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A Novel Method for Removal of Human Immunodeficiency Virus: Filtration with Porous Polymeric Membranes

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Abstract. We propose a new method to rid solutions of a virus by using a novel regenerated multilayered structured cellulose membrane (BMM). When the filtrate of human immunodeficiency virus (HIV) preparation was obtained through BMM it showed no infectivity. Electron microscopic observation revealed that HIV was completely caught by the multilayers of the BMM. Conveniently, BMM was seldomly found to adsorb protein molecules and also to have a high filtration rate. These characteristics may have a use in the removal of other variously sized pathogenic agents from plasma.

Introduction

Since the initial reports on acquired immunodeficiency syndrome (AIDS) in 1981 [2, 3] the number of cases reported each year has dramatically increased. Now, AIDS is a public-health problem of worldwide magnitude [6]. Several seroepidemiological studies have suggested that patients with hemophilia were at high risk to be infected with human immunodeficiency virus (HIV), a causative agent of AIDS from contaminated clotting factor concentrates [7, 8, 15]. We and others have previously reported on the inactivation of the virus by various means, such as heat and ultraviolet irradiation [11, 16, 19]. Because there is convincing evidence that HIV can be inactivated by heat, these blood products have been heat-treated before administration [4]. However, there are also controversial reports [20, 21], indicating that heat-treatment at 60 °C for 30 h is insufficient to inactivate HIV. Moreover, the possibility cannot be overlooked that serum proteins are altered by this treatment. Thus, it seems worthwhile to develop a new method by which HIV transmission in blood products can be prevented.

Many attempts to obtain a virus-free filtrate using polymeric membranes have been reported [18]. However, it is not yet practical to obtain virus-free products from blood plasma through filtration using a polymeric membrane. For a hypothetical system to collect HIV-free products from the plasma, there are many requirements, such as high filtration rate and high recovery of proteins in addition to the elimination of the virus. In this paper we demonstrate that our filtration method, with a novel porous regenerated cellulose hollow fiber, can remove HIV infectivity completely from the virus-rich culture supernatant while maintaining both a high filtration rate and a high permeability of proteins. In addition, we also show electron micrographs which reveal that HIV particles cannot filter through the membrane and are trapped by each thin layer constituting the multilayer structure of the membrane.

Materials and Methods

BMM Membrane

Cuprammonium solution with various concentrations of cellulose ranging from 5 to 9 weight%, was spun through a spinnerette having double cocentered holes. The microphase separation occurred before being taken up by a roller. A detailed description of the spinning method of BMM is given in the patient literature [22]. BMM will be commercialized in 1989 by Asahi Chemical Ind. Co. Ltd. The mean pore size $2r_f$ was obtained by the water flow method, while porosity was calculated using the apparent density method [13]. BMM was formed into a cylindrical module having an effective filtration area of 0.03 m^2. The characteristics of the pore's structure were determined through electron microphotometry.

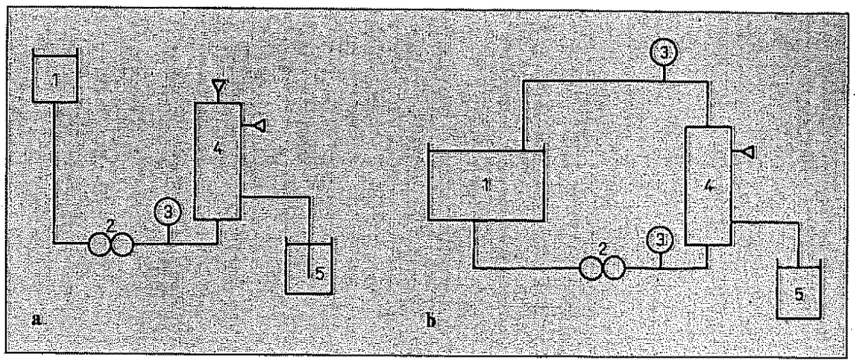

Fig. 1. Schematic representation of filtration equipment: 1 = original solution; 2 = pump; 3 = pressure gauge; 4 = BMM module; 5 = filtrate. **a** Perpendicular filtration (dead-end filtration); **b** parallel filtration (cross filtration).

