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Characterization of an Antiviral Agent Based on Nonionic Surfactants and It's Effects on Human Dermal Fibroblasts

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CHARACTERIZATION OF AN ANTIVIRAL AGENT BASED ON NONIONIC SURFACTANTS AND ITS EFFECTS ON HUMAN DERMAL FIBROBLASTS

by

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ABSTRACT

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The AIDS pandemic has directed various research endeavors towards finding an appropriate method for eliminating all potentially infectious material within bone allografts before implantation into a recipient. To that effect, Panavirocide was conceived by Medicine and Applied Sciences, Inc. This compound, in which three nonionic surfactants serve as the active agents, has been shown to inactivate HIV-1 particles within blood and blood products. Because Panavirocide has never been used with allografts, the purpose of the present research was to characterize the nonionic surfactants for the putative alteration of the formulation by determining the critical micelle concentration values and to determine the effects of Panavirocide on mammalian fibroblasts.

Modification of a protocol proposed by Vulliez-Le Normand and Eiselé (1993) allowed the determination of the critical micelle concentration values. These calculated values of 0.120 ± 0.026 mM for Brij 35, 0.062 ± 0.008 mM for Nonoxynol 9, and 0.234 ± 0.005 mM for Nonidet P40 corresponded quite well with the literature-derived values of 0.092 mM, 0.0812 mM, and 0.29 mM, respectively.

Using *in vitro* proliferation assays, the toxicity level was determined to be between 0.001X and 0.005X, diluted from a 1X stock solution of Panavirocide. Furthermore, as determined through the proliferation assays and by morphological changes, demineralized bone matrix, a commonly used allograft material, treated with Panavirocide did not adversely affect the human dermal fibroblasts used in this study.

DEDICATION

I dedicate this thesis to

Wolf and Wolf Gang

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I truly thank the venerable Dr. Lloyd Wolfinbarger, Jr., who has been my fatherly mentor and dear friend throughout the arduous years in his laboratory. He provided me with constant encouragement, support, laughter, and, when necessary, scoldings that bolstered me in the last three years and helped me to grow not only professionally but also personally. I owe him my first-born.

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INTRODUCTION

ALLOGRAFTS

Bone and connective tissue transplantation is becoming the established method for the repair of osseous defects. Traditionally, autografting, i.e., the transplantation of grafts derived from the same individual, has been the predictably effective protocol with high clinical success. However, autografts are fraught with certain complications that preclude their use in large grafting procedures. These complications include limited availability of graft materials and infections that can result from the secondary surgeries to harvest the autografts. Allografting, transplanting from within the same species, is therefore one of the major means by which bone and soft tissue grafting occurs. Allografts, however, are not exempt from their own set of complications. The grafting of fresh-frozen allografts from human cadavers, the most time-honored method of bone grafting, results in a high proportion of infections and fractures (Friedlaender,

1991; Berrey *et al.*, 1990; Enneking and Mindell, 1991; Stevenson *et al.*, 1991). In addition, fresh allografts tend to promote an immunogenic response within the recipient (Friedlaender, 1987; Friedlaender, 1991; Guo *et al.*, 1991; Horowitz and Friedlaender, 1991; Solomon, 1991; Stevenson *et al.*, 1991). This latter problem may be somewhat mitigated by deep-freezing, freeze-drying, or demineralizing the allograft before implantation (Friedlaender, 1987; Friedlaender, 1991; Guo *et al.*, 1991; Solomon, 1991; Stevenson *et al.*, 1991; Virolainen *et al.*, 1993). Furthermore, Berrey et al. (1990) determined that the incidence of allograft fractures may not differ significantly from autograft or synthetic metallic implant fractures. Possibly the most significant problem concerning the use of allografts is the potential transmission of blood-borne pathogens, particularly the human immunodeficiency virus (HIV) (Centers for Disease Control, 1988).

Despite the problematic aspects of bone grafting, an increasing number of bone and connective tissues are being transplanted. Within a span of about four years, the amount

of soft tissue and bone transplantation increased from 200,000 to approximately 500,000 currently (Friedlaender, 1991; Prewett *et al.*, 1991; Horowitz and Friedlaender, 1991; LifeNet, personal communication, 1994). The large amount of bone and soft tissue grafting in conjunction with a growing concern over the transmission of blood-borne pathogens has led to the consideration of synthetic implants as a viable and attractive alternative to allografts. However, allografts possess certain unique characteristics that are missing in these synthetic implants, including their natural shape and osteoinductive capabilities.

The inherent advantages of natural allografts have led researchers to explore the means of inactivating all potentially infectious material within these allografts before implantation into a recipient. Due to the AIDS pandemic, interest has focused on HIV and the ways of preventing its transmission via bone allografts.

HUMAN IMMUNODEFICIENCY VIRUS

1. Characterization

The human immunodeficiency virus (HIV) is a retrovirus that is the causative agent of the terminal illness known as acquired immunodeficiency syndrome (AIDS). Retroviruses are divided into three subfamilies -- lentiviruses, oncoviruses, and spumaviruses (Dalgleish and Weiss, 1990; Hehlmann, 1991; Roth, 1989). HIV belongs to the first subclassification and is closely related to the simian immunodeficiency virus (SIV) whose reverse transcriptase is Mg^{2+}, rather than Mn^{2+}, dependent (Gonda and Oberste, 1993; Gonda and Weislow, 1993; Myers *et al.*, 1993; Roth, 1989).

Two distinct HIV viruses have been isolated. HIV type 1 (HIV-1) is the typical virus that is associated with the advent of the characteristic AIDS symptoms. HIV type 2 (HIV-2) is less pathogenic (Hehlmann, 1991) and is seldom correlated with the manifestation of AIDS (Gonda and Oberste, 1993). Furthermore, HIV-2 is more closely related in amino

acid sequence to SIV (Gonda and Weislow, 1993; Roth, 1989).

HIV-1 is spherical in shape with a glycosylated outer membrane derived from the host cell (Gonda and Weislow, 1993). The glycoprotein gp120 is anchored to the bilayer lipid envelope via another glycoprotein, gp41, and also interacts with CD4 receptors on host cells to initiate HIV infection (Cohen, 1993b; Gonda and Oberste, 1993; Roth, 1989). The central bar- or cone-shaped capsid, composed mainly of the protein p24 (which can serve as a surrogate marker for the progression of AIDS (Lefrère *et al.*, 1992)), encloses the nucleic acid core (Fyfe *et al.*, 1991; Gonda and Oberste, 1993; Gonda and Weislow, 1993; Hehlmann, 1991; Wang and Barklis, 1993). Many proteins, including the reverse transcriptase, are encapsulated within the capsid and may be associated with the genomic RNA (Gonda and Oberste, 1993; Roth, 1989).

Attachment of gp120 to the CD4 differentiation antigen of T helper lymphocytes permits entry of HIV into the cell and initiates infection (Gonda and Weislow, 1993; Hehlmann, 1991). HIV penetrates the cell via receptor-mediated endocytosis in

which a clathrin-coated pit is formed (Gonda and Oberste, 1993; Roth, 1989). The single-stranded genomic RNA is reverse-transcribed within the cytoplasm of the host cell into double-stranded DNA, which is termed the provirus. The provirus then integrates into the host DNA where it can remain latent for an extended period of time (Gonda and Oberste, 1993). This dormant phase may be broken by various means, including infection by other viruses and normal growth factors secreted by white blood cells (Roth, 1989). Virus particles are assembled near the host plasma membrane and are released by a budding process (Gonda and Oberste, 1993; Gonda and Weislow, 1993; Wang and Barklis, 1993). The immature core structures develop within the extracellular environment, a process which may be essential for complete HIV infectivity (Gonda and Oberste, 1993).

2. Cell types infected by HIV

HIV preferentially infects memory helper T lymphocytes and macrophages via the CD4 cell surface antigen. Microglia and alveolar macrophages, which are macrophage-type cells

within the brain and lung, respectively, can also harbor the virus (Gonda and Oberste, 1993; Roth, 1989; Weiss, 1993). Furthermore, many $CD4^-$ cell types are susceptible to HIV infection (Dalgleish and Weiss, 1990). Galactocerebroside serves as the portal of entry in the $CD4^-$ astroglial, neuronal, and oligodendroglial cells from the central nervous system (Weiss, 1993). Other $CD4^-$ cells that can support HIV replication include endothelial cells, which line the capillaries within the brain, and epithelial cells (Hehlmann, 1991; Roth, 1989; Weiss, 1993).

Massive viral loads within the lymph nodes are sustained from initial infection to the appearance of AIDS. Indeed, follicular dendritic cells are associated with active viral replication. Furthermore, Langerhans cells, which are the immune cells within the skin and mucous membranes, and the related blood dendritic cells, which are distinct from follicular dendritic cells, may be able to support HIV replication (Roth, 1989; Weiss, 1993).

In short, many cell types are susceptible to HIV

infection. As long as HIV retains its mutability, the possibility that other cell types and tissues will be infected cannot be dismissed.

3. Deleterious effects of HIV on target tissues

HIV may damage the infected cells in many ways. One way is by directly killing the cell that it invades, i.e., the infection is cytopathic (Cohen, 1993b; Roth, 1989; Weiss, 1993). In addition, virus-infected cells can trigger an immune response which results in their destruction (Roth, 1989). Functions of these infected cells can become aberrant and result in interference with normal immunological activity (Weiss, 1993).

HIV may also be detrimental to uninfected cells. Certain HIV variants cause the host cells to form syncytia, or fuse, with uninfected neighboring cells (Cohen, 1993b; Gougeon and Montagnier, 1993; Hehlmann, 1991; Roth, 1989). Prevalence of the syncytium-inducing variant is thought to be an one of the

catalysts for the actual onset of the AIDS symptoms.

Helper $CD4^+$ cells may be destroyed via the abnormal activation of a cell death program. Intrathymic T cells undergo apoptosis, or programmed cell death, which is a normal phenomenon in the development of the immune system (Gougeon and Montagnier, 1993). However, HIV infection can induce anomalous apoptosis in infected cells and also in uninfected cells (Cohen, 1993b; Gougeon and Montagnier, 1993).

4. Methods to prevent the spread of HIV

A very important means by which HIV transmission can be impeded is in the modification of sexual behavior (Dalgleish and Weiss, 1990; Merson, 1993). This behavioral change should be effected by the implementation of intervention and prevention programs that include education about this disease.

Vaccines, particularly those that activate the cell-mediated portion of the immune system, have been suggested (Dalgleish and Weiss, 1990; Fyfe *et al.*, 1991; Haynes, 1993;

Hehlmann, 1991; Kurstak *et al.*, 1993; Merson, 1993; Roth, 1989; Salk *et al.*, 1993). Cell-mediated, rather than antibody-mediated, responses appear to be essential for restraining HIV infection (Salk *et al.*, 1993). Therefore, induction of cell-mediated immunity may control or prevent the viral epidemic.

HIV replication may be prevented by interrupting various stages in the virus' life cycle with therapeutic drugs. One important target is the viral reverse transcriptase. The agents 3'-azidothymidine (AZT), 2',3'-dideoxyinosine (ddI), and 2',3'-dideoxycytidine (ddC) disable the virus at this step (Cohen, 1993a; Dalgleish and Weiss, 1990; Gonda and Weislow, 1993; Hehlmann, 1991; Johnston and Hoth, 1993; Roth, 1989; Ruprecht *et al.*, 1992; Valentine *et al.*, 1992; Yarchoan *et al.*, 1989). Because the HIV protease splices a large pro-protein into viral components that are essential, damage to this enzyme could result in new viral particles that are noninfectious and malformed (Gonda and Weislow, 1993; Johnston and Hoth, 1993). Numerous other points in HIV's life cycle are being considered as potential replication control targets.

These include viral attachment, RNA packaging and virion construction, and the release of the virus (Cohen, 1993a; Johnston and Hoth, 1993).

PROTOCOLS TO INACTIVATE OR REMOVE HIV

Many methods that inactivate and/or remove viruses have been proposed and studied (AuBuchon and Dodd, 1992; Horowitz *et al.*, 1992; Kndryk *et al.*, 1992; Mellonig *et al.*, 1992; Prewett *et al.*, 1991; Rawal *et al.*, 1990; Salzman *et al.*, 1993; Sharma *et al.*, 1990). Horowitz et al. (1992) and other laboratories (AuBuchon and Dodd, 1992) demonstrated that a combined organic solvent and detergent treatment effectively inactivates HIV, the hepatitis B virus, and the hepatitis C virus. This treatment was performed on fresh frozen plasma with 1% tri(n-butyl) phosphate as the organic solvent and 1% detergent, typically Triton X-100, Tween 80, or sodium cholate, for 4 hours at 30° C. In fact, the methods used to remove the added reagents also remove additional virus. Because it was designed to inactivate viruses while maintaining protein integrity, this protocol is compatible

with viral disinfection of blood and blood products.

Normal tissue processing techniques used in the preparation of demineralized bone may also serve to inactivate viruses (Prewett *et al.*, 1991; Salzman *et al.*, 1993). For example, ethanol may be virucidal towards tissue-borne HIV and has already been demonstrated to penetrate cortical bone (Salzman *et al.*, 1991). Furthermore, the decalcification steps themselves (i.e., hydrochloric acid) are frequently assumed to destroy HIV as well as other viruses. Strongly acidic solutions are known to disrupt phosphodiester bonds within nucleic acids (Prewett *et al.*, 1991); therefore, RNA, the genetic material in the retrovirus HIV, would be affected.

Another method which has been proven to inactivate HIV is the preliminary procedure used in processing bone allografts (Salzman *et al.*, 1993). It involves the removal of the allograft's adventitious material followed by two cycles of freeze-thawing. Indeed, other studies determined that if the virus-laden plasma is first frozen, the amount of virus that can be successfully isolated in culture decreases from 50% to

5 - 10%. However, saline and freeze-drying do not inactivate the HIV (Buck *et al.*, 1989; Salzman *et al.*, 1993).

The first protocol, the combined organic solvent/detergent treatment, is an example of a method developed primarily for disinfection of plasma. Filtration techniques, which remove the vector leukocytes, are examples of other methods used for blood and blood products (AuBuchon and Dodd, 1992; Rawal *et al.*, 1990). The latter two methods are used in conventional allograft processing protocols.

A method designed specifically for the inactivation of viruses within allografts employs ethylene oxide (Friedlaender, 1987) and heat to render HIV noninfectious (Eastlund *et al.*, 1989). Because the use of ethylene oxide and heat involves wholesale cellular destruction, these agents would not be appropriate for the inactivation of viruses in plasma. In one particular experiment, Eastlund *et al.* (1989) spiked the medullary canals of human femur shafts with HIV and then treated them with ethylene oxide at 37° C for 250 minutes (4 hours, 10 minutes) or with heat, 56° C, for 5 hours. The

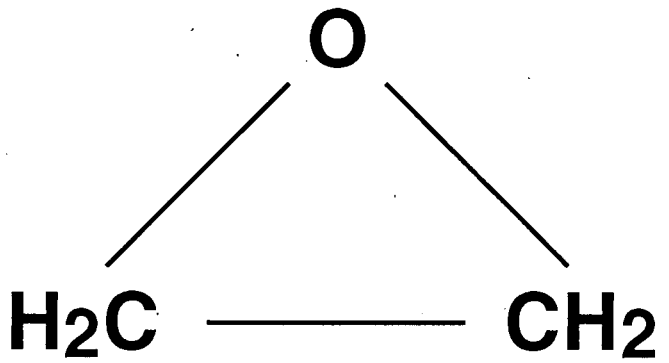
controls were held at 35° C. These procedures were found to inactivate the virus within the dense cortical bone.

Ethylene oxide (figure 1) is a highly irritating gas that can cause pulmonary edema. It is used in many capacities, including the fumigation of textiles and foodstuff, in the sterilization of surgical devices, as an agricultural fungicide, and as the starting material for the synthesis of nonionic surfactants (The Merck Index, 1976).

DETERGENTS

Soluble amphiphiles are phase-dependent polymorphic molecules which exhibit unique behavior at the interface of two immiscible substances (Helenius and Simons, 1975; Mukerjee and Mysels, 1970; Neugebauer, 1994; Neugebauer, 1990; Vulliez-Le Normand and Eiselé, 1993). Thus, these compounds are also known as surface active agents, or surfactants, and more commonly, as detergents. Detergents affect physical interfacial characteristics by reducing the surface tension of a solution (Helenius and Simons, 1975; Mukerjee and Mysels,

Figure 1. Structure of ethylene oxide.


O
H2C
CH2

1970; Neugebauer, 1994). This phenomenon is mediated by the inherent amphipathic nature of detergents. Their hydrophilic and hydrophobic moieties direct the alignment of these detergents into the most energetically favorable position, with the hydrophilic head in the aqueous phase and the hydrophobic tail in the air or the hydrocarbon phase. The detergent molecules in the resultant monolayer at the interface are in equilibrium with the detergent monomers in the bulk solution (Helenius and Simons, 1975; Neugebauer, 1994).

At very low concentrations, detergents exist in solution as these monomers. However, beyond a defined and narrow concentration range, the monomers spontaneously aggregate to form colloidal units called micelles. Micelles are globular structures in which the hydrophilic heads of the individual monomers form a cortex around the hydrophobic tail core. This transitional concentration of detergent is termed the critical micelle concentration (CMC). Typically, a higher CMC value denotes a broader concentration range at which this transformation occurs (Helenius and Simons, 1975; Neugebauer,

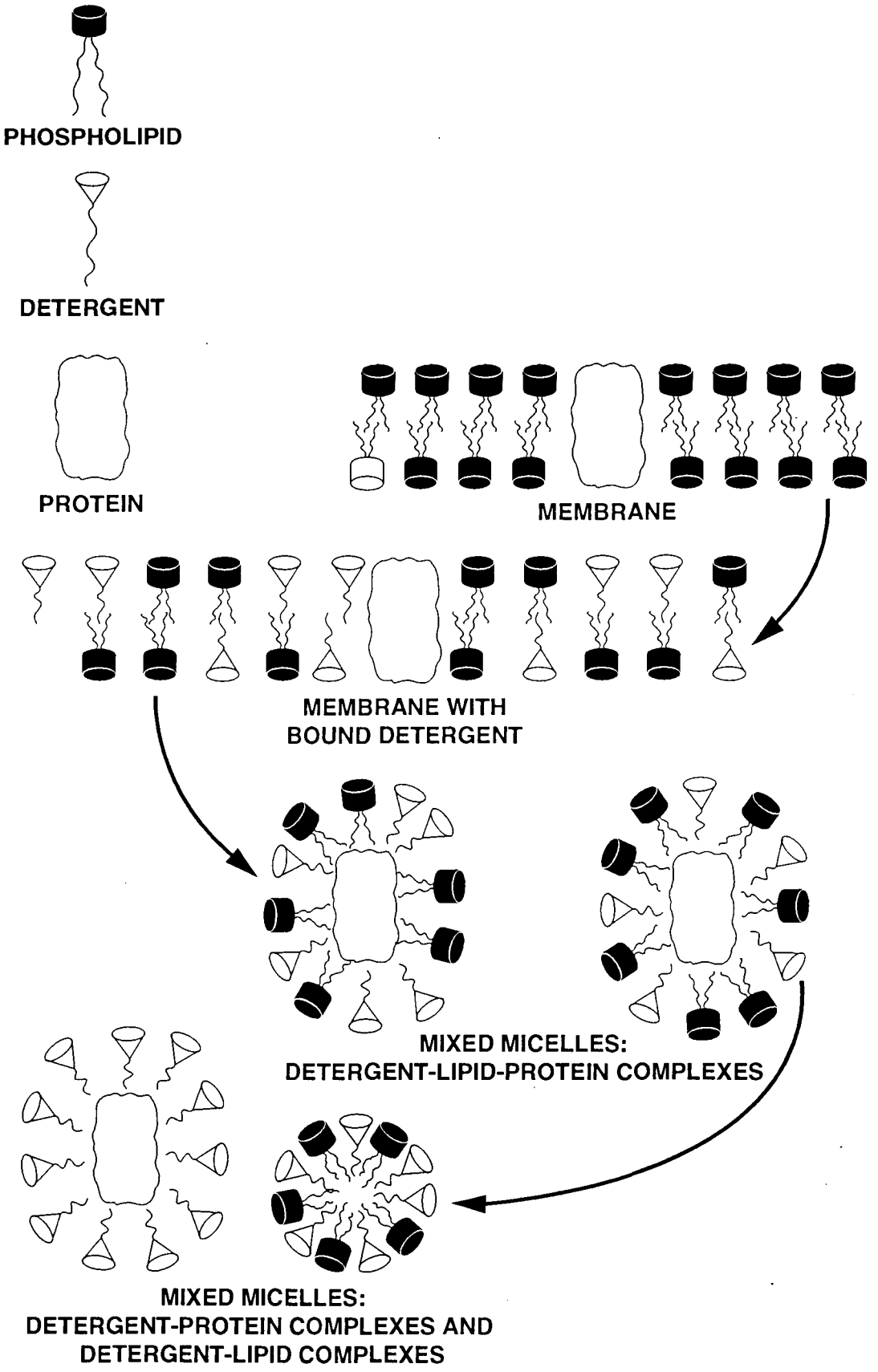
1994). Above the CMC, any additional detergent is incorporated into the micelles; therefore, the monomeric concentration remains generally stable. The established equilibrium between the constituent monomers and micelles is dynamic rather than static; monomers are constantly transferred from the bulk solution to the micelles and vice versa. Furthermore, the CMC value varies depending on the detergent. Indeed, several extraneous factors may contribute to the alteration of the CMC. These factors include temperature, ionic strength, pH, and the presence of impurities (Helenius and Simons, 1975; Hjelmeland, 1990; Neugebauer, 1994; Neugebauer, 1990).

