cells (virus control).

The cells were prepared, placed in HFs, implanted into mice and left in animals for 6 days. When the uninfected AZT-treated cells were observed for toxicity using the MTT assay, none of the values was significantly lower than the cell control samples (Table 2). When the same assay was performed and the cell control values were compared to the infected cells (virus controls), the cell control mean O.D. value was 1.780 and the virus control value was 0.262, showing that infection without treatment lowered the viability in cells by 85.3% (Table 2). The values for the AZT treated (150, 75, 37.5 mg/kg per day) animal (infected) HF samples were 0.970, 0.465 and 0.404. The two higher O.D. values are significantly different from the virus controls when the values were compared using the Mann-Whitney *U* test. The fact that O.D. values of cells from AZT-treated animals were reduced when compared to the cell controls indicates that HIV infection was taking place. Indeed, when these cells were observed microscopically, CPE was seen. Therefore, it appeared that AZT had an inhibitory effect when measured on day 6. We assume that there would have been eventual destruction of these cells from AZT-treated mice since CPE was observed. Consequently, the effect of AZT may have been to delay, but not totally inhibit infection in the cells. To some extent, this may reflect the patient situation in which AZT is beneficial for a while but does not continue. In the short time of the animal study, we don't expect that this finding resulted from development of resistant virus but from exhaustion of new uninfected target cells.

When p24 values were compared, the virus control cell samples had a mean value of 551 000 pg/ml and the three AZT groups (150, 75, 37.5 mg/kg per day) had values of 24 900, 33 200 and 154 667 pg/ml, respectively. All three treatment group values were significantly different from the virus control values when compared by the Mann-Whitney *U* test.

When RT values were compared, the virus control cell samples had a mean value of 74 821 and the three AZT groups had values of 4921, 5176 and 24 134 DPMs. All three treatment group values were significantly different from the virus control values when compared to the Mann-Whitney *U* test.

In addition to this experiment, we performed an earlier animal experiment in which two doses of AZT (135 and 96 mg/kg per day) were evaluated. For that experiment, the p24 concentration of the virus control group was 500 000 pg/ml, while the samples from animals receiving 135 mg/kg were 24 744 and those receiving 96 mg/kg were 130 500. For RT observations, the virus control group samples were 46 510 DPM, while the 135 mg/kg group samples were 2618 and the 96 mg/kg group were 3650.

We believe that these two studies in which AZT was administered orally show that drug was taken up in the alimentary tract and distributed by

circulation such that blood levels high enough to inhibit HIV were available to enter the HF.

In our hands, CEM cells grew well in HFs and HIV/1 virus infection progressed well in infected CEM cells placed in HFs. In early studies, we found that pressing the fibers flat with forceps to heat seal them resulted in some cracks and leakage of materials from the fibers, which led to some variation of results as seen in the MTT toxicity observations in the AZT animal efficacy experiment. We have solved this problem by placing a polyurethane cuff on each end of the fiber and the cuff is pressed shut and heat sealed.

Hollingshead et al. (1995) recently demonstrated the use of polyvinylidene fluoride (PVDE) hollow fibers as carriers of HIV-infected cells for implantation into SCID mice and evaluation of anti- AIDS drugs. In that paper, PVDE fibers with a 500 000 molecular weight cutoff were loaded with CEM-SS cells or cells infected with HIV/1, strain IIIb and implanted subcutaneously or intraperitoneally into SCID mice. In comparison, our model which uses HFs with a 70 000 molecular weight cut off allows us to perform similar studies in conventional outbred mice since mouse serum factors such as antibody and most complement components are excluded from the cavity of the HF. Only protein D with an approximate relative molecular weight of 24 000 Da would readily pass through our HF. Conventional mice are less expensive than SCID mice and require much less labor to appropriately care for them, therefore this model using polyacrylonitrile-polyvinylchloride is much less expensive. In addition, we use a higher MOI and a much shorter implantation (incubation) time which also reduces cost and makes the model more practical.

In conclusion, we have determined that HIV/1, strain RF will replicate in CEM-SS cells in polyacrylonitrile-polyvinylchloride hollow fibers and that this provides an inexpensive method of evaluating potential anti-AIDS drugs in conventional, outbred mice. In essence, we have taken an inexpensive in vitro assay and placed it in an animal. Addition of the animal to the assay adds the requirement that the drug must reach blood levels adequate to reach tissues and our artificial tissue, the hollow fiber, in order to inhibit the virus. For a drug to be active, it must be taken up (orally), metabolized and distributed in the blood for the model to work. We have confirmed the findings of Hollingshead et al. (1995), but have used a hollow fiber of lower molecular weight cutoff which allows the use of conventional animals in a less expensive model.

Acknowledgements

This work was supported in part by Public Health Service Contract NO1-AI-05086 from the National Institute of Allergy and Infectious Diseases and by Southern Research Institute.

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