Filtration Procedure with BMM

The hollow fiber module was set into the filtration system, which was designed to enable changes in transmembrane pressure (i.e. the difference between the pressure at the inner wall of the hollow fiber membrane BMM and that at the outer wall, ΔP; shear rate at the inner wall of the hollow fiber, $\dot{\gamma}$; filtration rate, u) and flow rate. Figure 1 shows a schematic representation of the filtration system. When the hollow fiber is blocked at the end (fig. 1a), $\dot{\gamma}$ approximates zero ('perpendicular filtration') [12]. Two typical filtration conditions of constant ΔP and constant u were employed. The values of $\dot{\gamma}$ and u were given by the feed speed of the 2 pumps in Figure 1b.

Proteins

The following proteins of various molecular weights (MW) were employed as model proteins of human plasma protein; thermolysin from bacillus thermoproteolytics (MW, 28,000; Serva Feinbiochemica GmbH & Co.), bovine serum albumin (MW, 65,000; Sigma Chemical Co.), bovine serum γ-globulins (MW, 150,000; Sigma Chemical Co.), urease (MW, 480,000; P-L Biochemicals Inc.), human IgM (MW, 900,000; Calbiochem-Behring Corp.), bovine serum β-lipoprotein (MW, 2,700,000; United States Biochemical Corp.), and hemocyanin from keyhole limpet (MW 3,000,000–7,500,000; Calbiochem-Behring Corp.). All proteins were of reagent grade or better. The protein concentration was determined by the use of Tonein®-TP manufactured by Otsuka Assay Laboratories, Japan [1].

Cells

In this study HTLV-1-carrying MT-4 cells were used. The cells were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml (complete medium) at 37°C in a CO_2 incubator while subcultured twice a week.

Virus and Virus Infection

The HTLV-III_B isolate of HIV was obtained from the culture supernatant of its producer the MOLT-4/HTLV-III_B cells, as described previously [10]. In one specific experiment (table 2), the culture supernatant of MOLT-4/HIV-III_B was centrifuged at 40,000 rpm in an SW41Ti rotor (Beckman) at 4°C for 1 h. The virus pellets produced were then resuspended in pooled human plasma, kindly provided for by the Green Gross Corp., Osaka, Japan. This HIV-spiked plasma was also used as a virus preparation. The virus preparation was filtered using BMM (ΔP, 200 mm Hg; $\dot{\gamma}$, 0–2,200 s^{-1}) with filtrate and filtrand being stored at –80°C until use. In each well of a 24-well plastic tray, 6×10^5 MT-4 cells were suspended in 1.8 ml of complete medium. Then, 0,2 ml of sample solution was added to each well. After incubation, at 37°C in a CO_2 humidified atmosphere, the cells were investigated for HIV-induced CPE and for HIV antigen synthesis. By means of the trypan blue dye exclusion method the CPE was analyzed by measuring the decrease in the number of viable cells. The viral antigen expression was determined by IF as described previously [10].

Plaque Assay

The plaque assay has been fully described previously [9]. All experiments were carried out in triplicate. The detection limit of this method is 3.4 PFU ml^{-1} of the virus titer.

Assay for HIV Antigen

HIV p24 (gag) antigen, in a cell-free culture medium of infected cells, was examined at pre- and postfiltration with BMM by a solid-phase sandwich-type enzyme immunoassay (Abbott Laboratories, North Chicago), according to the manufacturer's instructions.