Information about the CMC value of a specific surfactant provides a practical means for determining the minimum detergent concentration that is necessary for the solubilization of a hydrophobic entity, such as a membrane protein or the membrane itself. In a purely lipid system, solubilization can be defined as the formation of mixed micelles. However, the complexity of membranes precludes the use of this simplistic definition. In its most basic terms,

solubilization of a membrane proceeds in four relatively distinct steps. First, the detergent attaches to and integrates into the membrane. The lamellar integrity of the membrane breaks down and detergent-lipid-protein complexes are formed. Finally, these complexes are further solubilized into detergent-protein and detergent-lipid aggregates (Helenius and Simons, 1975; Hjelmeland, 1990; Neugebauer, 1994) (figure 2). In the initial steps of solubilization, detergents increase the permeability of cellular membranes (Weisman *et al.*, 1989).

The CMCs of numerous detergents have been determined through a variety of methods which may be divided into two broad categories, those that require an additive and those that do not (Mukerjee and Mysels, 1970). A popular protocol that does not require the addition of any substance involves the measurement of surface tension (Helenius and Simons, 1975; Mukerjee and Mysels, 1970; Neugebauer, 1994; Vulliez-Le Normand and Eiselé, 1993). Surfactants depress the surface tension of a solution; therefore, with increasing detergent concentration, the surface tension decreases. Because micelles remain within the bulk solution and do not affect

Figure 2. Solubilization of cell membranes with detergents (Neugebauer, J. *A Guide to the Properties and Uses of Detergents in Biology and Biochemistry*, p. 12. Calbiochem-Novabiochem International, 1994.)


PHOSPHOLIPID
DETERGENT
PROTEIN
MEMBRANE
MEMBRANE WITH
BOUND DETERGENT
MIXED MICELLES:
DETERGENT-LIPID-PROTEIN COMPLEXES
MIXED MICELLES:
DETERGENT-PROTEIN COMPLEXES AND
DETERGENT-LIPID COMPLEXES

surface tension, the values for surface tension diminish to a specific baseline with increasing detergent concentration. The surfactant concentration at the surface tension value that first achieves the baseline is considered the CMC.

Another procedure for calculating CMC is the classical light scattering technique in which the intensity of light passing through a detergent solution is measured at normally a 90° angle to the incident beam (Helenius and Simons, 1975; Mukerjee and Mysels, 1970; Neugebauer, 1994). In this method, the turbidity of the detergent solution is measured, with turbidity increasing with elevated detergent concentrations. As in the surface tension measurements, a graph of observed data vs. detergent concentration exhibits two slopes. The point of intersection from the regression analyses of these two lines represents the CMC.

Changes in the spectral signatures of dyes and measurements of interfacial tension are also used to provide CMC values (Mukerjee and Mysels, 1970; Neugebauer, 1994; Vulliez-Le Normand and Eiselé, 1993). Both methods are

examples of procedures that involve the use of an additive. The former is based on the concept that the spectral characteristics of a dye that is added to a detergent solution are extremely different in the area above and below the CMC value. The latter technique requires the addition of an immiscible liquid to the detergent solution. The interfacial tension between the two liquids is then measured.

Numerous other techniques exist to measure the CMC of a surfactant, and each of them, including those mentioned above, possesses its own set of drawbacks. Many of the methods are tedious and/or rely on subjective analysis of the data, which may lead to variations in CMC values depending on the individual conducting the research. Furthermore, some techniques require unavailable and nonstandard laboratory equipment.

In view of these difficulties, Vulliez-Le Normand and Eiselé (1993) proposed an alternate technique that is a modification of the dye solubilization procedures. Their protocol is based on the principle that hydrophobic dyes are

only solubilized by detergent solutions containing micelles. The amount of dye solubilized is therefore directly correlated with the concentration of micelles. Dye intensity is determined on a standard spectrophotometer or on an ELISA plate reader.

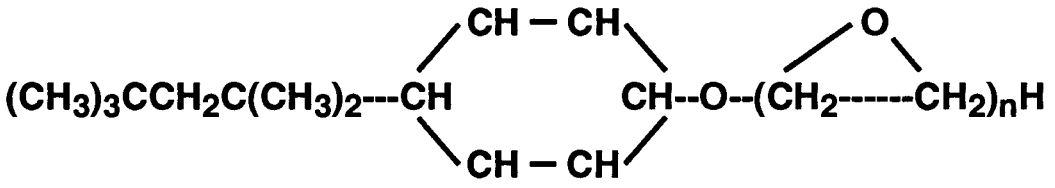
PANAVIROCIDE

Panavirocide (PV) is a liquid panoply of various nonionic detergents that has been shown to inactivate HIV-1 particles. It was originally designed to inactivate HIV and other viruses in blood and blood products. PV has also been incorporated in various cleansers to be used as a skin and surface disinfectant (Sharma *et al.*, 1990).

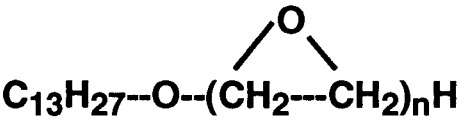
The main active ingredients in PV are the three nonionic detergents polyoxyethylene-p-t-octylphenol (Nonidet P40), polyoxyethylene 2,3-lauryl ether (Brij 35), and nonylphenolethoxylated polyethylene glycol (Nonoxynol 9) (figure 3). These polyoxyethylene amphiphiles are derived from ethylene oxide, a toxic compound which has been shown to

Figure 3. Structures of the three nonionic detergents in PV.
a) Nonidet P40 b) Brij 35 c) Nonoxynol 9

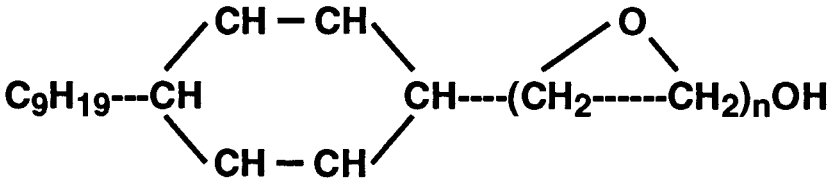
a.



b.



c.



inactivate HIV-1 particles as measured by various parameters of HIV infection (Sharma *et al.*, 1990).

The objectives of the present study were i) to characterize PV by refining the method proposed by Vulliez-Le Normand and Eiselé for determining CMC and to establish its routine use in the laboratory, ii) to determine the toxic effects of PV towards mammalian cells using *in vitro* proliferation assays, and iii) to determine the toxic effects of PV-disinfected demineralized bone matrix towards mammalian cells as defined by *in vitro* proliferation assays and by morphological changes.

The ultimate goals are to delineate the concentration of PV and of its active components with which to flush bone allografts used in specific tissue transplant operations and to determine the innocuous residual detergent levels that may remain within the allografts.

MATERIALS AND METHODS

A. Determination of critical micelle concentration values for detergents used in the study

Hydrophobic dye was obtained from the Sanford Corporation (2711 Washington Boulevard, Bellwood, IL 60104) which produces waterproof pens. The blue ink is normally available in their permanent fine point overhead projector pen K-21, ink code 03. The ink was not extracted from the pen with 50 ml ethanol as per Vulliez-Le Normand and Eiselé (1993). Rather, the ink, stock number 02103, was acquired, in bulk, directly from the Sanford Corporation.

Aliquots of 100 - 250 μl of ink were placed in microcentrifuge tubes. The pipet tip was placed at the bottom point of the tube, and the pipet was gently and slowly depressed so that the ink was evenly distributed at the bottom of the tube. Care was taken to prevent splattering of the ink at the microcentrifuge tube opening. The ink was evaporated

at room temperature, between 24 - 26° C, for at least 3 - 4 days. Nonidet P40 (Sigma, St. Louis, MO), Brij 35 (Sigma, St. Louis, MO), and Nonoxynol 9 (Rhone-Poulenc, Cranbury, NJ) were prepared fresh in 20 mM Tris-HCl with 0.1 M NaCl, pH 8.0 at room temperature, and used within 48 hours (the buffer was prepared in deionized water). In the 250 μl evaporated ink tubes, aliquots of 500 μl of detergent solution or buffer without detergent were added. For the 100 and 150 μl ink tubes, 250 μl samples were added. The detergent solutions were incubated in the presence of the ink film for approximately 16 hours. The tubes were vortexed twice -- once after adding the sample and again before transferring a portion of the sample to an ELISA plate. After the incubation period, 60 μl of the sample were added to an ELISA plate. The solution within the microcentrifuge tube was drawn up slowly to prevent particulate matter from being taken up as well. Furthermore, care was taken to prevent the pipet tip from scraping the inside wall of the microcentrifuge tube which might result in ink fragments that would affect subsequent optical density readings (figure 5).

A spectral scan was performed on the ink in order to determine the optimal wavelength with which to measure micelle formation. A drop of ink was placed in the Tris buffer used in the experiment. The blanks contained the buffer alone. The spectral scan was performed on a Shimadzu UV160U spectrophotometer (Kyoto, Japan) from 280 to 800 nm in 10 nm increments. The results revealed that the absorbance peaked at about 616 nm (figure 4). The Bio-Tek EL 312 ELISA reader (Bio-Tek Instruments, Winooski, VT) that was available to our laboratory contained two absorbance filters that were potential wavelength choices -- 595 nm and 650 nm. Because the absorbance values were higher at 595 nm, this lower wavelength appeared to be better suited for the study (figures 4 and 5). Two controls of buffer alone were also pipetted into the plate.

B. Initiation and maintenance of human dermal fibroblasts

Biopsy samples of full-thickness skin, provided by LifeNet Transplant Services, were procured from an adult human organ donor (identified only by the LifeNet code of 92-792).

Figure 4. Spectral scan of the bulk ink obtained from the Sanford Corporation. A drop of ink was mixed with the Tris buffer that was used in subsequent CMC calculation experiments. The absorbance peaked at about 616 nm as per Vulliez-Le Normand and Eiselé (1993).

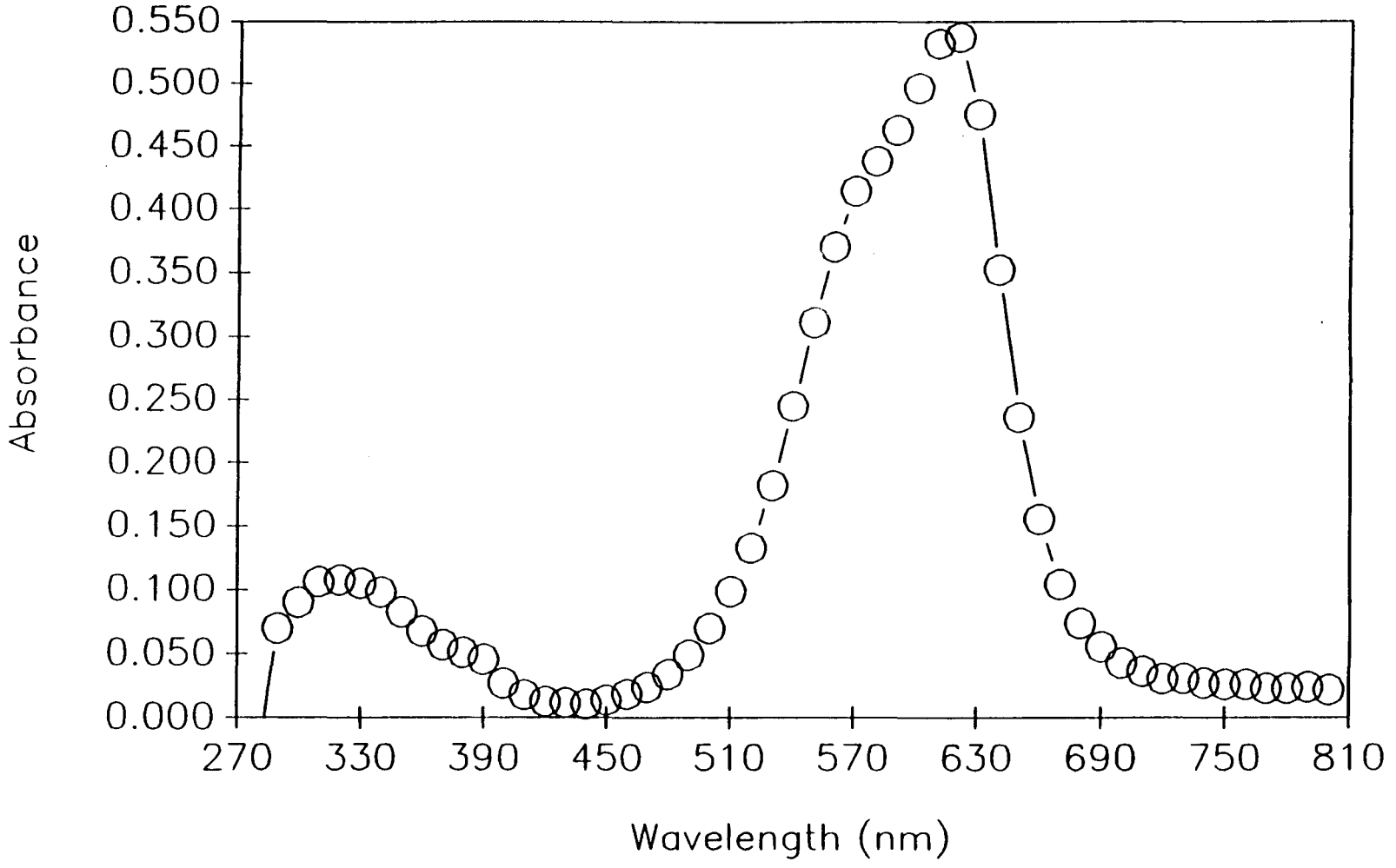
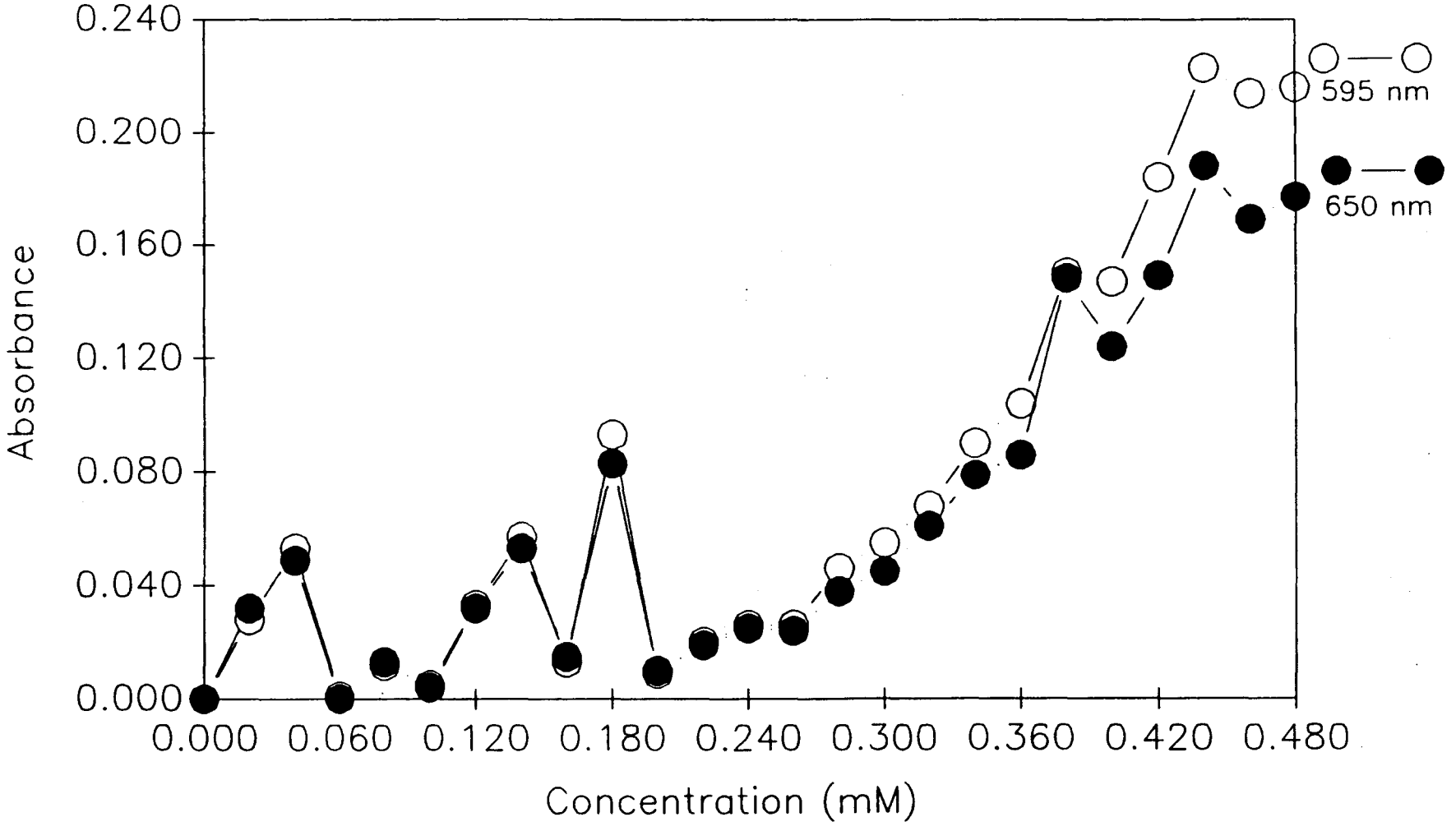

0.550
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
Absorbance
270
330
390
450
510
570
630
690
750
810
Wavelength (nm)

Figure 5. Optical densities of Nonidet P40 solubilized dye from 0.02 mM to 0.48 mM as determined at two wavelengths, 595 nm and 650 nm. Detection of a blue color indicates initial formation of micelles. Color was not visually noticeable until about 0.24 mM. The erratic absorbance patterns at the lower concentrations are due to slivers of ink that affected the readings.


0.240
0.200
0.160
0.120
0.080
0.040
0.000
Absorbance
0.000 0.060 0.120 0.180 0.240 0.300 0.360 0.420 0.480
Concentration (mM)
595 nm
650 nm

The tissue was transported on ice from LifeNet in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with penicillin and streptomycin, final concentration equaling 400 units/ml and 80 μg/ml, respectively, and used for culture initiation within 24 hours of procurement. Once within the laboratory, the tissue was rinsed 2 - 3 times with fresh alpha-modified MEM supplemented with 10% (v:v) fetal bovine serum (FBS) and a penicillin and streptomycin mixture, final concentration equaling 200 units/ml and 200 μg/ml, respectively. The dermis was dissected aseptically from the epidermis with forceps and scissors and divided into approximately 0.5 cm X 0.5 cm pieces. The pieces of tissue were placed in T-25 tissue culture flasks (Corning, NY) containing 3 ml of the rinse medium. The tissue pieces were allowed to adhere to the flasks, and the flasks were inverted and placed in a 37° C humidified incubator gassed with carbon dioxide to maintain a 5% CO_2 environment. After two days, the flasks were rotated daily so that the tissue pieces were routinely bathed in the antibiotic-containing medium. Once the tissue fragments had attached firmly to the flask surface, the flasks were turned to their normal position. Cells typically migrated out of the

tissue in approximately 2 weeks, whereupon the tissue pieces were discarded in bleach and the medium was changed. The primary cultures were allowed to grow to subconfluence, trypsinized (Sigma, St. Louis, MO), and passaged into T-75 flasks (Corning, NY). Cells were fed every 2 - 3 days with alpha-modified MEM supplemented with 10% FBS and maintained in a 37° C, 5% carbon dioxide humidified incubator. Depending on the cell density, cells in a given flask were subcultured into 2 or 3 flasks, i.e., 1:2 or 1:3, before reaching confluence.

Penicillin and streptomycin, final concentration equaling 200 units/ml and 200 μg/ml, respectively, were only used in the medium for initiation of primary cultures and on aliquots of cells that were thawed and reestablished in culture after cryopreservation. All procedures were performed under sterile conditions.

C. Trypsinization and cell passage

Cultures were harvested and passaged using 0.05% Trypsin-EDTA (Sigma, St. Louis, MO). Briefly, media from the flasks

were discarded into bleach. The flasks were rinsed with 3 ml of trypsin to remove residual media, specifically the FBS within the media. To T-75 flasks, 7 ml of trypsin were added; to T-25 flasks, 3 ml of trypsin were added. The cultures were incubated with the trypsin and monitored under a light microscope. When the cells had become "rounded" (i.e., when the trypsin had disrupted the cells' protein connections to the flask surface), the flasks were tapped firmly to dislodge the cells. Trypsin incubation times vary with cell line and cell densities. For the human dermal fibroblasts used in this study, the incubation period was from 2 to 4 minutes.