Electron Microscopy

After filtration with BMM (mean pore size; 30 nm), filters were fixed with a 2% glutaraldehyde/paraformaldehyde mixture in cacodylate buffer (pH 7.4) for 30 min. This was done immediately after the supernatant from HIV-infected cell culture was filtered. After cutting, small pieces of the filters were postfixed with 2% osmic acid for 2 h, dehydrated with a graded acetone, and embedded in an Epon 812. Ultrathin sections were made with an LKB Ultrotome III, using a diamond knife (Diatome). Thereafter they were stained with uranyl acetate and lead citrate and finally examined with a JEOL 200CX transmission electron microscope. Some parts of the filters were also examined by scanning electron microscopy.

Results

Pore Characteristics and Permeability of BMM

The pore structure of BMM can be represented by a compile of several layers, each with a thickness of approximately 0.2 μm, and each showing a network-like struc-

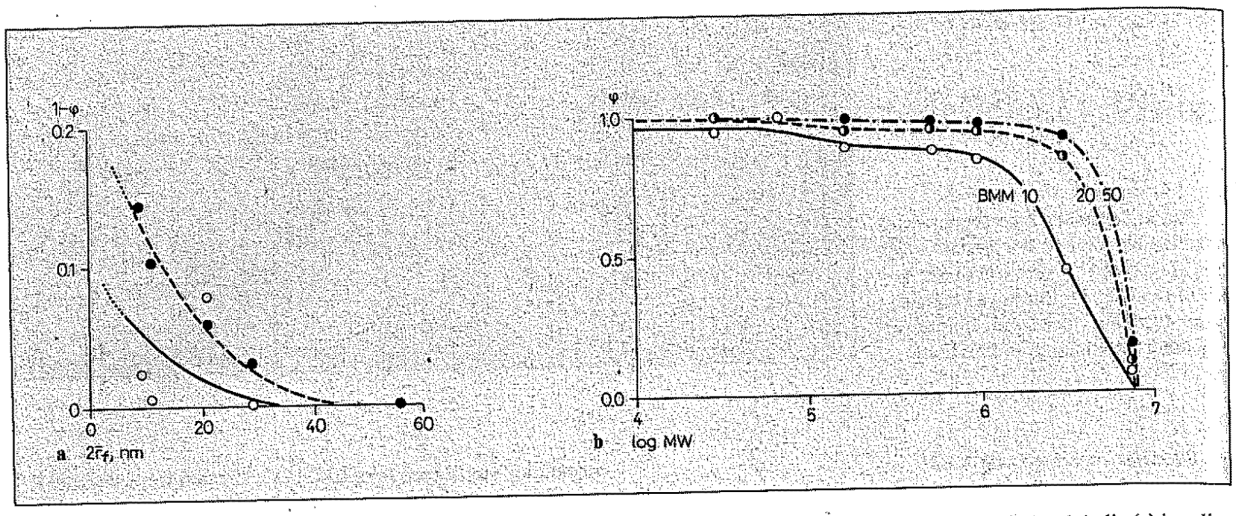

Fig. 2. Permeation properties of BMMs: **a** $2\bar{r}_f$ dependence of rejection coefficient $1-\varphi$ for bovine albumin (○) and γ-globulin (●) in saline water (1 weight%, $\Delta P = 200$ mm Hg). Here, φ was the concentration ratio between filtrate and filtrand. **b** Plots of sieving coefficient φ of the protein molecules in an aqueous solution of a 200 ppm in concentration against their log MW (MW = molecular weight) for BMMs with various mean pore sizes $2\bar{r}f$ ($\dot{\gamma} = 0$ sec^{-1}). Filtration procedure is described in Materials and Methods.

Table 1. Characteristic values and filtration rate of regenerated cellulose hollow fiber (BMM) employed

Sample code	Diameter, μm		Mean pore size $2\bar{r}_f$, nm	Porosity %	Filtration rate (ml m^{-2} h^{-1} mm Hg^{-1})		
	inner	outer			water	culture sup	
					$\dot{\gamma} = 0 \sim 2{,}000$, s^{-1}	$\dot{\gamma} = 0$, s^{-1}	$\dot{\gamma} = 2{,}000$, s^{-1}
BMM 9	327.6	327.8	9.1	50.4	40.0	22	33
BMM 10	279.2	340.8	11.6	54.9	51.8	28	39
BMM 20	272.8	330.2	20.2	48.6	148.9	72	110
BMM 30	257.1	306.3	28.6	43.2	311.8	160	290
BMM 50	274.2	328.2	56.0	51.9	1,306	340	710
BMM 105	206.5	265.1	103.3	52.7	4,218	1,600	3,120

sup = Supernatant.

ture [12]. The rejection coefficient $1-\varphi$ (φ; sieving coefficient) of γ-globulin was larger than that of albumin as shown in figure 2a. The rejection coefficient of protein decreased with protein concentration, ΔP, and $\dot{\gamma}$. The large value of the filtration rate for BMM (table 1) was closely related to poor adsorption of proteins by BMM. As shown in figure 2b, proteins of a molecular weight less than 2.7×10^6 could easily transport through the pores in BMM whose pore sizes were larger than 10 nm. The filtration rate for the culture supernatant was about one half of that for water. This rate was twice as large as commercially available membranes such as the cellulose acetate membrane having the same mean pore size as BMM. This rate decreased in the case of pooled plasma and was about 6 liters/m^2 h for BMM50 under constant ΔP of 100 mm Hg and $\dot{\gamma}$ of 0 s^{-1}. The sieving coefficient of BMM50 for total protein of pooled plasma was more than 95% and decreased with an increase in the filtration volume. When this volume was more than 3 liters/m^2, the decrease was distinguishable.

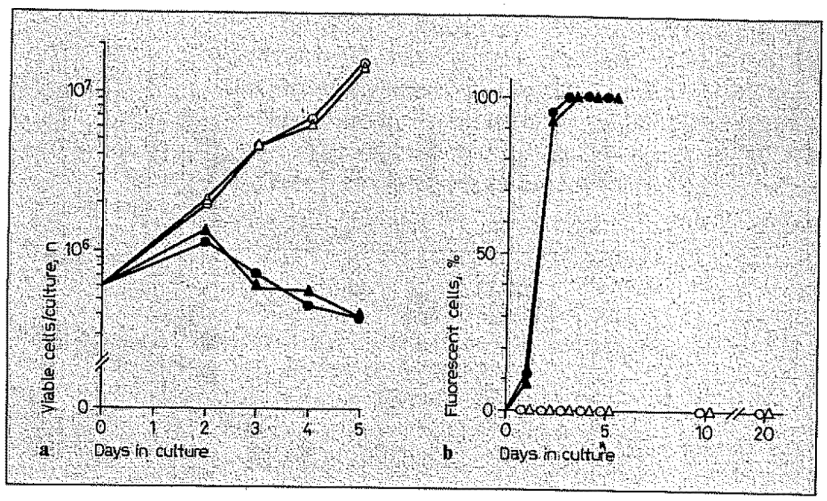

Fig. 3. Effect of the filtration on HIV infectivity detected by CPE (a) and induction of virus-specific antigen (b). MT-4 cells were cultured in a complete medium inoculated with 10% original virus preparation (●), filtrate (△) or filtrand (▲) obtained through BMM having a mean pore size of 105 nm, or without inoculation (○). a Representation of the number of viable cells; b representation of the percentage of fluorescent cells.