D. Cryopreservation of human dermal fibroblasts

Cells from various passage numbers were cryopreserved and stored at -155° C in order to provide a uniform cell population for use during this study. Flasks of cells were trypsinized with trypsin-EDTA, neutralized with 10% serum-containing alpha-MEM, and the cell suspensions were placed in sterile 15 ml centrifuge tubes. The tubes were centrifuged at 2000 rpm in an IEC table-top centrifuge for 5 minutes at room

temperature. Cell pellets were resuspended in the cryopreservation solution which consisted of 10% FBS and 5% sterile dimethyl sulfoxide (v:v) (Tera Pharmaceuticals, Inc., Buena Park, CA) in alpha-modified MEM. The cell suspension was dispensed as 1 ml aliquots into sterile 2 ml Cryogenic freezing vials (Corning, NY) to a final concentration of approximately 0.5 - 1 million cells per ml. Vials of cells were placed in a styrofoam container and frozen gradually by placing the container in a refrigerator at 4° C for 1 hour, then in a freezer at -20° C for 1 hour, and finally in a freezer at -70° C overnight. Long-term storage of the cells was in a Harris Ultra-Low freezer that was maintained at -155° C. As needed, aliquots of cells were quickly thawed in a 37° C water bath and reestablished in culture, prior to being used, by pipetting the thawed cell suspension into T-75 flasks containing 10 - 15 ml alpha-MEM supplemented with 10% FBS. Cells from passage 7 to passage 9 were used in the experiments.

E. Characterization of human dermal fibroblast cell morphology

The cellular morphologies of fibroblasts in various passages were determined using an Olympus phase contrast light microscope (Tokyo, Japan) at a magnification of 100. Light photomicrographs (ISO 100 and 200) were taken with an Olympus camera (Tokyo, Japan).

F. Preparation of Panavirocide (PV)

Panavirocide (Patent Pending, Medicine and Applied Sciences, Inc.) is a formulation of three nonionic detergents that act as the active ingredients in this product. Depending on specific formulations, glutaraldehyde or sucrose may be present as a membrane stabilizer. For this study, glutaraldehyde was present in the formulation. In a 1X stock solution of PV that is obtained from Medicine and Applied Sciences, Nonidet P40 is present at 0.02% (vol/vol), Nonoxynol 9 is present at 0.02% (vol/vol), Brij 35 is present at 0.066% (vol/vol), and glutaraldehyde is present at 0.01% (vol/vol).

When PV was made in our laboratory, the percentage of each detergent remained constant; however, the detergents were weighed in order to obtain a weight-to-volume, rather than a volume-to-volume, ratio. Glutaraldehyde was added in some experiments and deleted in others. The laboratory-made formulations that were used in each experiment are indicated as appropriate.

G. Post exposure *in vitro* proliferation assays

Cells were seeded at 3000 cells per cm^2, permitted to attach and grow for 11-18 hours, and then incubated in the presence 0.02X, 0.01X, 0.005X, 0.001X, and 0.0005X PV, dissolved in Dulbecco's phosphate buffered saline (DPBS), for 12, 24, 36, and 48 hours. The controls were exposed to a final concentration of 0.02X (2%) DPBS lacking PV in order to mimic the addition of DPBS to cell culture media. The PV and DPBS were added directly to the flasks containing attached cells. After the designated treatment interval, the cell cultures were rinsed 3 times with 3 ml of FBS- and PV-free alpha-modified MEM. Alpha-modified MEM supplemented with 10%

FBS was then added to the flasks such that the flasks that were counted on days 1 and 2 contained 5 ml, the flasks that were counted on days 3 and 4 contained 10 ml, and the flasks that were counted on days 5 and 6 contained 15 ml. Exactly 24 hours later (i.e., day 1), cultures were harvested by trypsinization and the cells counted. Triplicate cultures were counted in this manner every 24 hours for a total of 6 days. Total cell number was plotted against days in cultures. The significant results were normalized to percentage of cells present in the control cultures.

H. Continuous exposure *in vitro* proliferation assays

Cells were seeded at 3000 cells per cm^2 and allowed to attach and grow for 11-18 hours. The volumes of alpha-modified MEM supplemented with 10% FBS within the flasks were such that the flasks that were to be counted on days 1 and 2 contained 5 ml, the flasks that were to be counted on days 3 and 4 contained 10 ml, and the flasks that were to be counted on days 5 and 6 contained 15 ml. After the attachment period and without changing the media, PV dissolved in DPBS was added

to the flasks to a final concentration of 0.02X, 0.01X, 0.005X, 0.001X, and 0.0005X. At 24 hour intervals, triplicate flasks of cells were trypsinized and the cells counted. The cellular counts were plotted against days in culture. The significant results were normalized to percentage of cells present in the control cultures.

I. Assessment of the cytotoxic effects of PV-treated demineralized bone matrix in culture

Demineralized bone matrix (DMB), 250-710 μm particle size range, was exposed to a 1X stock solution of PV lot (1), obtained from Medicine and Applied Sciences, for 30 - 40 hours in 15 ml centrifuge tubes at room temperature. PV was in much greater excess than DMB. Control DMB was exposed to a similar volume of DPBS for the same length of time. The 15 ml tubes were centrifuged for 2 minutes at approximately 2500 rpm at room temperature. The liquid was decanted. A volume of 10 ml of DPBS, which served as the rinse solution, was added to the tubes. The tubes were vortexed well for several seconds and then centrifuged again for 2 minutes at about 2500 rpm at room

temperature. The rinse steps were repeated three times for a total of four rinse cycles.

Human dermal fibroblasts were prepared in the same manner as previously described. Briefly, cells were seeded at 3000 per cm^2 and allowed to attach and grow for 11 - 18 hours. Then 10 μg of the PV- and DPBS-treated bone were added to and mechanically dispersed over each culture flask containing 10 ml alpha-MEM supplemented with 10% FBS. Triplicate flasks were counted daily for four days.

J. Electron microscopic studies

Sterile tissue culture cover slips, 24 x 33 mm in size (Miles Scientific, Naperville, Il), were cut in half and added to multiwell plates (Becton Dickinson and Company, Lincoln Park, NJ). PV-treated DMB was added to the experimental wells. DPBS-treated DMB was added to the control wells. Cells were seeded at 3000 cells per cm^2. The multiwells have a total area of 9.62 cm^2; therefore, a total of 28,860 cells were added in 10 ml α-MEM. The cultures were allowed to grow

to extreme confluence (approximately 2 weeks) with the media changed only once.

The media was aspirated after about 2 weeks, and a primary fixative of 2% paraformaldehyde and 2.5% glutaraldehyde in a 0.1 M cacodylate (CaCO) buffer, pH 7.4, was added. The cells may be exposed to this fixative for 1 - 2 hours at room temperature or overnight at 4° C. The latter option was used. The fixative was discarded the next day and the cells were exposed to the fixative buffer (CaCO, pH 7.4) three times for 10 minutes each time at 4° C. The cultures were then exposed to a secondary fixative consisting of osmium tetroxide in 0.1 M CaCO, pH 7.4. This osmication step may be performed for about 1.5 - 2 hours at 4° C or for 1 hour at room temperature. In this study, the second condition was used. After the secondary fixation, the cells were rinsed 3 times with the osmium tetroxide buffer for 10 minutes each time at 4° C.

The dehydration steps followed with increasing concentrations of ethanol. The specimens were exposed to 30%

ethanol at 4° C for 10 minutes, followed by 50% ethanol at 4° C for 10 minutes, followed by 70% ethanol at 4° C overnight. Alternatively, the specimens may be treated with 70% ethanol for 10 minutes at 4° C. The dehydration steps continued the next day with 95% ethanol for 10 minutes at 4° C, then with two washes of 100% ethanol at 4° C for 10 minutes each time. The final step consisted of three washes with 100% acetone at 4° C for 10 minutes each time. The samples were transported to the critical point dryer in the third acetone wash.

Because the specimens were fragile cell monolayers over bone, the coverslips on which they were growing were wrapped in filter paper and placed in a carrier before being transferred to the bomb in the critical point dryer. After securing the bomb, the dryer was vented strongly for 4 minutes. The container was allowed to fill with liquid carbon dioxide for 4 minutes, then the dryer was vented slowly for 4 more minutes. It was filled and vented for a total of three times for 4 minutes each time. After the third fill phase, the bomb was placed in hot water, between 50 to 60° C. The pressure was monitored until it reached approximately 1500

psi. Finally, it was vented at a rate of 100 psi per minute until the pressure reached 0 psi. This slow rate prevented crenation of the cells. After the pressure dropped to about 500 psi, the dryer was vented more quickly. The outer portion of the bomb was completely dry when it was opened to prevent sample rehydration.

The thoroughly dehydrated samples were mounted on metal studs with double stick carbon tape. They were coated with gold palladium to a thickness of 100 Å in a Polaron sputter coater, model E5200 (BioRad), and then viewed with a Cambridge StereoScan 100 electron microscope (Cambridge, United Kingdom).

K. Statistical analysis

Data points contained a minimum of three replicates. Linear regression analyses were performed on EnerGraphics 3.0 (Enertronics Research, Inc.) in order to determine CMC values. The university SAS statistical program was used to analyze data via a model I analysis of variance (ANOVA) to test for

significance among treatment groups, followed by the Bonferroni or REGWF multiple comparison procedure. Significance was set at $\alpha = 0.05$.

RESULTS

A. Basic morphology

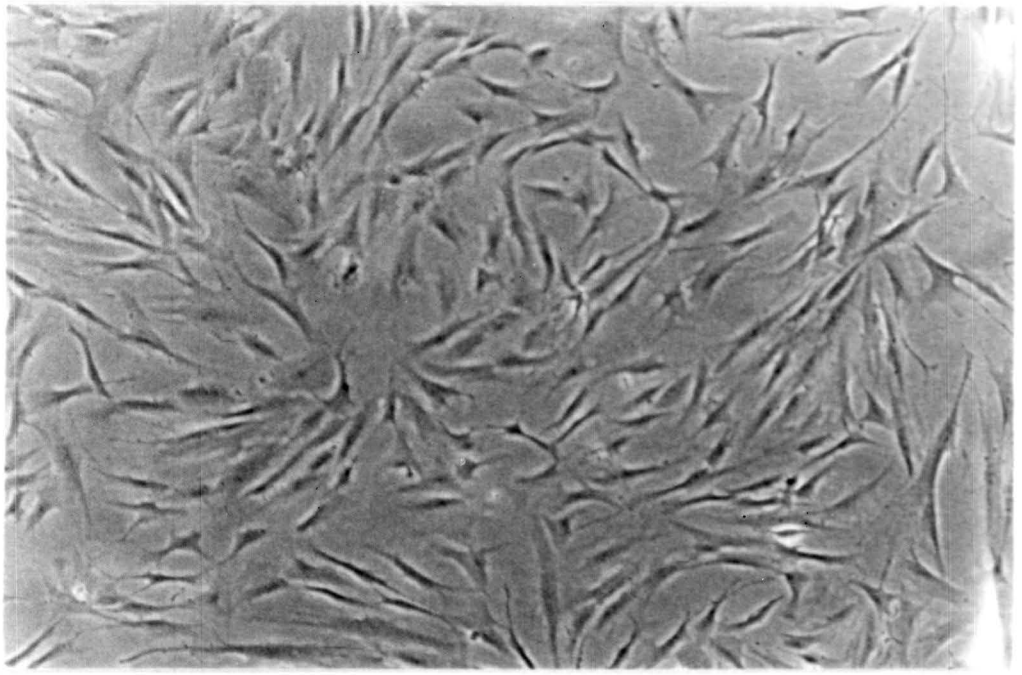
The human dermal fibroblasts used in this study were elongated, spindle-shaped cells that became more granular with subsequent passages. With increasing passage number, these bipolar cells tended to become diffuse in character and gained long, thin processes. The nuclei were typically oval or round in nature. Upon achieving confluence, human dermal fibroblasts aligned in circular and parallel arrays. This characteristic confluence pattern became vague in later passages (figure 6).

B. Determination of critical micelle concentration values

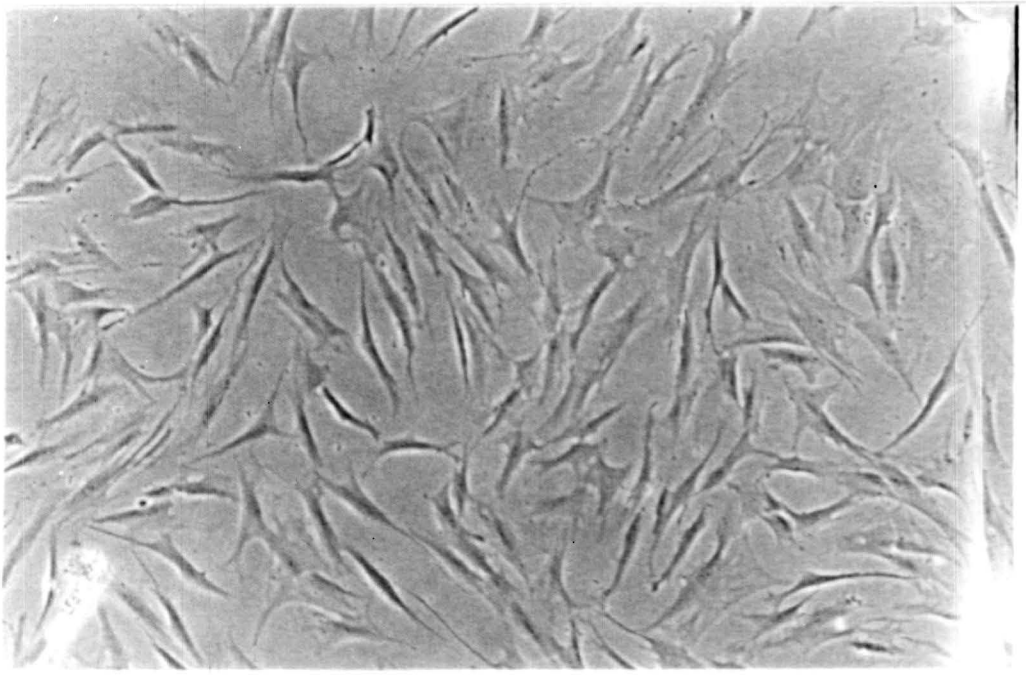
Many of the preliminary assays were performed in an effort to resolve the problem of obtaining the ink in bulk instead of extracting it from the pens. Vulliez-Le Normand and Eiselé performed the assay with 100 μl of evaporated ink

Figure 6. Photomicrographs of subconfluent human dermal fibroblasts cultured in alpha-modified minimum essential media supplemented with 10% FBS. Identical quantities of cells were seeded on the same day and allowed to grow for two days (magnification = 100X). a) Passage 5 b) Passage 7

a.



b.



to 100 μl of sample. However, at these volumes, even with centrifugation, particles of ink were drawn up with the sample extract and interfered with the absorbance readings (figure 5). Using higher volumes of sample and ink while maintaining the one-to-one ratio also created problems. At 250 μl of ink and 250 μl of sample, the color became very dark. Even the lower concentrations of detergent solution appeared to solubilize more dye than was expected. These problems may have been caused by the high dye concentration relative to the sample and/or by an insufficient evaporation period. Due to its concentrated nature, the bulk ink had to be fully evaporated before use in the study. Preliminary studies determined that if 250 μl of ink is used, then 500 μl of sample should be added. The better alternative is to evaporate 100 or 150 μl of ink and then add 250 μl of sample. The lower ink volume required only 3 or 4 days to result in evaporation and drying of the ink. In addition, the larger sample volume should be at least 250 μl to avoid pipetting up ink particles when transferring 60 μl of the sample to the ELISA plate.

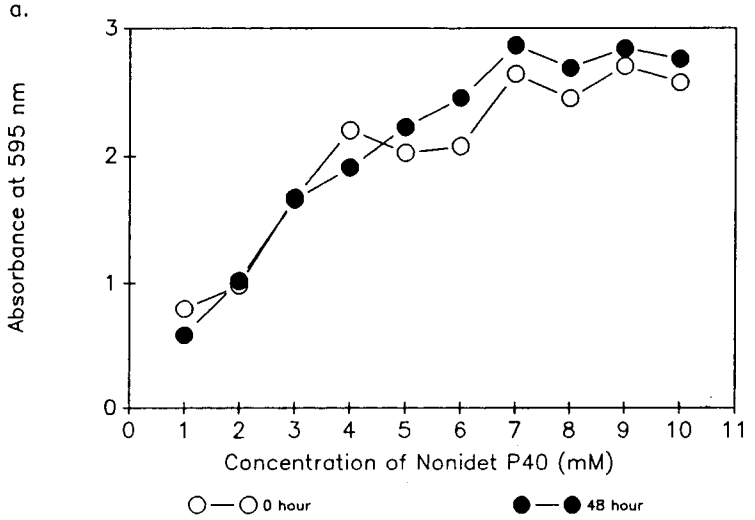
The detergent solutions should be used within 48 hours of preparation. In 48 hours, Nonidet P40, Brij 35, and Nonoxynol 9 did not degrade. Consequently, the absorbance patterns were unaffected (figure 5). Initial results of assays using old detergent solutions, i.e., solutions prepared approximately six months earlier, indicated that the absorbance still may not change after an extended storage period (results not shown).

Preliminary screenings of the detergents from 1 mM to 10 mM implied that the CMCs for all three are below 1 mM (figure 7). Therefore, further screenings were performed between 0.1 mM to 1.0 mM (figure 8). Figure 8a shows the absorbance versus concentration pattern for Nonidet P40. According to this graph, the CMC is between 0.1 mM and 0.2 or 0.3 mM. Brij 35, shown in figure 8b, appears to possess a CMC value between 0 and 0.3 mM. Finally, figure 8c indicates that the CMC value of Nonoxynol 9 is between 0 and 0.1 mM.

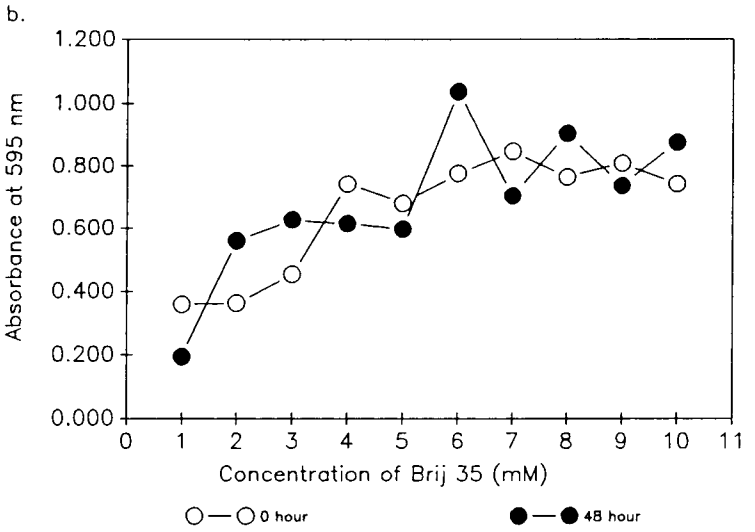
More data were collected within this narrowed range in order to specify the CMC values for the three detergents.

Figure 7. Optical densities of the detergent solubilized dye as a function of detergent concentrations from 1 mM to 10 mM. The results indicate that the CMCs are below 1 mM. Furthermore, the detergent solutions may be used within 48 hours of preparation without compromising the results. The dye and sample volumes were 250 ul and 500 ul, respectively. Analyses of detergents within 1 hour of preparation are represented as "0 hour" and analyses of the detergents 48 hours after preparation are represented as "48 hour".
a) Nonidet P40 b) Brij 35 c) Nonoxynol 9

a.


Absorbance at 595 nm
0
1
2
3
0 1 2 3 4 5 6 7 8 9 10 11
Concentration of Nonidet P40 (mM)
0 hour
48 hour

b.


Absorbance at 595 nm
0.000
0.200
0.400
0.600
0.800
1.000
1.200
0 1 2 3 4 5 6 7 8 9 10 11
Concentration of Brij 35 (mM)
0 hour
48 hour

c.

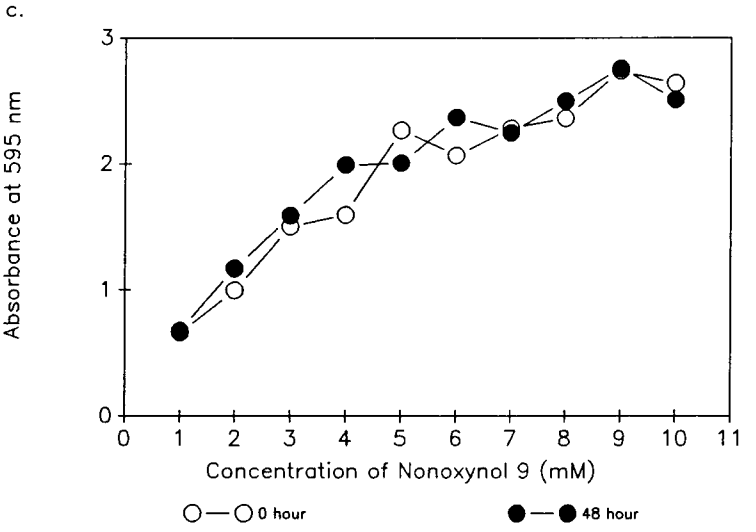
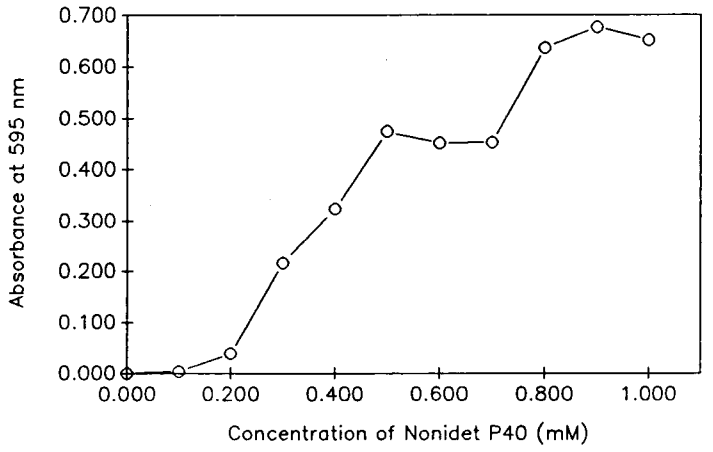
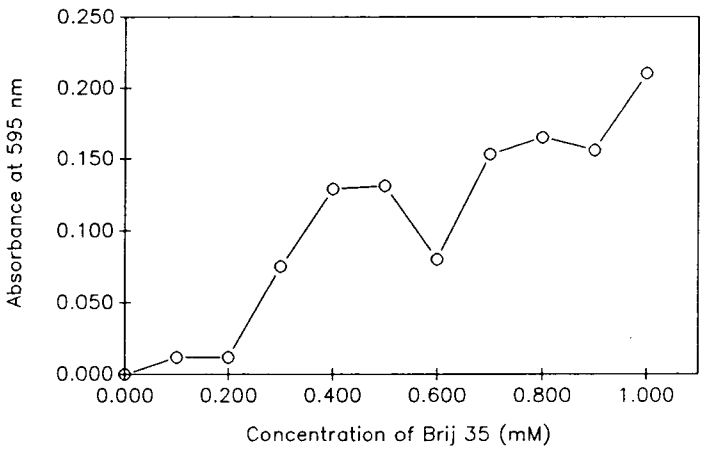

Absorbance at 595 nm
0
1
2
3
0 1 2 3 4 5 6 7 8 9 10 11
Concentration of Nonoxynol 9 (mM)
0 hour
48 hour

Figure 8. Optical densities of the detergent solubilized dye as a function of detergent concentrations from 0.1 mM to 1.0 mM. The dye and sample volumes were 250 ul and 500 ul, respectively. a) Nonidet P40 b) Brij 35 c) Nonoxynol 9

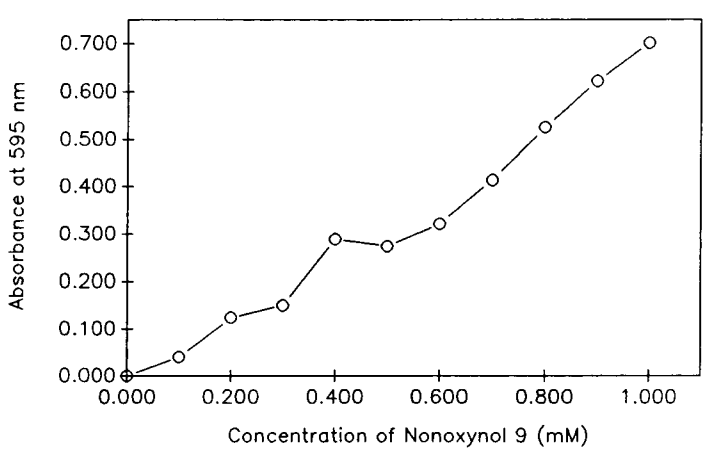
a.


Absorbance at 595 nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0.000
0.200
0.400
0.600
0.800
1.000
Concentration of Nonidet P40 (mM)

b.


Absorbance at 595 nm
0.250
0.200
0.150
0.100
0.050
0.000
0.000
0.200
0.400
0.600
0.800
1.000
Concentration of Brij 35 (mM)

c.


Absorbance at 595 nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0.000
0.200
0.400
0.600
0.800
1.000
Concentration of Nonoxynol 9 (mM)

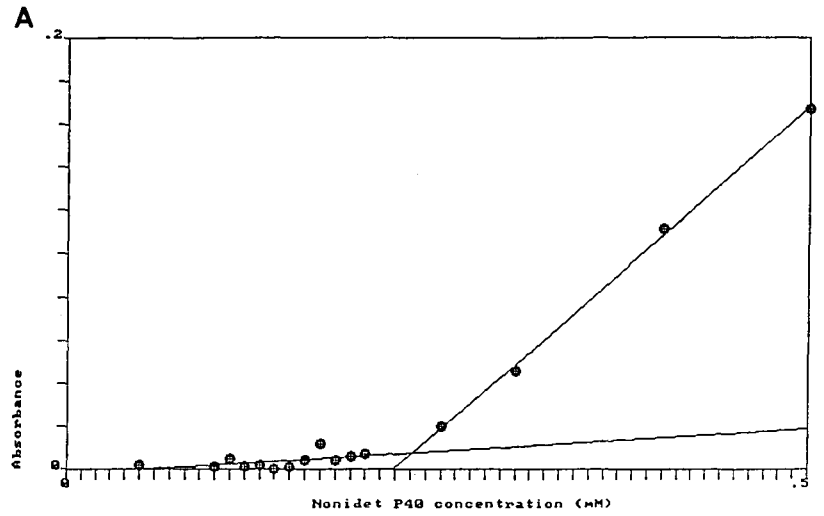
Figures 9 - 11 represent the results. The CMC assay was performed three times for each detergent and the values were calculated from the intersection of the two distinct slopes. The calculated values corresponded quite well with the literature-derived values (table 1). The resolved value of 0.234 mM +/- 0.005 for Nonidet P40 was closer to the literature value of 0.29 mM. The concentrations of the individual detergents in the various PV dilutions were also determined (table 1).

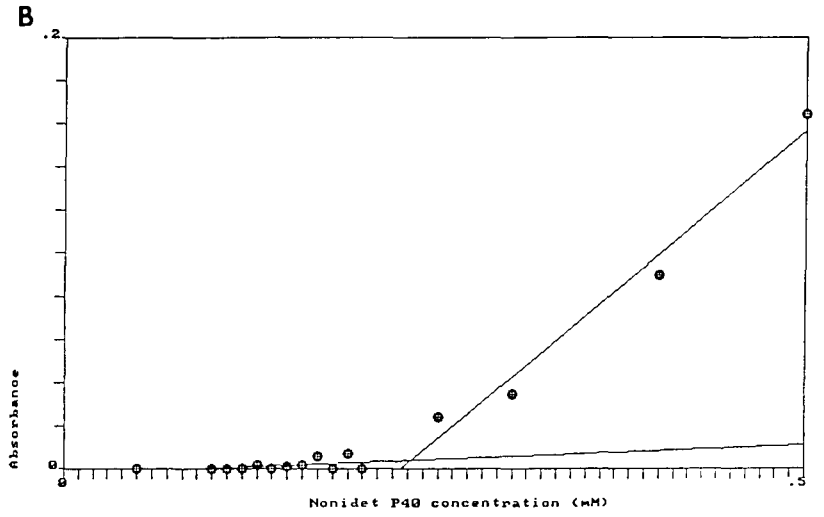
C. Validation of conditions used in cellular proliferation assays

1. The purpose of the first validation assay was to assure that Dulbecco's phosphate buffered saline (DPBS), in which the detergents in PV are dissolved, and the manipulation of the flasks would not affect cellular proliferation, i.e., that PV would be the sole factor responsible for the potentially variable cellular division rate.

The cells were seeded at 3000 cells per cm^2 and allowed

Figure 9. Optical density of Nonidet P40 solubilized dye as a function of detergent concentrations from 0.05 mM to 0.5 mM. CMC is determined from the point of intersection of the regression analyses of the two distinct slopes. a) CMC = 0.23 mM b) CMC = 0.232 mM c) CMC = 0.24 mM


A
.2
0
Absorbance
0
.5
Nonidet P40 concentration (mM)


B
.2
0
Absorbance
0
.5
Nonidet P40 concentration (mM)

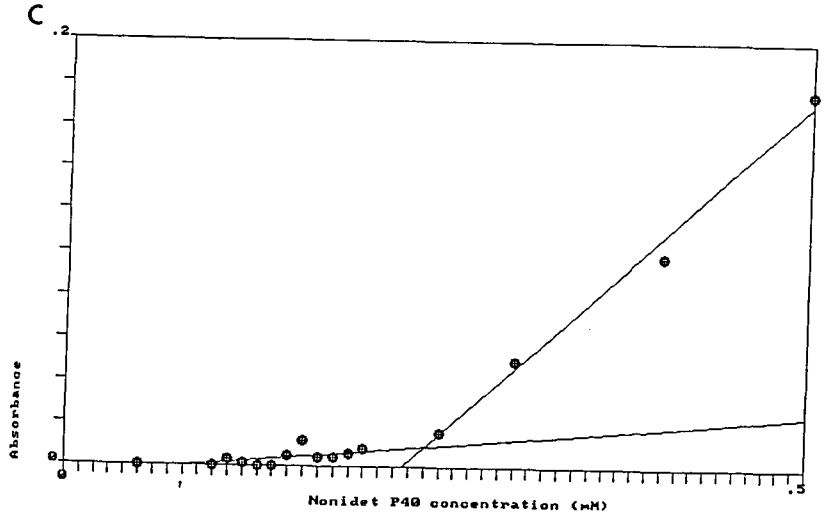
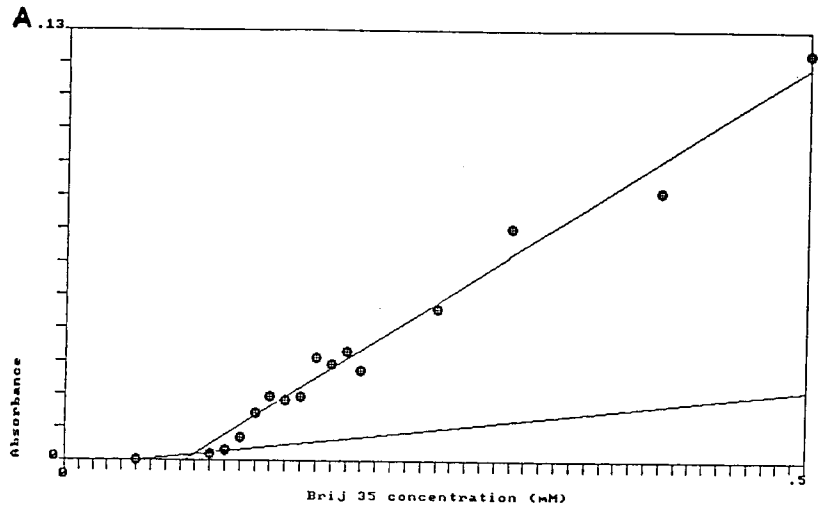
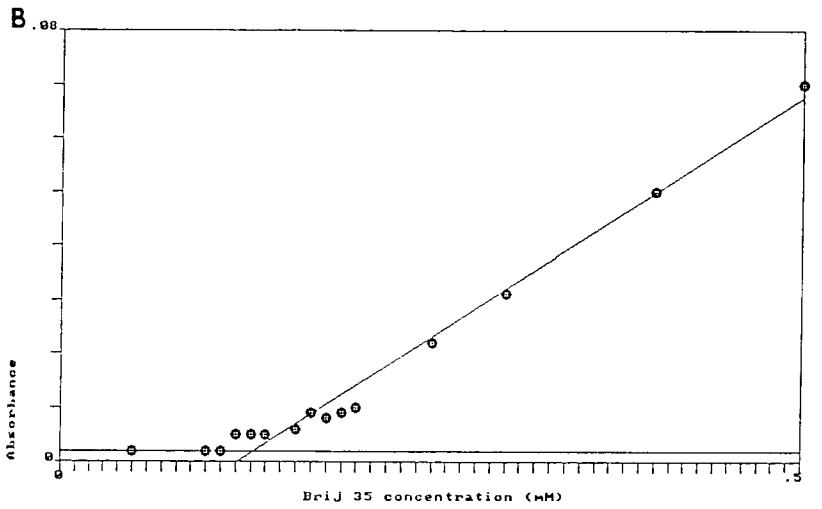

C
.2
0
Absorbance
0
.5
Nonidet P40 concentration (mM)

Figure 10. Optical density of Brij 35 solubilized dye as a function of detergent concentrations from 0.05 mM to 0.5 mM. CMC is determined from the point of intersection of the regression analyses of the two distinct slopes. a) CMC = 0.09 mM b) CMC = 0.132 mM c) CMC = 0.138 mM


A
.13
Absorbance
0
0
.5
Brij 35 concentration (mM)


B
.08
Absorbance
0
0
.5
Brij 35 concentration (mM)

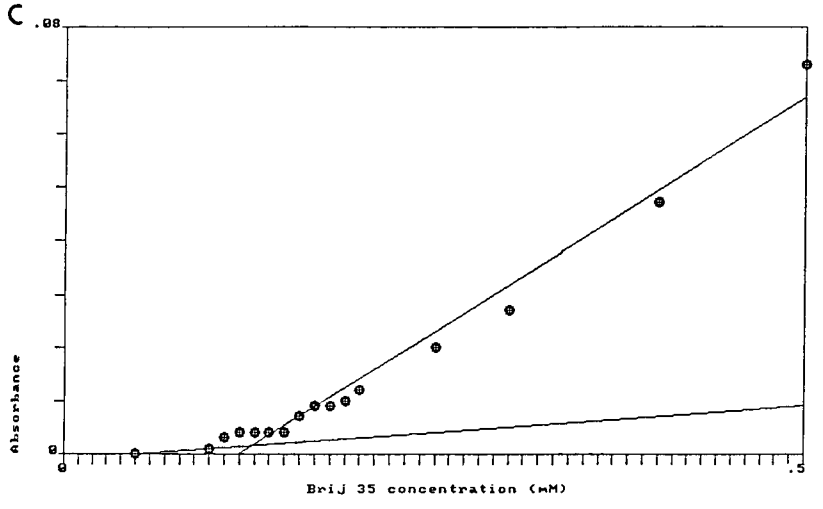
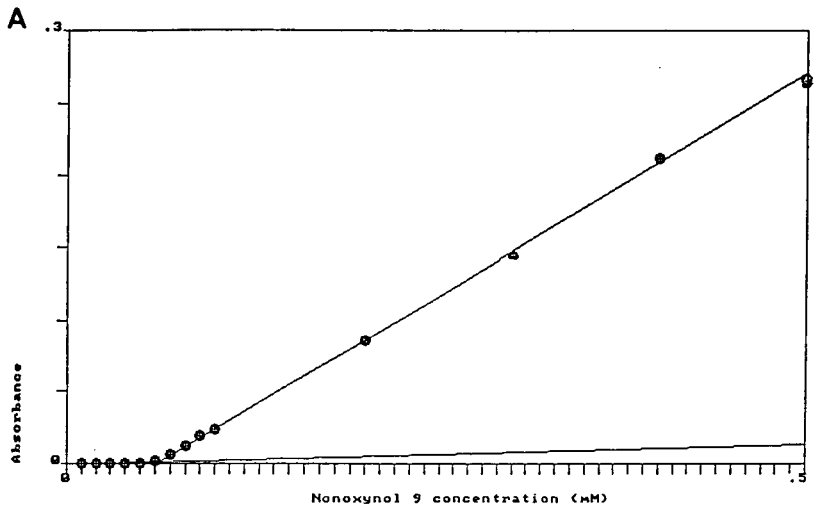
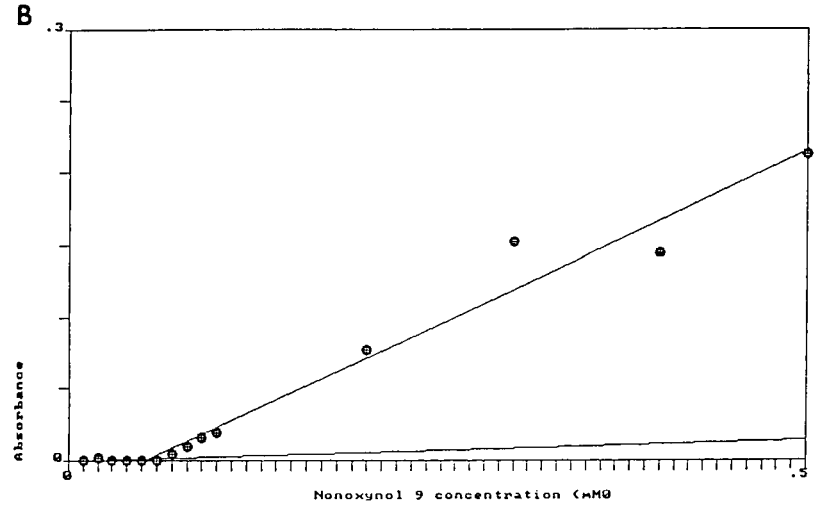

C
.08
Absorbance
0
0
.5
Brij 35 concentration (mM)

Figure 11. Optical density of Nonoxynol 9 solubilized dye as a function of detergent concentrations from 0.01 mM to 0.5 mM. CMC is determined from the point of intersection of the regression analyses of the two distinct slopes. a) CMC = 0.06 mM b) CMC = 0.055 mM c) CMC = 0.07 mM


A
.3
Absorbance
0
.5
Nonoxynol 9 concentration (mM)


B
.3
Absorbance
0
.5
Nonoxynol 9 concentration (mM0

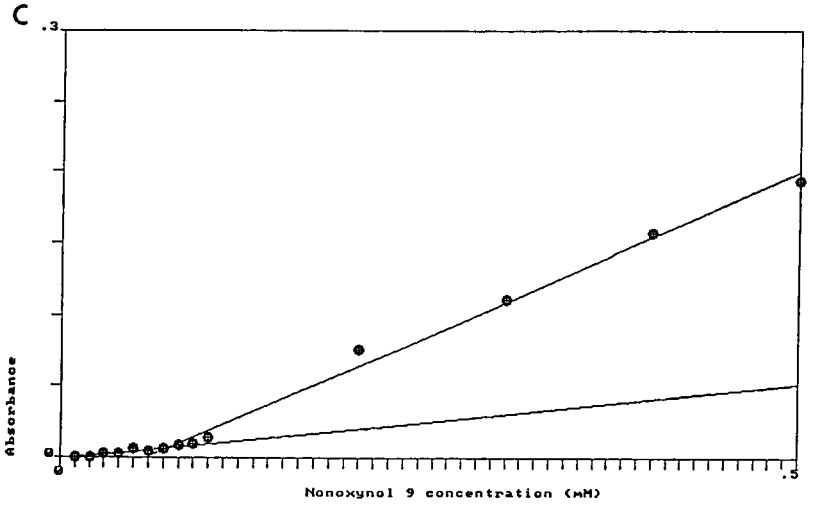

C
.3
Absorbance
0
.5
Nonoxynol 9 concentration (mM)

Table 1. Literature-derived and calculated CMC values of the three nonionic detergents and the individual concentrations of these detergents within various dilutions of PV.