Assay for HIV Infectivity

To evaluate the effect of filtration, virus-induced cell damage or CPE was examined in MT-4 cells, by culture in a complete medium containing 10% (vol/vol) of the filtrates or filtrands, for up to 5 days. At the same time, HIV-specific antigens were assessed by IF. MT-4 cells cultured with the filtrate, obtained through BMM whose pore size was 105 nm, were as proliferative as cells cultured in the complete medium, used as a control group (fig. 3a). The viability of such cells exceeded more than 90% throughout the culture. In addition, even 20 days after the culture process IF-positive cells were not detectable at all (fig. 3b). In contrast, as seen in the culture with the original virus preparation, significant growth inhibition was observed in MT-4 cells cultured with the filtrand from the same BMM (fig. 3a). Cell viability also decreased to less than 20% on day 5 after being cultured due to the HIV-induced CPE. Moreover, nearly 100% of the cells were fluorescent as early as day 2 of culture (fig. 3b). Essentially the same results ware obtained with BMMs having mean pore sizes of 50, 30, 20, 10 and 9 nm (data not shown). These results clearly showed that the filtration with BMM caused a complete elimination of HIV infectivity in the filtrates when mean pore size was smaller than 105 nm, as judged by these parameters. On the other hand, HIV in the filtrand seemed to be scarcely inactivated but rather concentrated.

To determine exact virus titer in the filtrates and filtrands, a plaque assay was performed. When the mean pore size of BMM was smaller than 105 nm, no plaque was formed with the filtrates, although the titer of the filtrands showed 10^6 PFU ml^{-1} (table 2). The results of a plaque assay confirmed that HIV particles could not filter through the BMMs whose mean pore size was smaller than 105 nm. When we used pooled human plasma containing 1×10^5 PFU ml^{-1} of HIV in the same study, essentially identical results were obtained (table 2).

Electron Microscopy

The wall of the membrane, constructed of electron-transparent components, revealed a spongy network-like appearance with numerous irregular narrow spaces upon transmission electron microscopy of the cross section perpendicular to the fiber axis of BMM. Upon filtration of the virus preparation a large number of HIV particles were observed through transmission electron microscopy (fig. 4a), in the network of the BMM and on the luminal surface by scanning electron microscopy (figure not shown). However, localization of matured HIV particles were restricted to an area of approximately 5 μm in depth from the luminal surface of the membrane (fig. 4b). Smaller granules suggesting either cores or debris of viruses and cells were observed in the deeper area of the membrane. They rapidly became more infrequent towards the outer wall area. These findings indicated that HIV was completely caught by BMM through the combination of sieving by all layers.

Filtrability of HIV Antigen

It has been shown that there was a considerable amount of HIV antigens which were not associated with the virion in the cell-free culture supernatant [17]. Thus