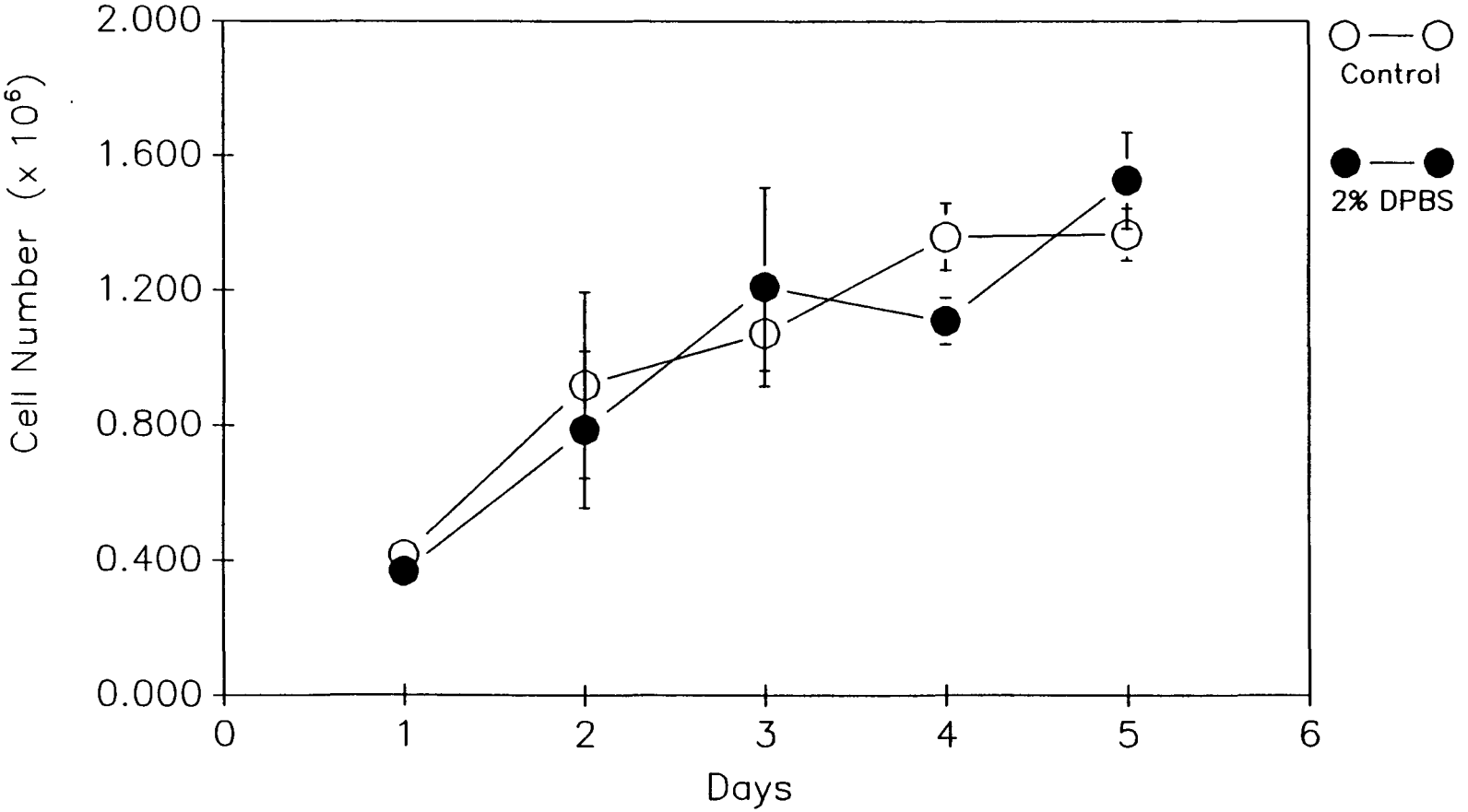
CONCENTRATION	DETERGENT (mM)		
	Brij 35	Nonoxynol 9	Nonidet P40
CMC Literature	0.092	0.0812	0.29 or 0.11
CMC Calculated	0.120 +/- 0.026	0.062 +/- 0.008	0.234 +/- 0.005
in 1X PV	55	32	33
in 0.02X PV	1.1	0.64	0.66
in 0.01X PV	0.55	0.32	0.33
in 0.005X PV	0.275	0.16	0.165
in 0.001X PV	0.055	0.032	0.033
in 0.0005X PV	0.0275	0.016	0.0165

to attach and grow for 11-18 hours. DPBS was added to the media within the experimental flasks to a final concentration of 2% (v/v) and exposed to the flasks of cells for 48 hours. Because 48 hours was the longest PV exposure period used in these experiments, this length of time was used in the validation assay. Similarly, 0.02X was the largest PV concentration used in these studies; therefore, this concentration was used in the validation assay. The control flasks contained media without DPBS.

After the prescribed length of time (48 hours), the DPBS-containing medium was discarded, and the experimental flasks of cells were rinsed with 3 ml FBS-free alpha-MEM 3 times. The media in the control flasks were discarded, but the flasks were not rinsed. Both the control flasks and the experimental flasks were replenished with 10 ml of alpha-MEM supplemented with 10% FBS. Cells were grown in a 5% carbon dioxide humidified incubator at 37° C. Triplicate flasks were harvested daily via trypsinization for 5 days and counted.

As noted in figure 12, the growth characteristic of

Figure 12. First validation assay showing the comparative growth characteristics of cells exposed to 2% DPBS and of cells that were not manipulated. The results were not statistically significant.


2.000
1.600
1.200
0.800
0.400
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
Days
Control
2% DPBS

fibroblasts exposed to 2% DPBS was not significantly different from that of the controls, i.e., the flasks that were neither handled nor exposed to DPBS. However, in order to be prudent and to keep flask manipulation constant from the control group to the treatment groups, DPBS was added to all controls in subsequent assays to a final concentration of 2% (v:v); furthermore, the controls were rinsed with 3 ml FBS-free alpha-MEM 3 times in the same manner that the treatment groups were. Thus, both the control cultures and the experimental cultures were handled in the same manner.

2. The second validation assay was performed in order to determine the potential adherence of PV to the flask surface. If PV showed some affinity for the flask surface, then the cells would be continuously exposed to PV even after rinsing, thus altering the experimental parameters in the post exposure proliferation assays.

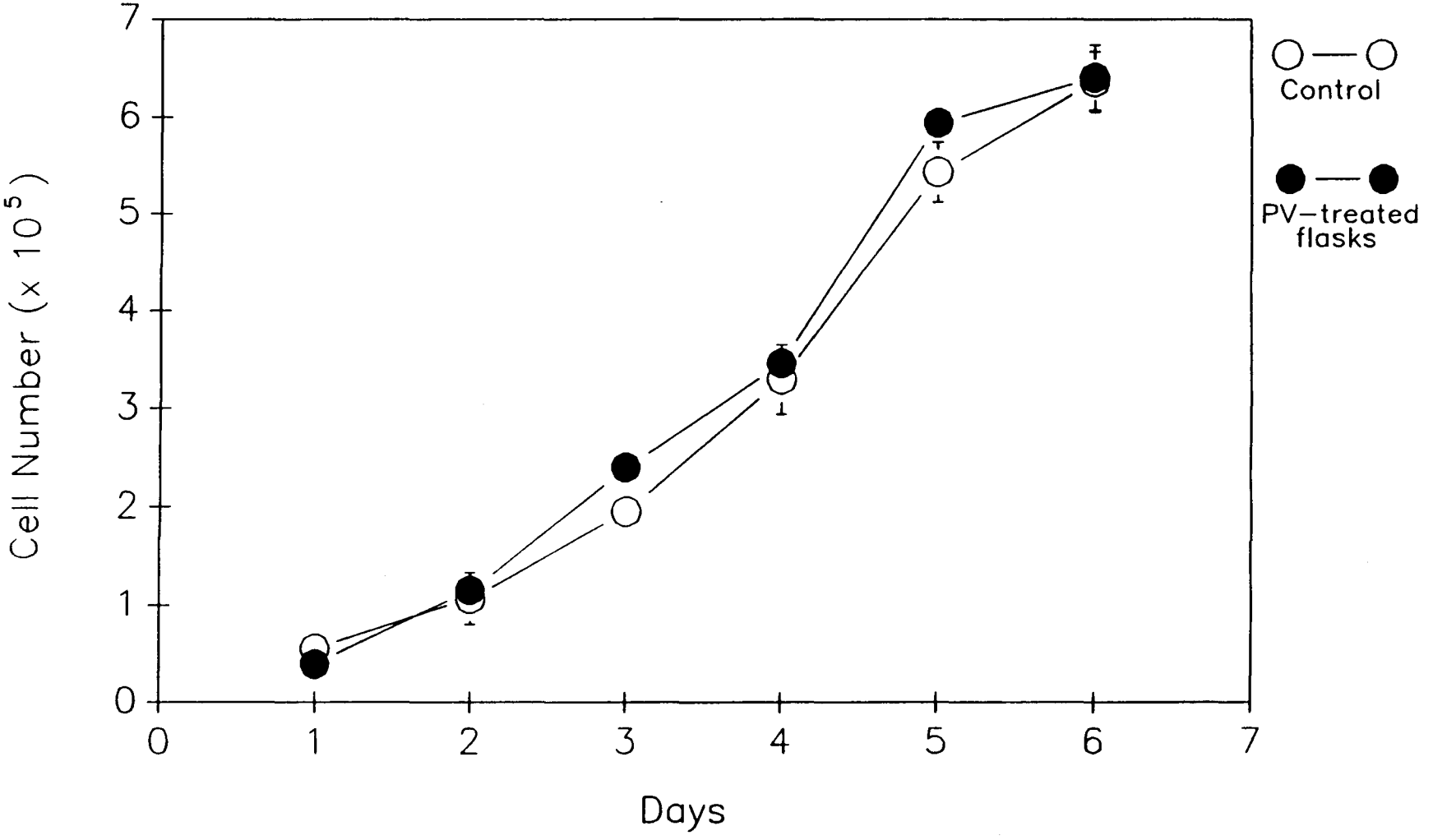
The treatment flasks were exposed for 48 hours to 0.02X PV dissolved in DPBS in a humidified carbon dioxide incubator maintained at 37° C at 5% CO_2. As in the first validation

assay, 48 hours and 0.02X PV were chosen because they represented the longest exposure interval and greatest PV concentration used in the post exposure proliferation assays. After 48 hours, the experimental flasks were rinsed 3 times with 3 ml of FBS-free alpha-MEM. Cells were then seeded into treated and untreated flasks and allowed to attach and grow as described previously. Triplicate flasks were harvested by trypsinization and counted using a hemocytometer every 24 hours for 6 days.

Figure 13 represents the cellular counts plotted against the number of days in culture. The data show that no significant difference was detected between the control and treatment groups, i.e., PV treatment of the flasks did not alter subsequent growth of cells.

3. The final validation experiment explored the effects of FBS on the toxicity of PV towards human dermal fibroblasts. Detergents are traditionally used for the isolation of membrane proteins; therefore, the reasonable assumption is that increasing FBS, which possesses a high protein content,

Figure 13. Second validation assay showing the comparative growth characteristics of cells grown in untreated flasks and PV-treated flasks. No significant difference was detected between control and treatment groups.

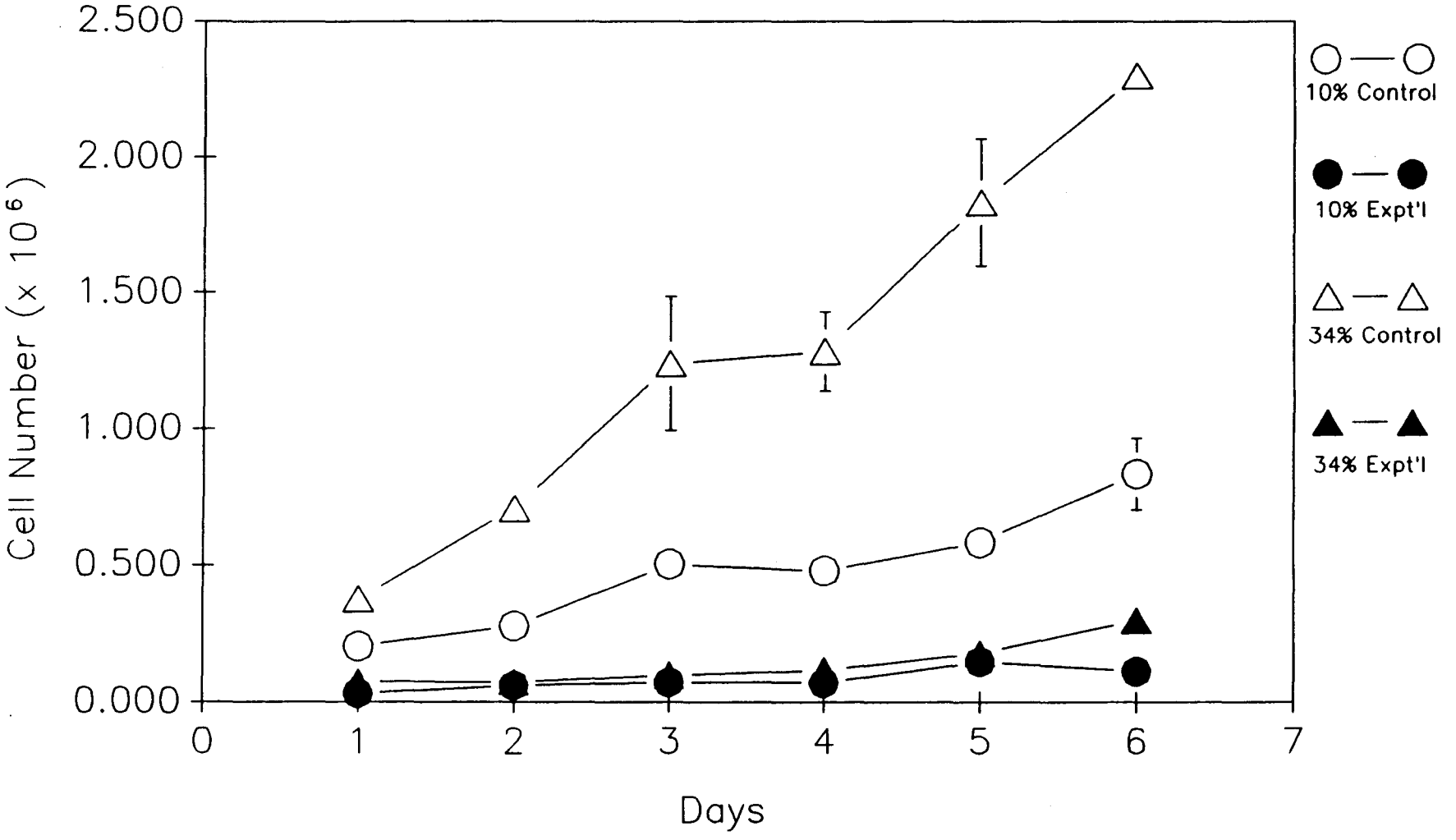

Cell Number (x 10^5)
0
1
2
3
4
5
6
7
Days
Control
PV–treated flasks

might decrease the toxic effects of PV by binding to it and effectively reducing the soluble detergent concentration. Thus, less detergent molecules would be available to react with the cellular membranes of the fibroblasts.

Cells were seeded at 3000 per cm^2 and grown as described above. After 11-18 hours, the media in one group was changed to alpha-modified MEM supplemented with 10% FBS. In contrast, the media in the second group was changed to alpha-modified MEM supplemented with 34% FBS. Experimental subgroups differentially treated with FBS were exposed to 0.005X PV for 48 hours at 37° C. The control cells within the two FBS groups were exposed to DPBS without PV. The resultant treatment groups were i) 10% FBS with no PV (control), ii) 10% FBS with 0.005X PV, iii) 34% FBS with no PV (control), and iv) 34% FBS with 0.005X PV. Triplicate flasks of cells were harvested daily for 6 days and the cells were counted using a hemocytometer.

Figure 14 illustrates the data collected. The 10% FBS control group is designated as A, the 10% FBS PV-treated group

Figure 14. Third validation assay showing the comparative growth curves of cells grown in the presence of 10% or 34% FBS and in the presence or absence of 0.005X PV.


2.500
2.000
1.500
1.000
0.500
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
10% Control
10% Expt'l
34% Control
34% Expt'l

is designated as B, the 34% FBS control group is designated as C, and the 34% FBS PV-treated group is designated as D. Because the higher FBS concentration provides more nutrients to the cells, group C exhibited a higher growth rate than the other groups, as expected. Group A, grown in the presence of 10% FBS, proliferated more quickly than the two PV-treated groups but did not proliferate as quickly as group C. However, the growth characteristics of groups B (10% FBS) and D (34% FBS), the two PV-treated groups, did not significantly differ from each other despite the different FBS concentrations.

One way to interpret this data would be to conclude that higher concentrations of FBS actually heighten the toxicity of PV towards mammalian fibroblasts. A more logical assumption would be that the increased FBS levels do not impinge upon the actions of PV on the cellular membrane as seen by the similar growth characteristics of groups B and D. In the absence of other factors, higher concentrations of FBS can accelerate cellular proliferation rate; therefore, the experimental cellular counts cannot be normalized to percent of control

counts for comparison.

D. Post exposure *in vitro* proliferation assays

The upper PV limit used in the *in vitro* post exposure and continuous exposure proliferation assays was determined through preliminary toxicity studies in which the mammalian fibroblasts were exposed to 0.02X PV, 0.04X PV, and 0.06X PV. These concentrations were chosen arbitrarily. The PV concentrations of 0.04X and 0.06X were extremely toxic to the cells, resulting in eventual cell death (data not shown). Therefore, 0.02X PV was chosen as the upper limit in subsequent assays.

Altered proliferation characteristics of cells is one of the main indicators of aberrant cell function. Therefore, the post exposure proliferation assay was used to determine changes in cellular function after exposure to PV. These experiments also determined the capability of mammalian fibroblasts to recover from PV exposure. The toxicity level of PV was determined concurrently.

Figures 15 - 19 show the graphed data obtained from 24 hour PV exposure. The concentrations of PV were 0.02X, 0.01X, 0.005X, 0.001X, and 0.0005X. The PV concentrations of 0.02X and 0.01X were toxic to varying degrees as represented by the lower rate of cellular proliferation. However, 0.005X, 0.001X, and 0.0005X were not significantly different from the controls. The toxic PV levels were normalized to percent of the controls (figure 20).

When the cells were exposed to the same PV concentrations for 48 hours, the results differed in a slight, yet critical, manner. The PV concentrations of 0.02X and 0.01X were still toxic (figures 21 and 22); however, in the 48 hour exposure interval, 0.005X also exerted a negative effect on cellular proliferation (figure 23). As in the 24 hour period, 0.001X and 0.0005X PV did not significantly affect the rate of cellular division (figures 24 and 25). The crucial results are again represented as percent of control in figure 26.

In the 24 and 48 hour studies, the PV that was used was obtained from Medicine and Applied Sciences, lot (1), in a 1X

Figure 15. Comparative growth characteristics of cells exposed to 0.02X PV for 24 hours and control cells.

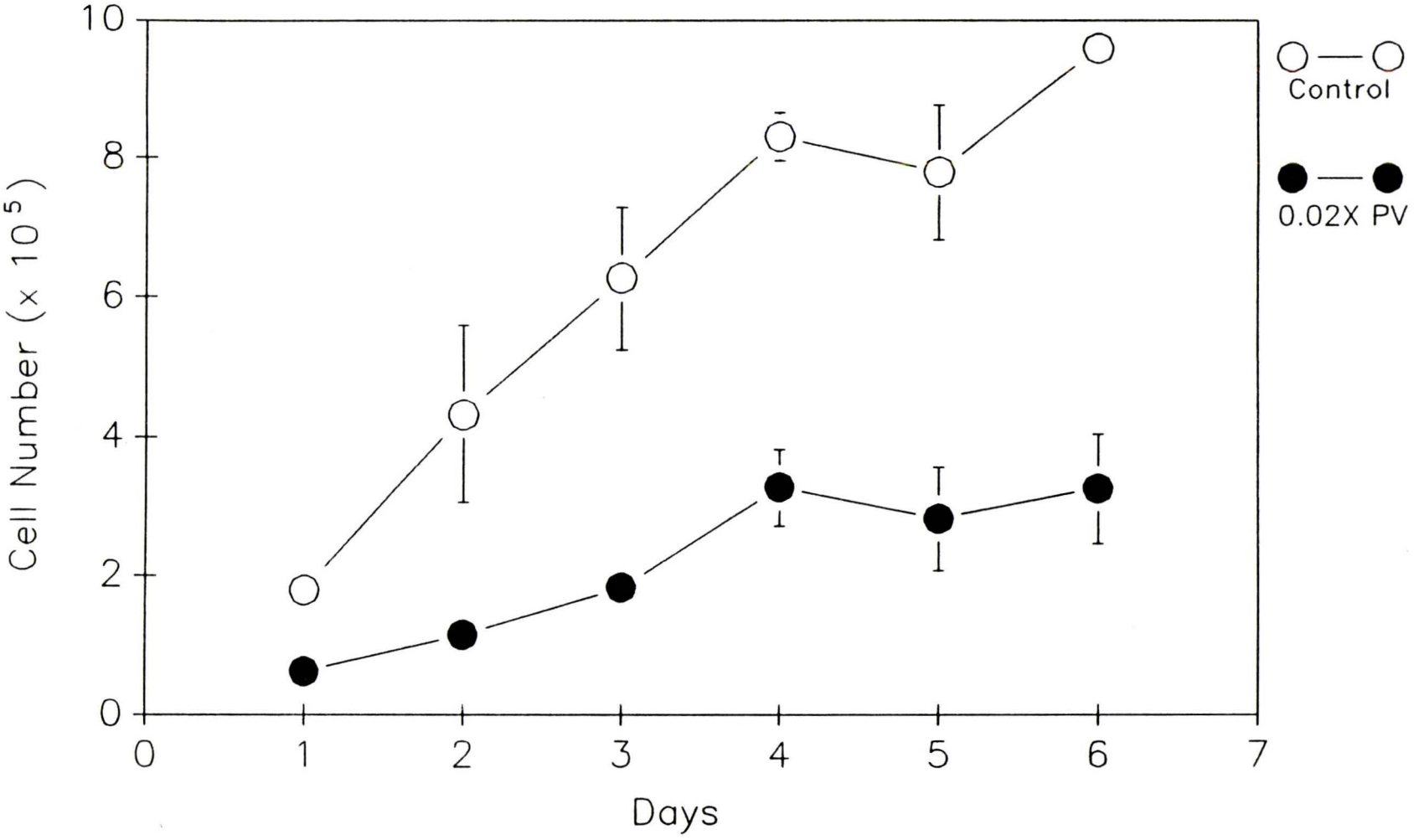

10
8
6
4
2
0
Cell Number (x 10^5)
0
1
2
3
4
5
6
7
Days
Control
0.02X PV

Figure 16. Comparative growth characteristics of cells exposed to 0.01X PV for 24 hours and control cells.