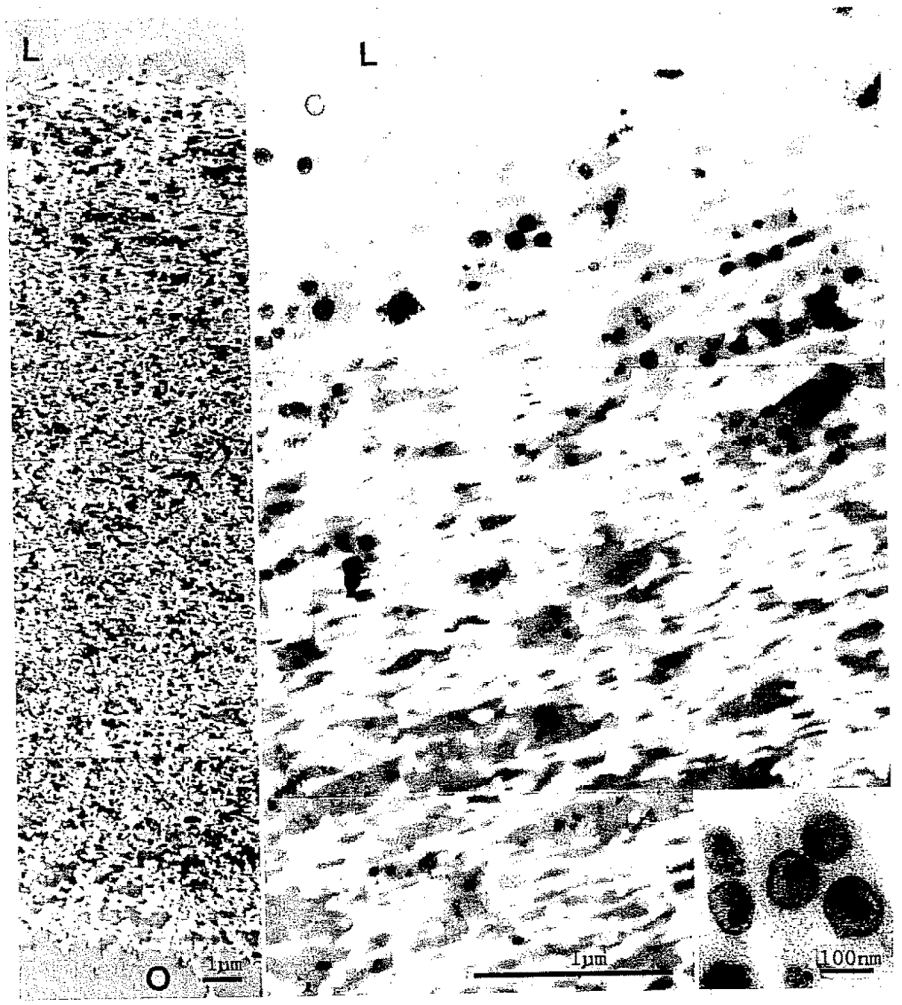

Fig. 4. Transmission electron micrographs of BMM after the virus preparation has been filtered. L = lumen of BMM; O = outside of BMM. Bars show 1 μm in a and b, and 100 nm in c. a A large number of black particles are seen at the inner wall of the membrane. Dark irregular areas of this figure reveal numerous narrow spaces of the membrane filled with the supernatant. × 8,000. b Enlargement of a. Many matured HIV particles are localized in the narrow spaces of the membrane. The distribution of matured HIV is restricted to an area of approximately 5 μm in depth from the luminal surface. Smaller granules, suggesting either cores or debris of viruses and cells, are also observed at the deeper area. × 40,000. c High magnification of HIV caught by BMM. A characteristic structure of HIV demonstrating eccentric or rod-shape cores is seen. ×110,000.

Table 2. Determination of biological activity of HIV by a plaque assay

Mean pore size, nm	Titer[a], PFU ml^{-1}			
	Filtrate		Filtrand[b]	
	culture sup	plasma	culture sup	plasma
105	<3.4	<3.4	$(2.0 \pm 0.85) \times 10^6$	$(1.1 \pm 0.20) \times 10^5$
50	<3.4	<3.4	$(1.6 \pm 0.64) \times 10^6$	$(1.4 \pm 0.22) \times 10^5$
30	<3.4	<3.4	$(2.4 \pm 0.20) \times 10^6$	$(1.2 \pm 0.05) \times 10^5$
20	<3.4	<3.4	$(1.9 \pm 0.20) \times 10^6$	$(1.0 \pm 0.15) \times 10^5$
10	<3.4	<3.4	$(3.0 \pm 0.55) \times 10^6$	NT

NT = Not tested.

[a] Figures represent the means ± SD (n = 3).

[b] The titer of original virus preparation in cell-free culture supernatant (sup) and in plasma was $(3.8 \pm 0.41) \times 10^6$ PFU ml^{-1} and $(1.3 \pm 0.25) \times 10^5$ PFU ml^{-1}, respectively.

Table 3. Filtrability of HIV p24 antigen

Fraction[a]		HIV p24 antigen, %	
		Filtrate[b]	Filtrand
Ultracentrifugation			
Before	(A)	66	100[c]
After			
Supernatant	(B)	54	60
Pellet	(C)	0.3	41
Loss[d]		11.7	−1

[a] Fractionation by ultracentrifugation was done as described in the text.
[b] Filtration procedure with BMM is described in Materials and Methods.
[c] The concentration of p24 in filtrand was 183 ng/ml. The limit of detection of this method is 100 pg/ml.
[d] Loss = A − (B + C).

we examined the HIV antigens in the filtrate of the virus preparation filtrated with BMM (pore size, 50 nm). The filtrate and the filtrand were centrifuged at 100,000 rpm in a TLA-100.2 rotor (Beckman) for 1 h at 4°C in order to separate HIV particles from HIV antigens free of virions. Thereafter the supernatants and the resulting virus pellets, which were resuspended in the same volume of phosphate-buffered saline as that of the initial virus preparation used, were assayed for the HIV p24 antigen.