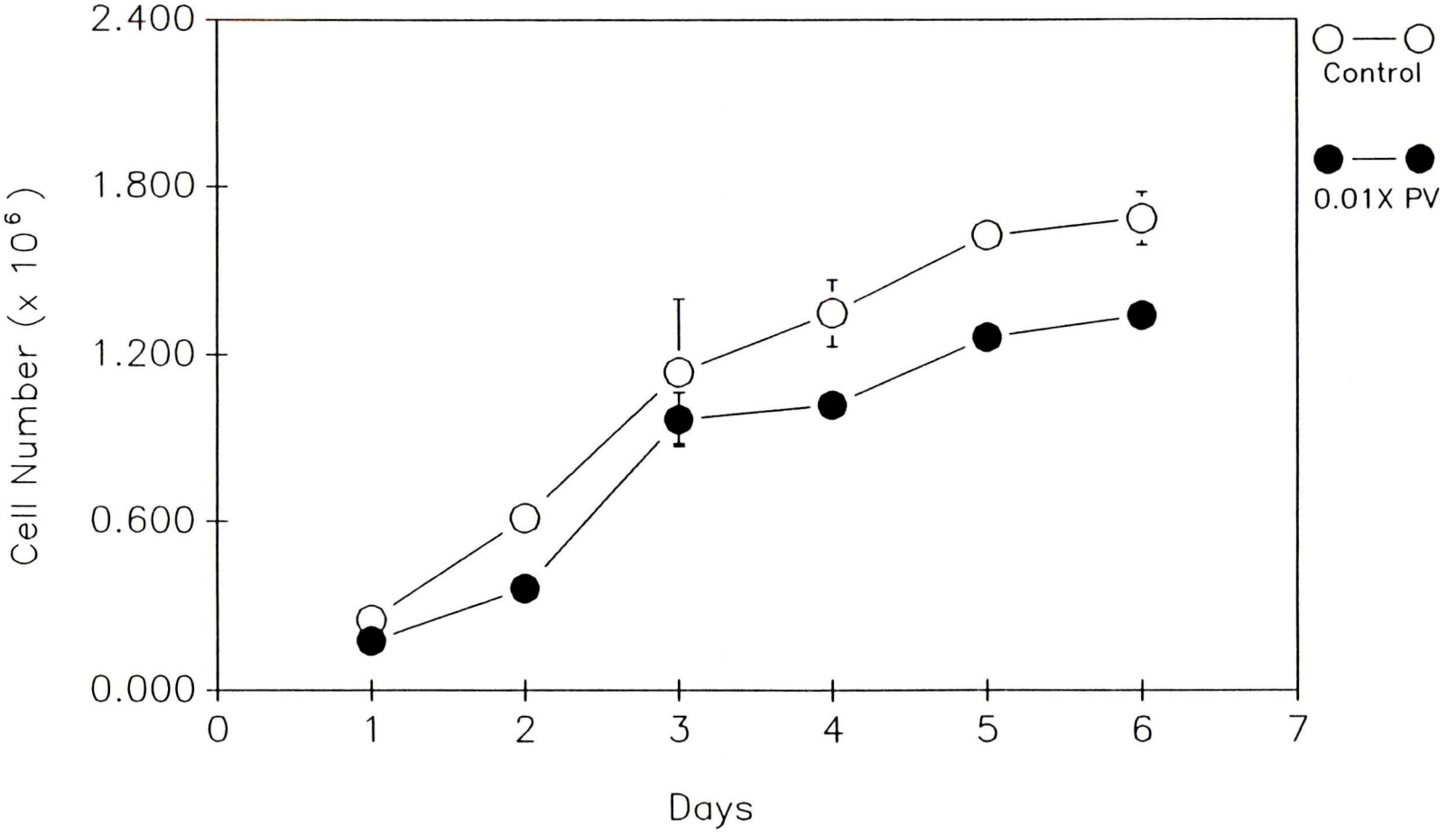

2.400
1.800
1.200
0.600
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
Control
0.01X PV

Figure 17. Comparative growth characteristics of cells exposed to 0.005X PV for 24 hours and control cells.

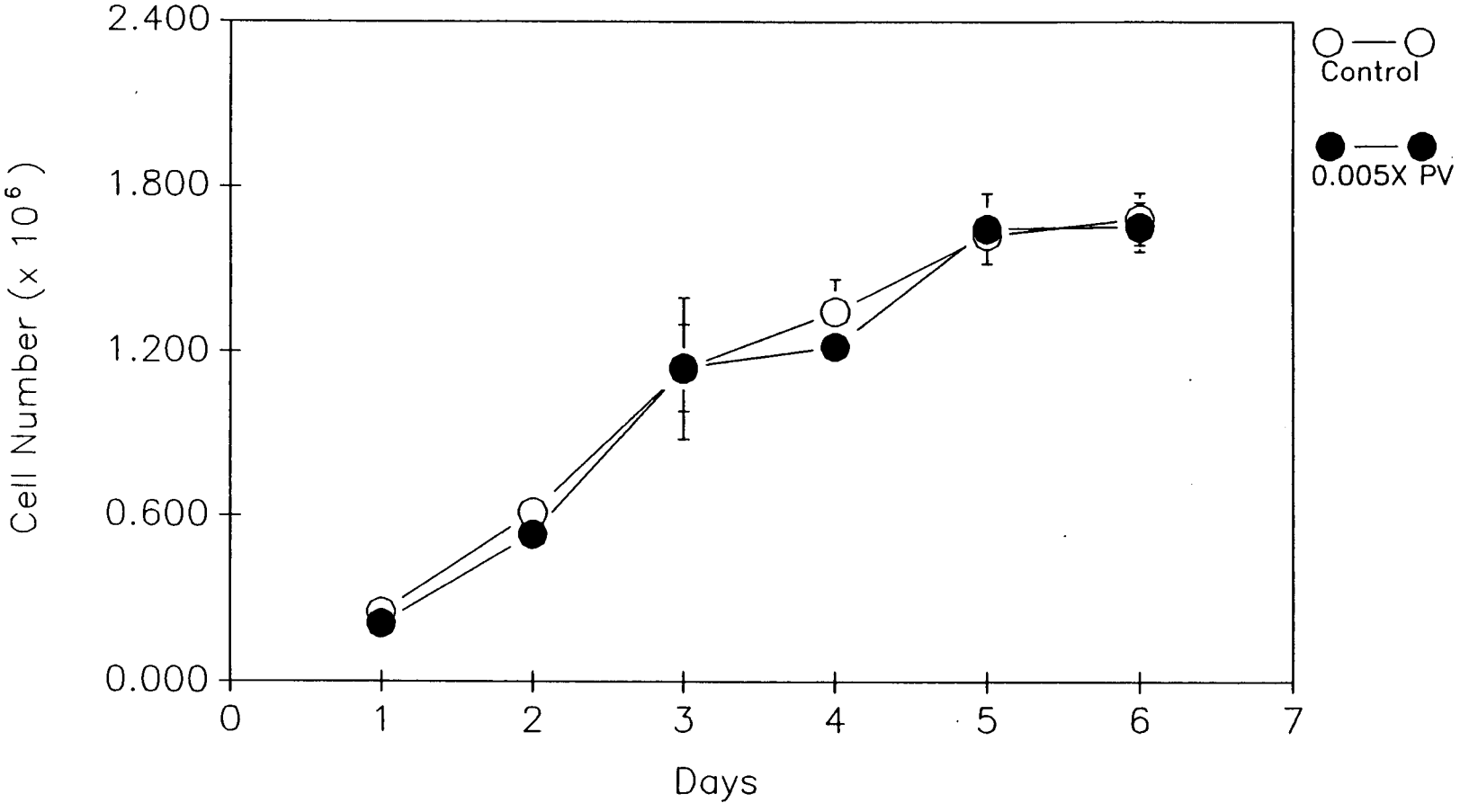

2.400
1.800
1.200
0.600
0.000
Cell Number (x 10^6)
0
1
2
3
4
5
6
7
Days
Control
0.005X PV

Figure 18. Comparative growth characteristics of cells exposed to 0.001X PV for 24 hours and control cells.

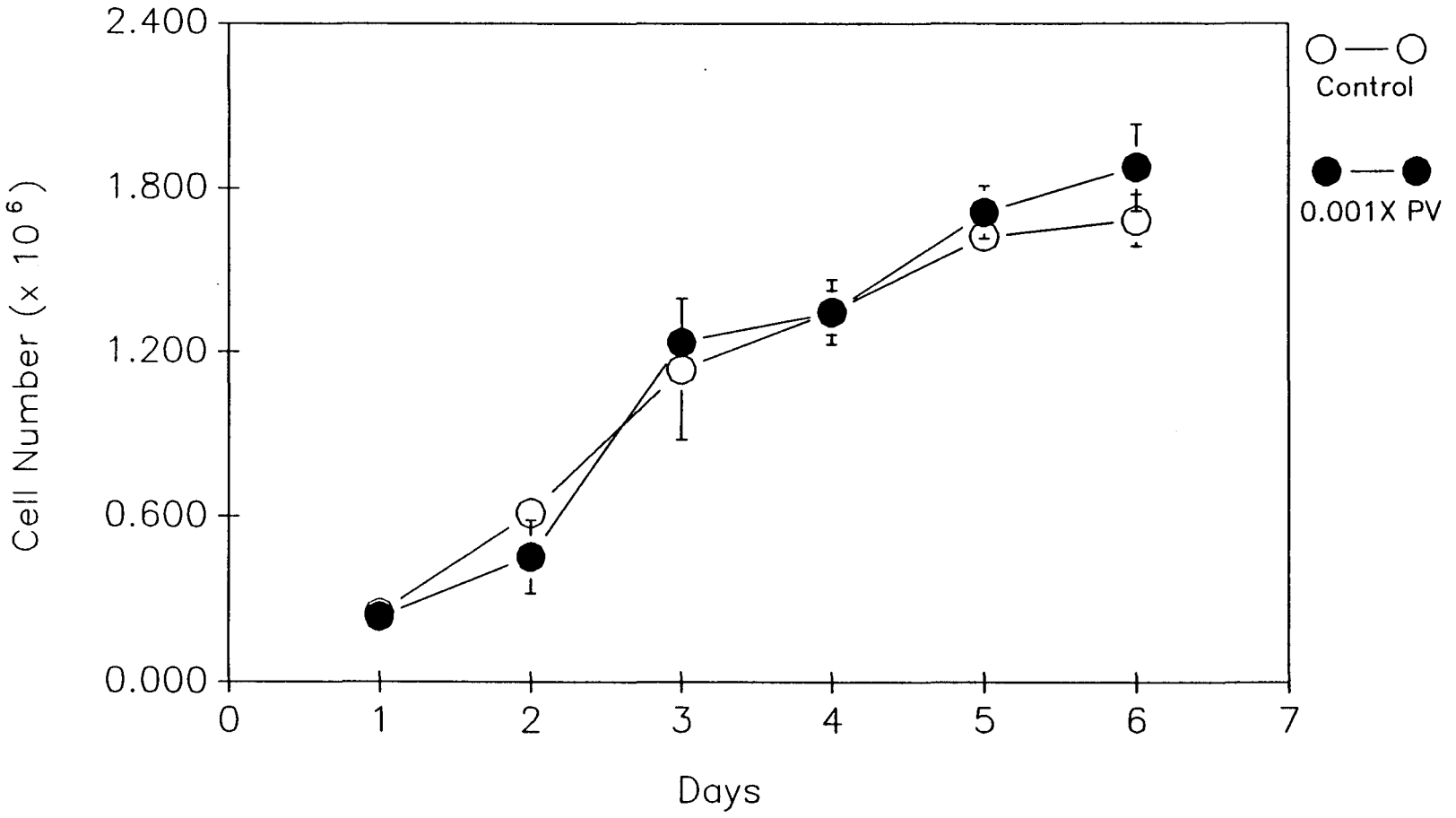

2.400
1.800
1.200
0.600
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
Control
0.001X PV

Figure 19. Comparative growth characteristics of cells exposed to 0.0005X PV for 24 hours and control cells.

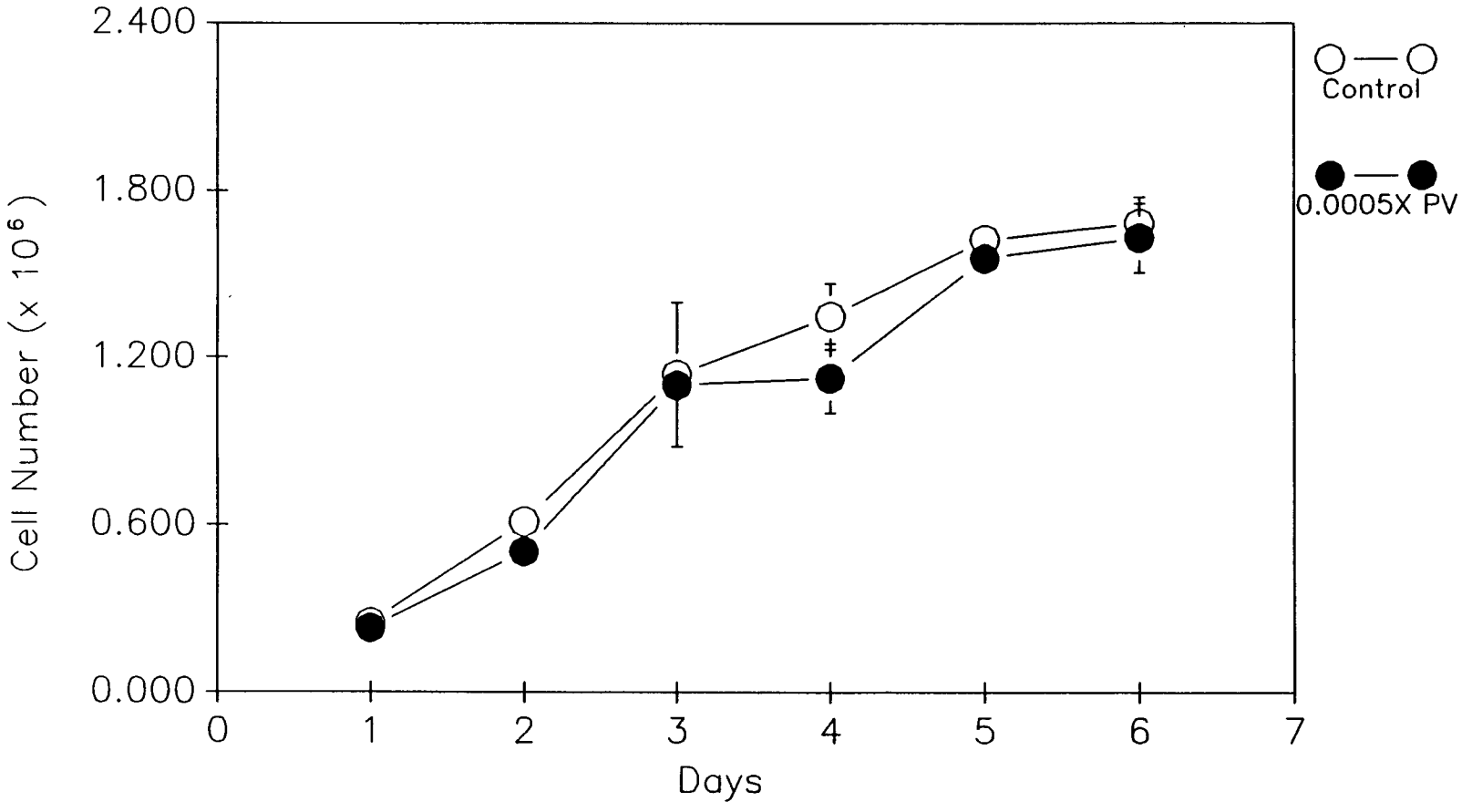

2.400
1.800
1.200
0.600
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
Control
0.0005X PV

Figure 20. The significantly different 24 hour post exposure proliferation assay results represented as percent of control cellular counts. The toxic PV levels were at 0.02X and 0.01X. The PV concentration of 0.005X was not toxic at the 24 hour exposure interval; however, because 0.005X PV affects cells in subsequent assays, it was included in this figure.

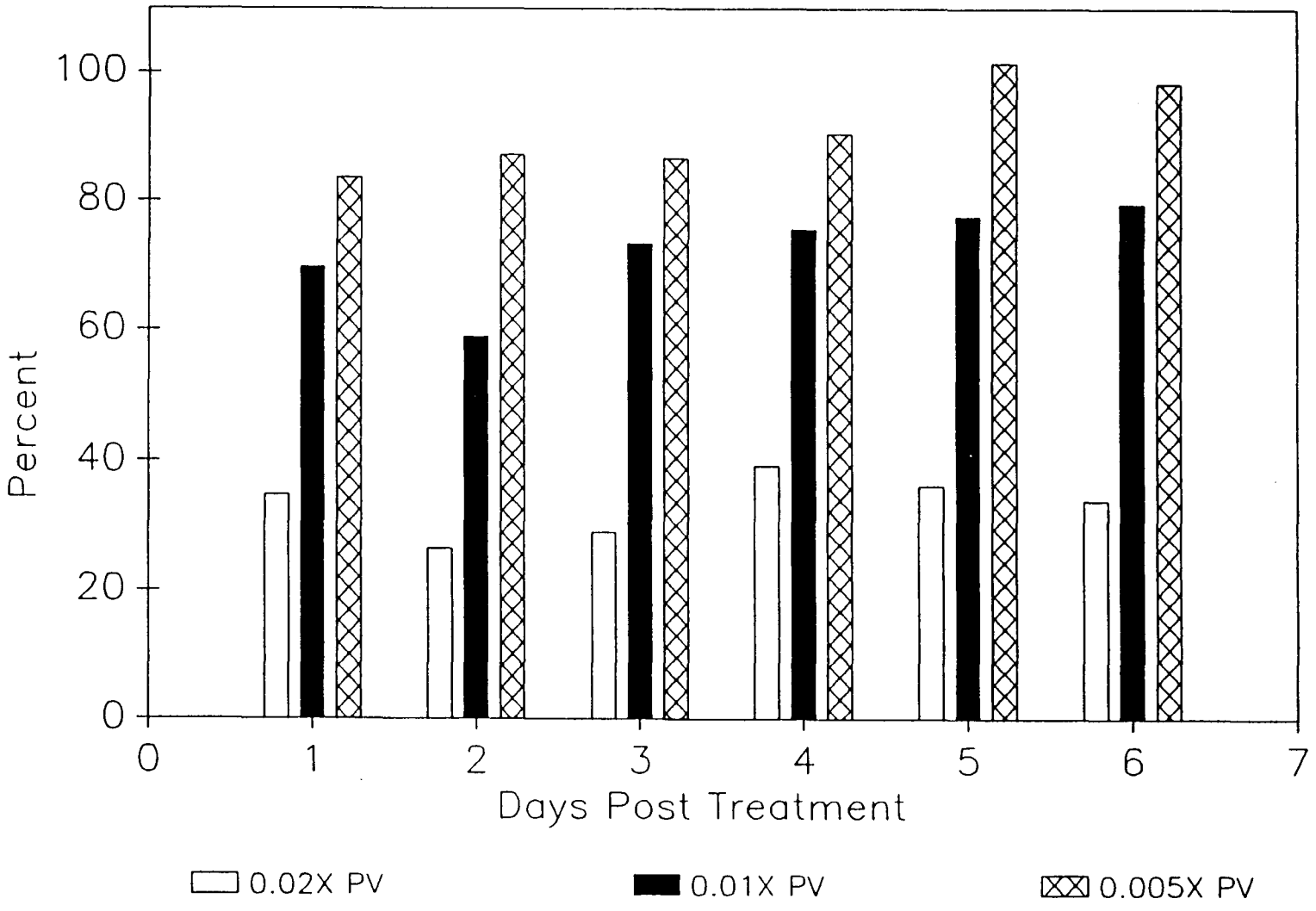

100
80
60
40
20
0
Percent
0
1
2
3
4
5
6
7
Days Post Treatment
0.02X PV
0.01X PV
0.005X PV

Figure 21. Comparative growth characteristics of cells exposed to 0.02X PV for 48 hours and control cells.

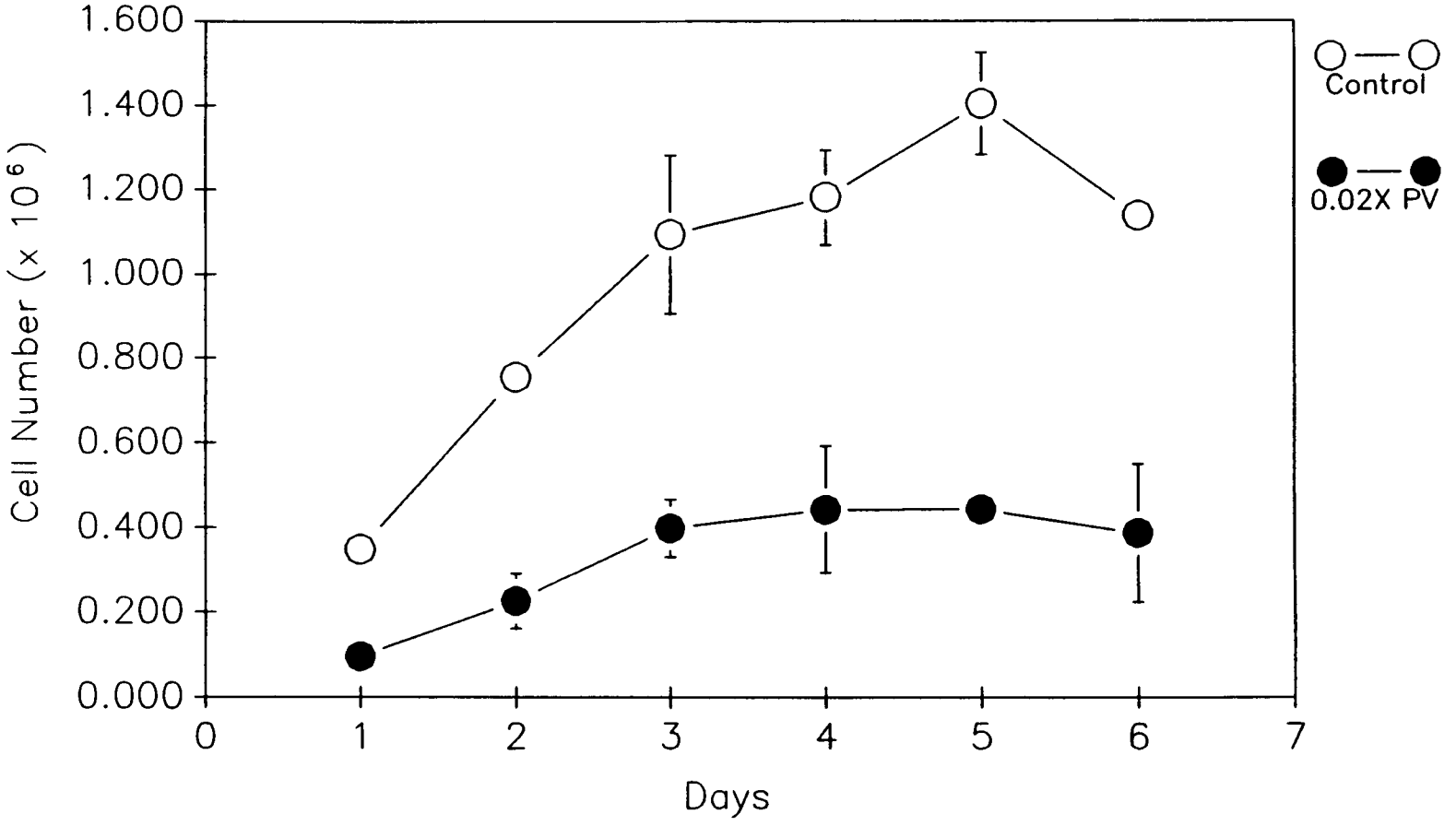

1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
Control
0.02X PV

Figure 22. Comparative growth characteristics of cells exposed to 0.01X PV for 48 hours and control cells.

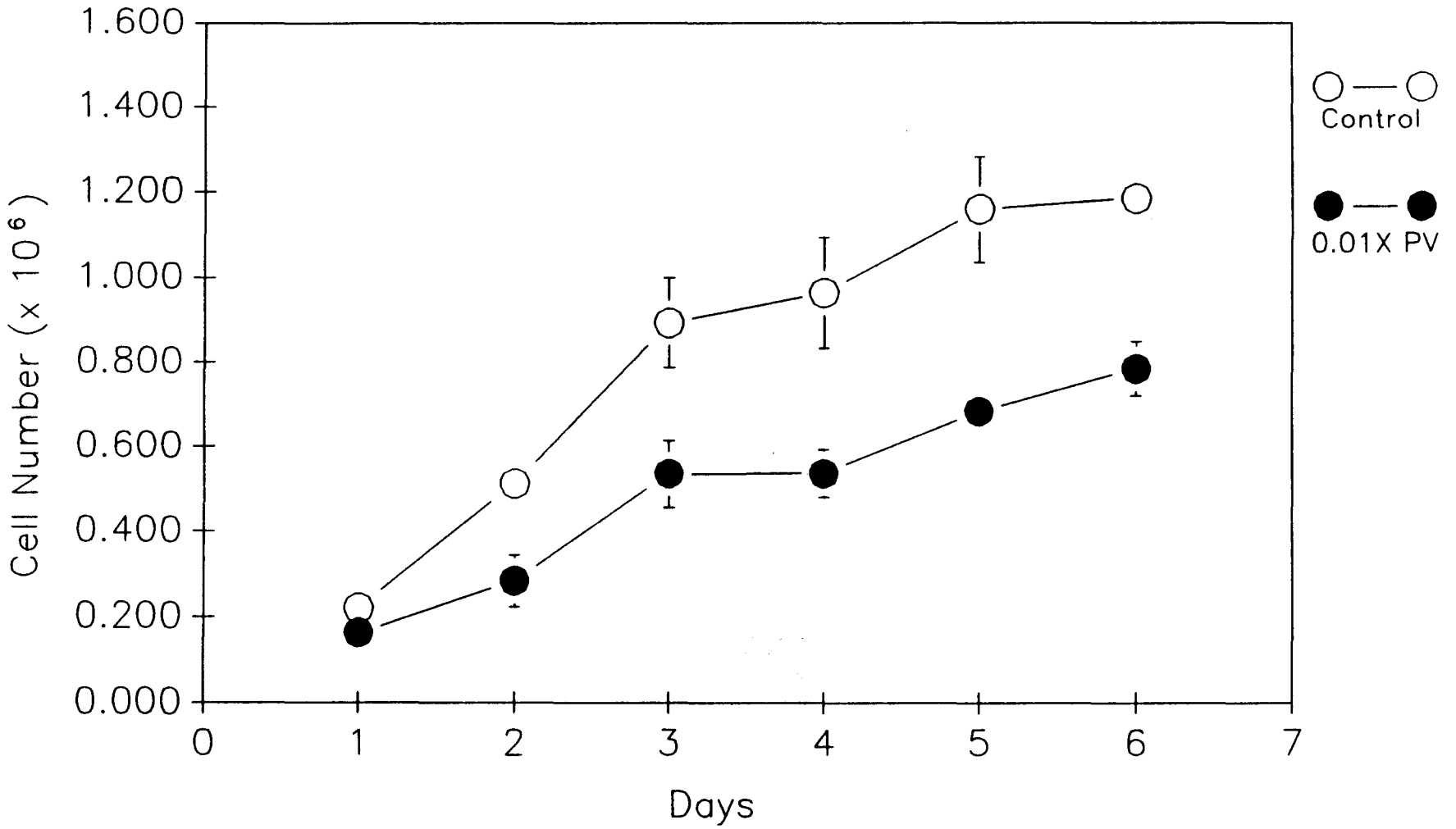

1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
Control
0.01X PV

Figure 23. Comparative growth characteristics of cells exposed to 0.005X PV for 48 hours and control cells.

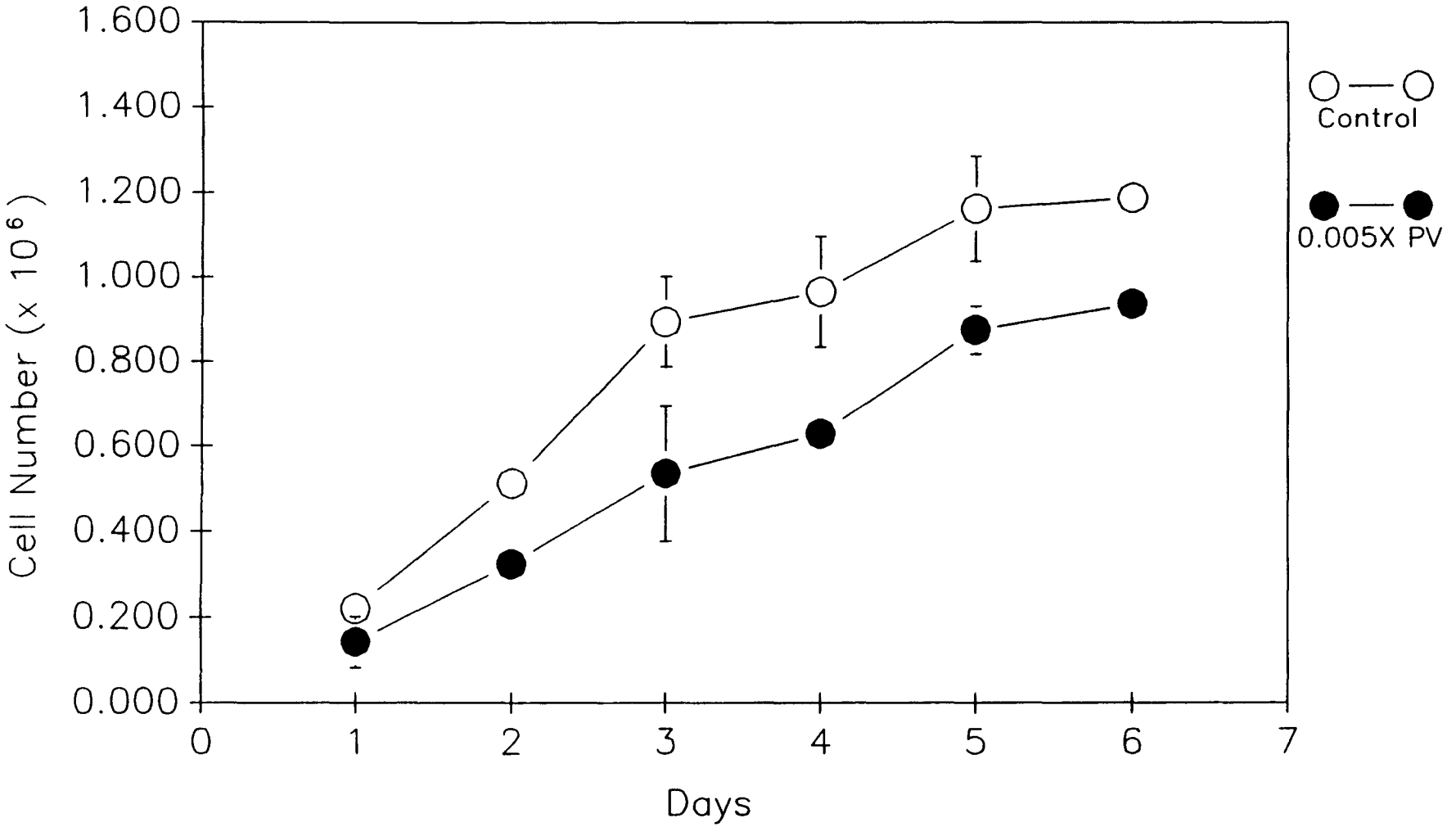

1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
Control
0.005X PV

Figure 24. Comparative growth characteristics of cells exposed to 0.001X PV for 48 hours and control cells.

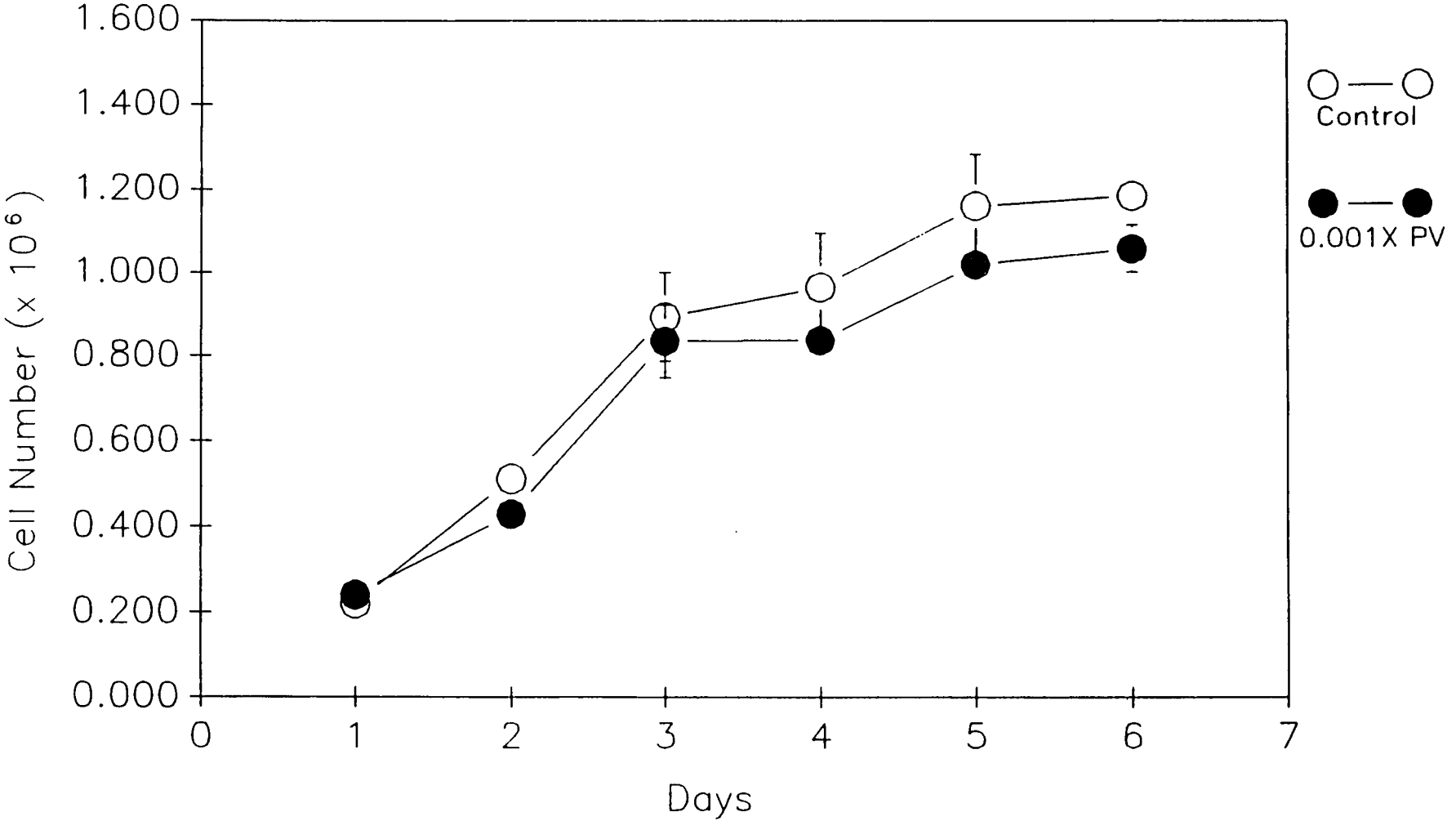

1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Cell Number (x 10^6)
0
1
2
3
4
5
6
7
Days
Control
0.001X PV

Figure 25. Comparative growth characteristics of cells exposed to 0.0005X PV for 48 hours and control cells.

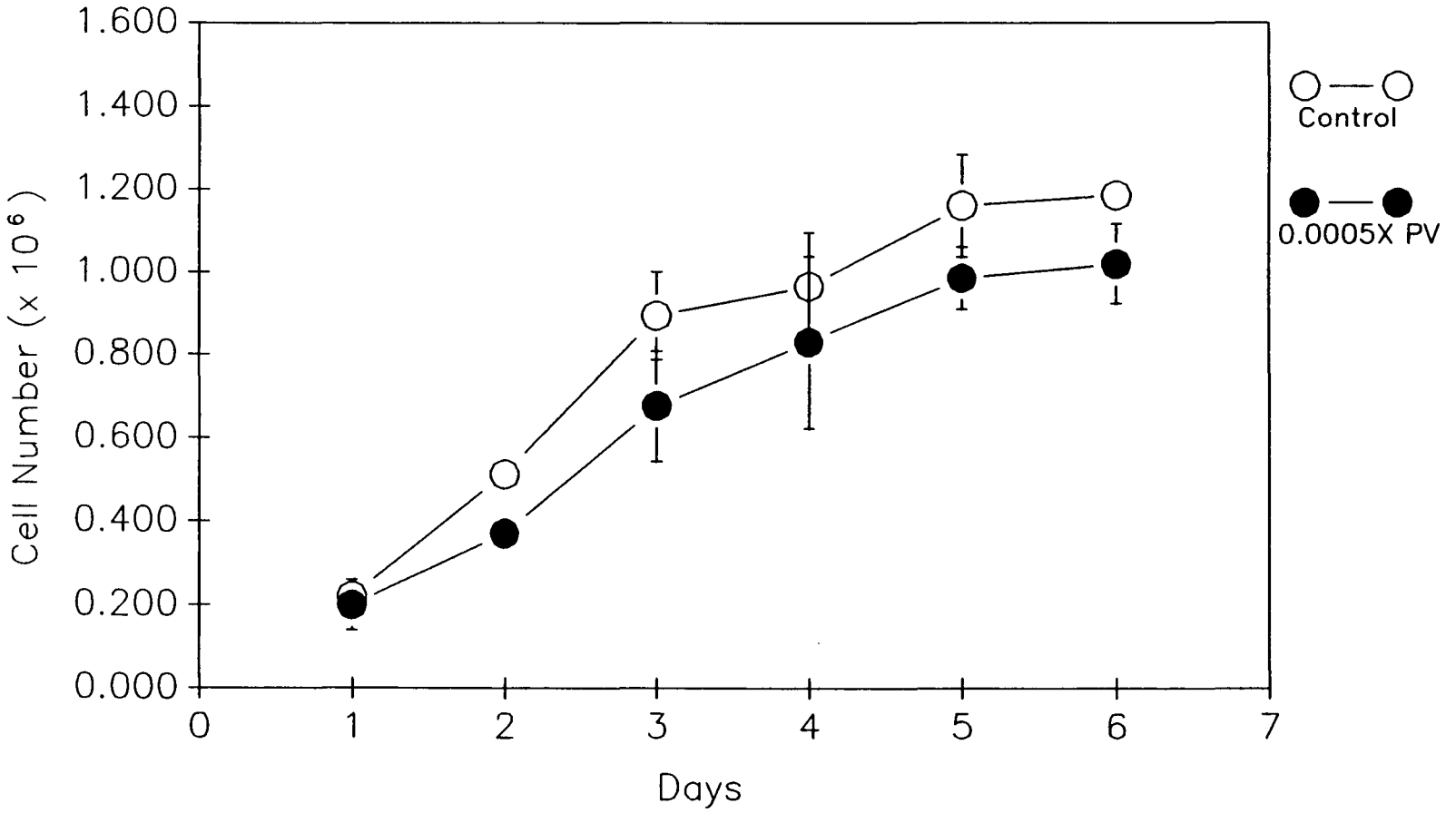
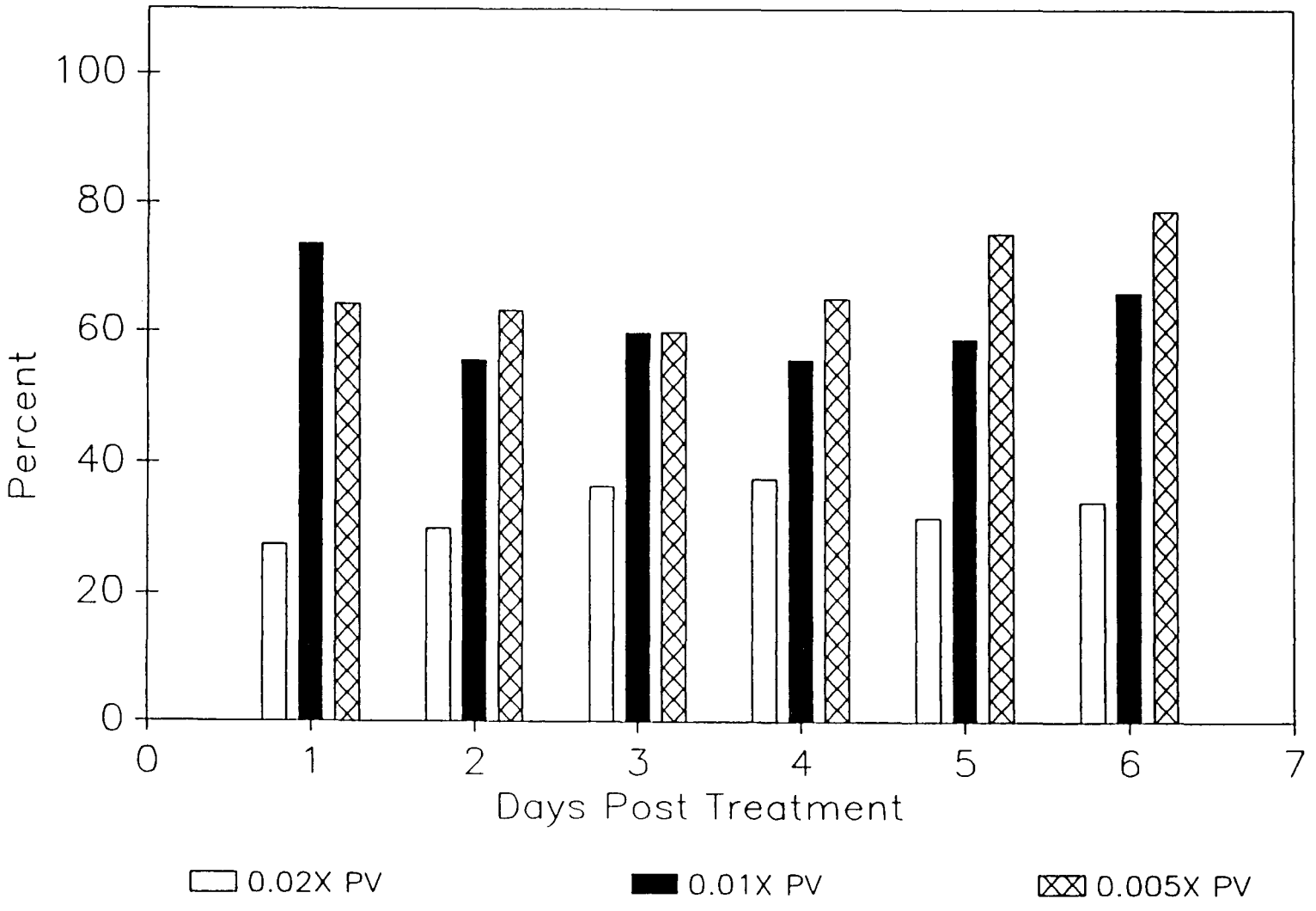

1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
Control
0.0005X PV

Figure 26. The significantly different 48 hour post exposure proliferation assay results represented as percent of control cellular counts. The toxic PV levels were at 0.02X, 0.01X, and 0.005X.