As shown in table 3, 60% of HIV p24 antigen was present in the virus-free supernatant after ultracentrifugation in the filtrand. Also, 66% of the antigen was filtered through the membrane. In the filtrate almost all amounts of filtered antigen were detected in the supernatant obtained by the ultracentrifugation, whereas only 0.3% of antigen was detected in the resulting precipitate fraction of the filtrate. These results showed that HIV p24 antigen was filtered through BMM as those of other protein molecules employed in this study (fig. 2).

Discussion

With blood and blood products there is a possibility of contamination with various pathogenic agents, either known or unknown. In the present study we used HIV preparations where the virus titer was 10^6 level of PFU ml^{-1}, as a contaminant and a filtration procedure with BMM as the elimination method.

The assay systems [9, 10] employed here for HIV infectivity are based on the observation that MT-4 cells are extremely susceptible to infection with HIV. These cells express virus-specific antigens and release numerous virus progeny, resulting in the death of infected cells. Released virus progenies expand rapidly by infecting neighboring uninfected MT-4 cells in the culture. Therefore, the IF method and the detection of CPE with this infection system seem to be very sensitive. As for quantitation, the plaque assay is superior to and more objective than IF. Using these sensitive assays, we showed that HIV infectivity in the filtrates obtained through BMMs whose pore sizes were smaller than 105 nm was not detectable (fig. 3, table 2). This strongly suggests that, under such conditions, HIV particles are barely filtrable, if not completely unfiltrable. Electron microscopic observation also supported this finding (fig. 4). Moreover, essentially the same results were obtained with pooled human plasma as shown in table 2, which may strengthen the practical importance of our procedures.

It seems curious that HIV, whose particle size is known as approximately 100 nm, cannot pass through BMM having a mean pore size $2\bar{r}_f$ of 105 nm; however, this is due to the multilayer structure of BMM. The total number of layers was estimated by electron microscopy to be 100 or more, so that even in the case that each layer can sieve about 15% of the virus, the rejection coefficient of BMM for the virus is greater than 0.9999991 ($=1-0.85^{100}$). This enables BMM having a mean pore size $2\bar{r}_f$ of 105 nm to filter HIV effectively. Moreover, BMM has a superior ability for catching virus with a high rate of filtration, when compared to other commercially available materials. It should be stressed that several biologically active proteins were filtrable with the BMM without loss of their activities when the mean pore size of BMM was larger than 20 nm. Consequently, BMM may have wide applications in the pharmaceutical field related to the blood industry. This was also the case for HIV antigens showing that some amounts of HIV antigens were apparently detected in the filtrates (table 3). More detailed results (e.g. the nature of the antigens) will be presented in subsequent papers.

Recently, novel human retroviruses, which have been termed HIV-II [5] or HTLV-IV [14], have been isolated and found to be similar to but distinct from HIV. The standard enzyme-linked immunosorbent assay (ELISA) for antibodies to HIV-I was negative for the above 2 viruses. Therefore, blood donation from those who are infected with HIV-II or HTLV-IV cannot be excluded by the screening of HIV-I antibodies using current methods.

Thus, viral inactivation methods, such as heat treatment, should be implemented if applicable to the processing of plasma derivatives. However, if such methods cannot be found or are not available, our filtration method may be very useful. Moreover, the method is also valuable as an additional step to achieve complete elimination of viral infectivity, even if one viral inactivation procedure, such as heat treatment, has already been performed. Since our procedure for elimination of HIV is based on a physical means, it is likely that the filtration method can remove other pathogenic agents of various sizes.

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