100
80
60
40
20
0
Percent
0
1
2
3
4
5
6
7
Days Post Treatment
0.02X PV
0.01X PV
0.005X PV

stock solution containing glutaraldehyde. When the experiments were repeated using 12 and 36 hour exposure periods, another PV solution from Medicine and Applied Sciences was used. This second detergent formulation was a 10X stock solution, lot (2). It was gradually introduced into the study. PV lot (2) was diluted to 1X and combined with PV lot (1) for the 36 hour exposure study. The 12 hour exposure study was performed last with only PV lot (2). The results are graphed in figures 27 - 34.

Because 0.02X and 0.01X PV were both toxic, the PV concentration of 0.02X was not utilized in the 36 and 12 hour studies and 0.01X PV was considered the upper limit. The PV concentration of 0.01X was more detrimental to cellular proliferation than was expected (figure 27). Indeed, it prevented some cells from attaching to the flask surface. Those cells that managed to attach never divided and were essentially nonviable. When the concentration of the mixed lots (1) and (2) was at 0.005X, the cells were again affected more than they were with the unadulterated PV lot (1) (figure 28). However, 0.001X and 0.0005X still did not significantly

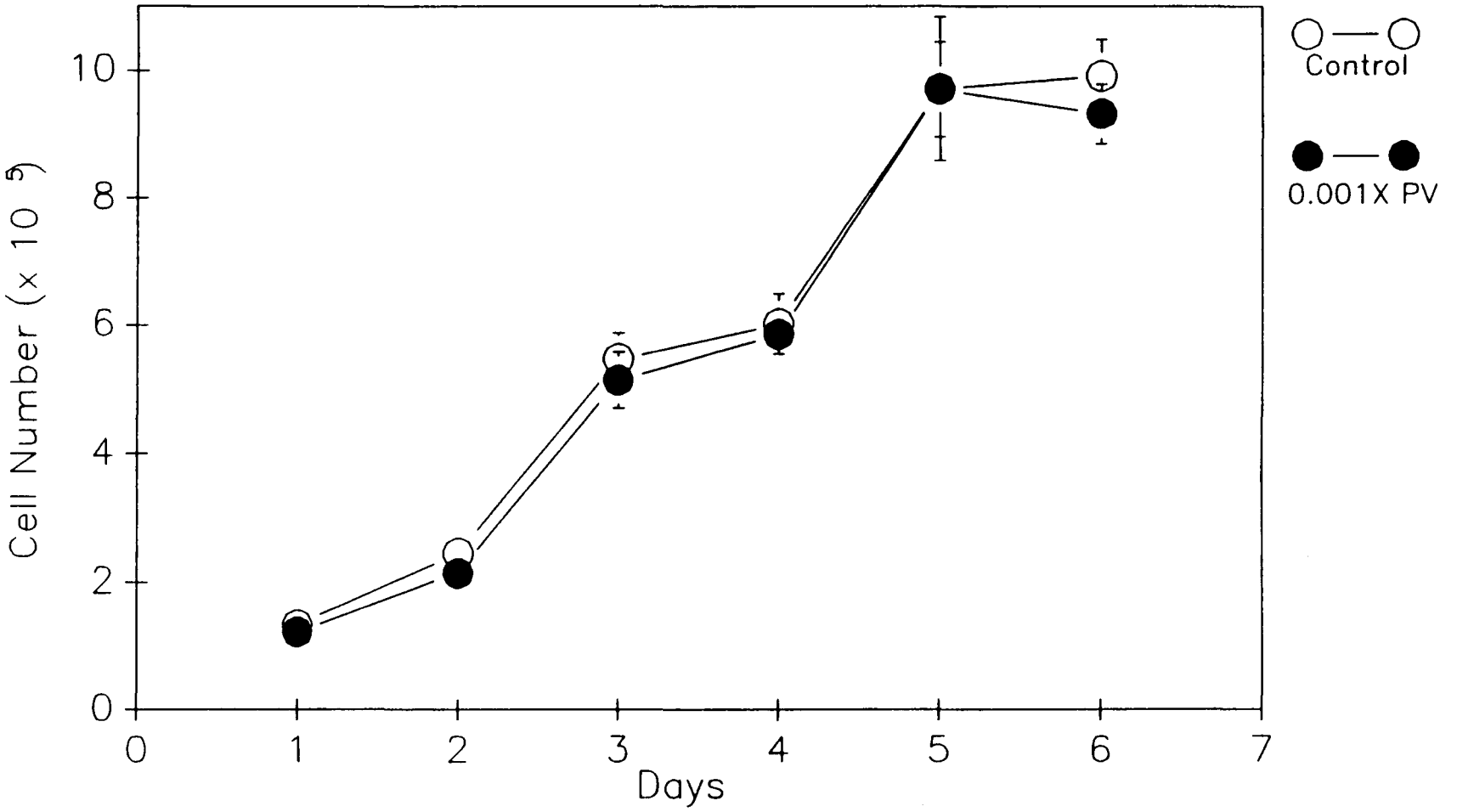
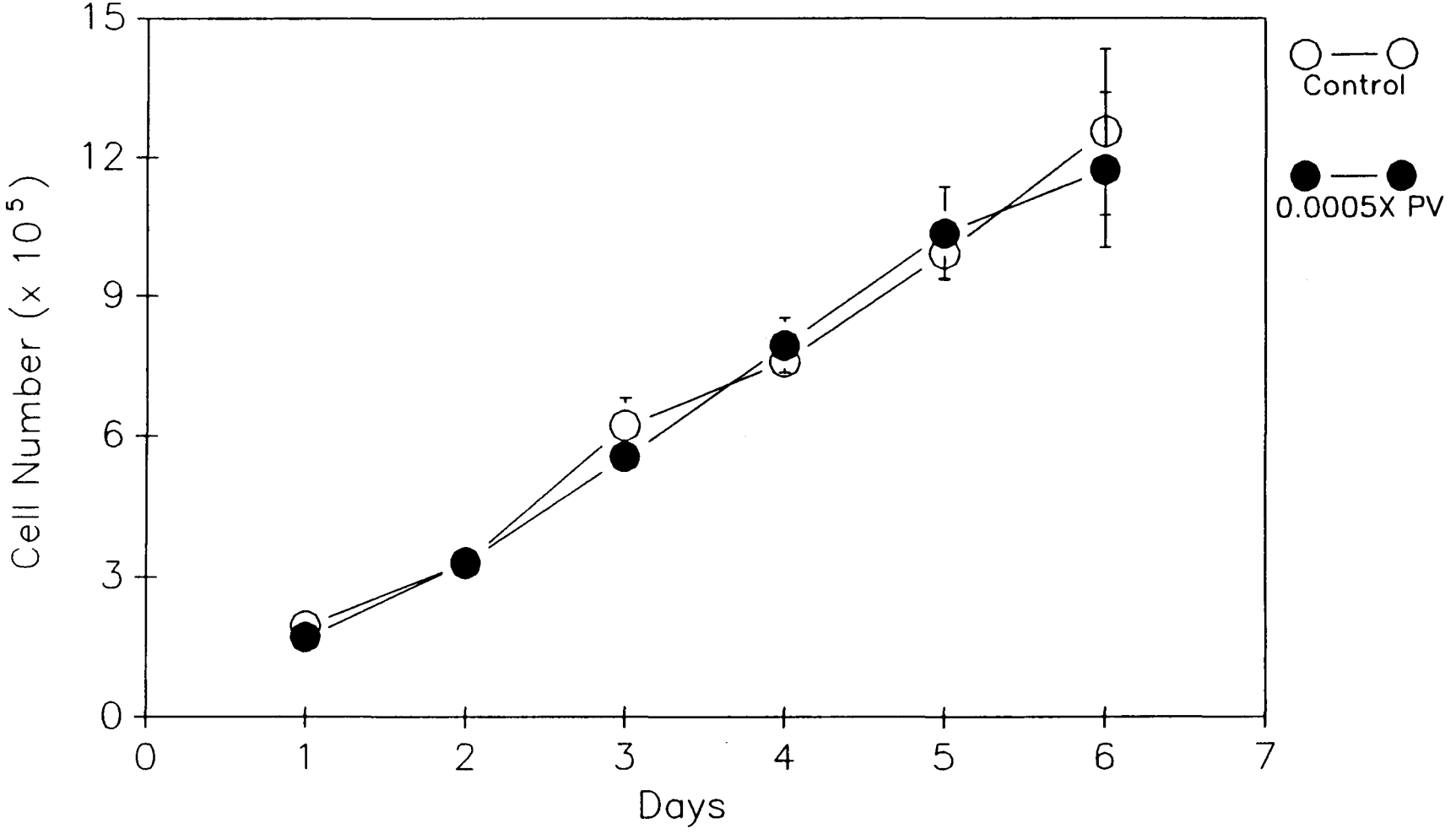

Cell Number (x 10 5)
10
8
6
4
2
0
0
1
2
3
4
5
6
7
Days
Control
0.001X PV

Figure 38. Comparative growth characteristics of cells exposed to 0.0005X PV for 36 hours and control cells. The PV was made in-house.


15
12
9
6
3
0
Cell Number (x 10^5)
0
1
2
3
4
5
6
7
Days
Control
0.0005X PV

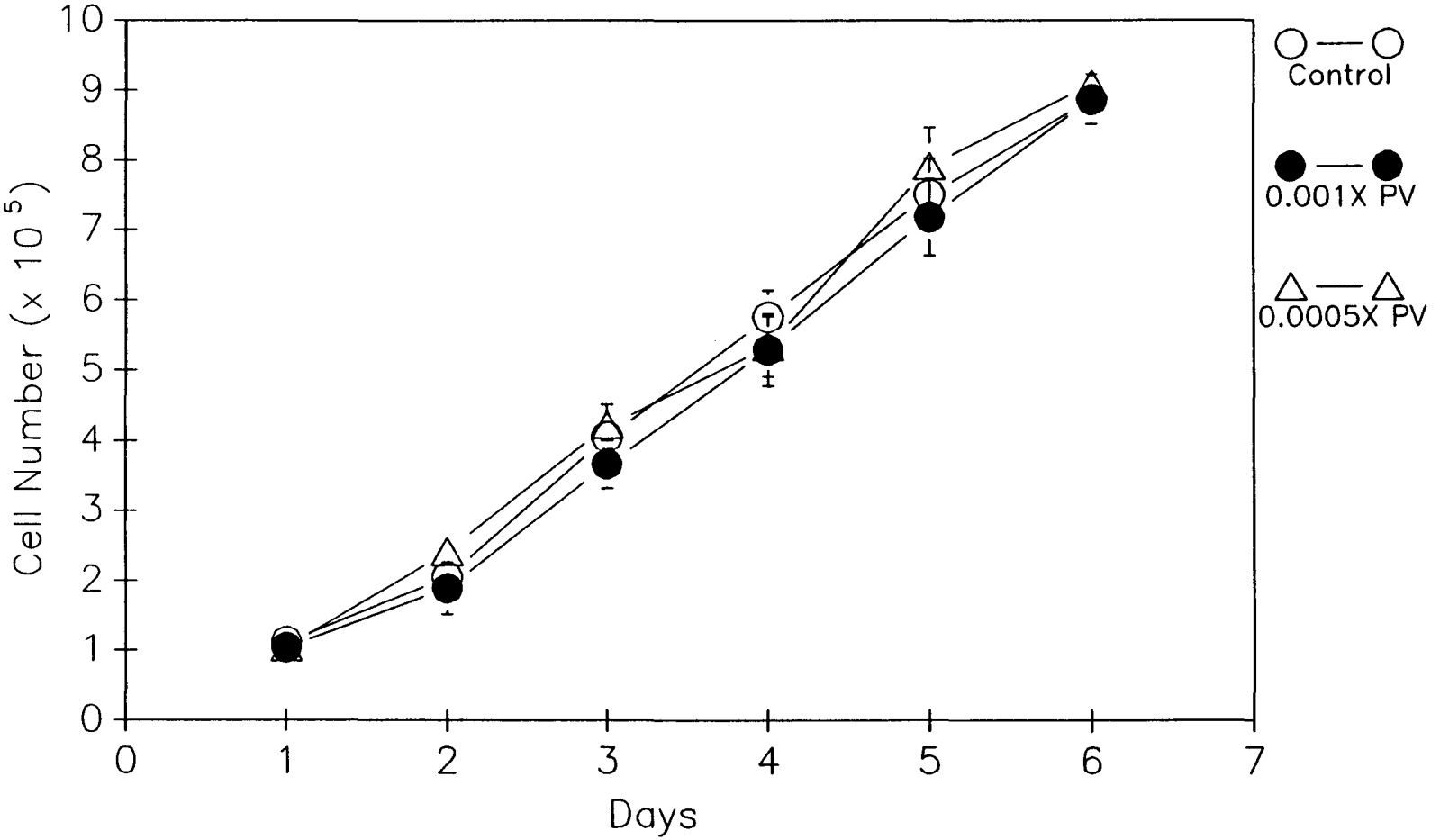
different from the controls (figures 37 and 38).

The results from the 12 hour exposure studies were different from the previous ones. As in the 36 hour exposure studies, 0.01X and 0.005X PV were more toxic to the cells than expected. Indeed, these concentrations effectively destroyed cellular viability (data not shown). The cellular proliferation rates of the cells grown in the presence of 0.001X and 0.0005X PV were, however, not significantly different from those of the controls (figure 39).

E. Continuous exposure *in vitro* proliferation assays

Residual detergents remaining within tissues upon implantation into a recipient may represent a form of continuous exposure to host cells which infiltrate the graft material. Therefore, this second type of cell proliferation assay was used to determine any changes in cellular function during continuous exposure to the varying concentrations of PV. The identical concentrations employed in the post exposure proliferation assays were used in the continuous exposure proliferation studies. These experiments were

Figure 39. Comparative growth characteristics of cells exposed to 0.001X and 0.0005X PV for 12 hours and control cells. The PV was made in-house.


Cell Number (x 10 5)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Days
Control
0.001X PV
0.0005X PV

essentially a continuation of the post exposure investigation and represented what might be considered to be the most harmful PV exposure interval.

PV lot (1) was used in these studies. The PV levels of 0.02X and 0.01X obliterated the fibroblasts (figure 40). Even though 0.005X PV was also very toxic, 0.001X and 0.0005X PV did not affect cellular proliferation (figure 41). The toxic concentrations are represented as percent of the control in figure 42.

The effects of glutaraldehyde, a membrane stabilizer that was included in the two PV formulations obtained from Medicine and Applied Sciences and in the laboratory-made PV, were determined using continuous exposure proliferation assays. The same investigation also determined the effects of laboratory-made PV on cellular viability and proliferation.

The results from the post exposure and continuous exposure studies taken together indicate that the defined toxicity level of PV is between 0.005X and 0.001X. Therefore,

Figure 40. The effects of continuous exposure of 0.02X and 0.01X PV on cellular proliferation.

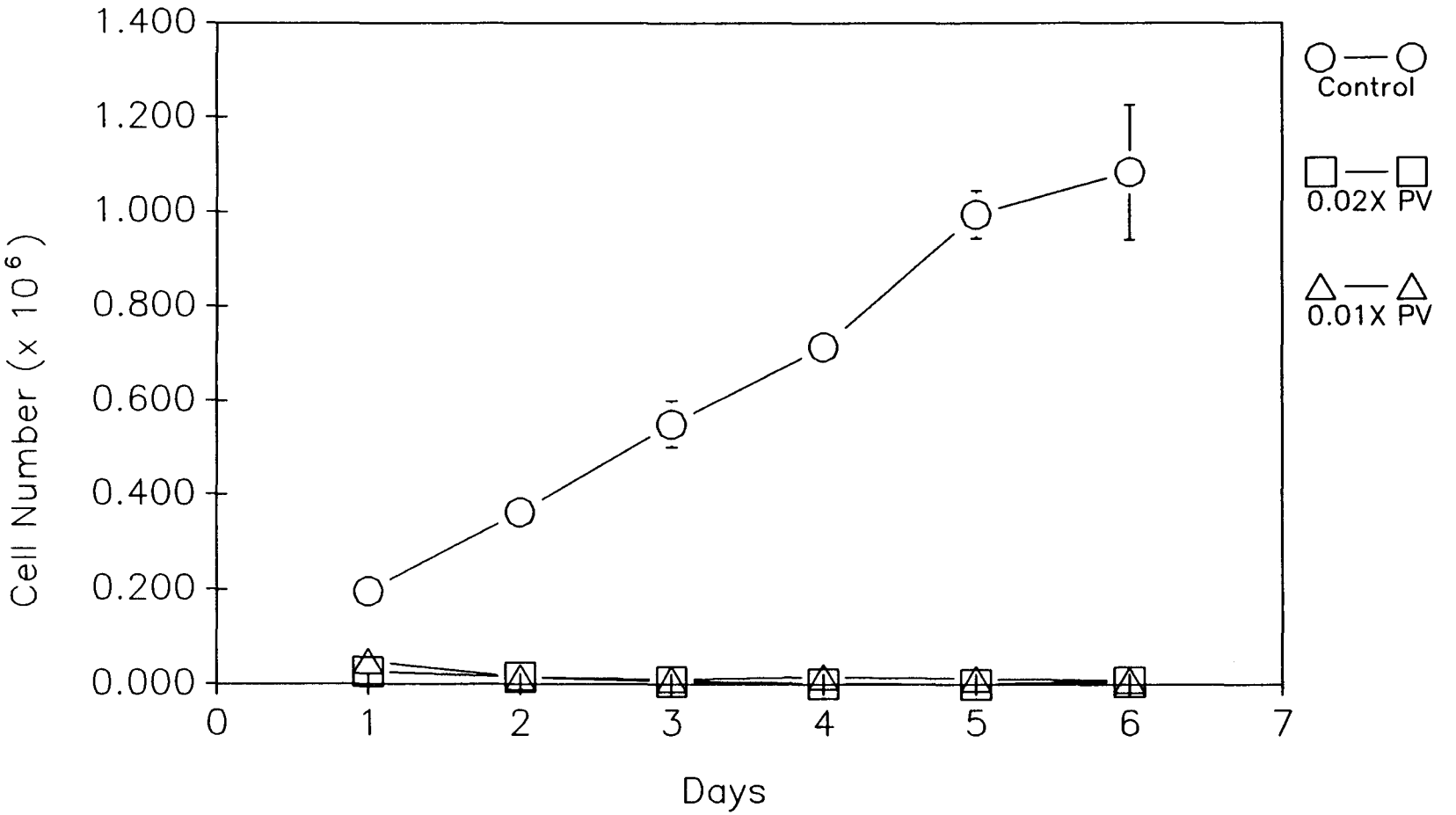

1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Cell Number (x 10^6)
0
1
2
3
4
5
6
7
Days
Control
0.02X PV
0.01X PV

Figure 41. The effects of continuous exposure of 0.005X, 0.001X, and 0.0005X PV on cellular proliferation.

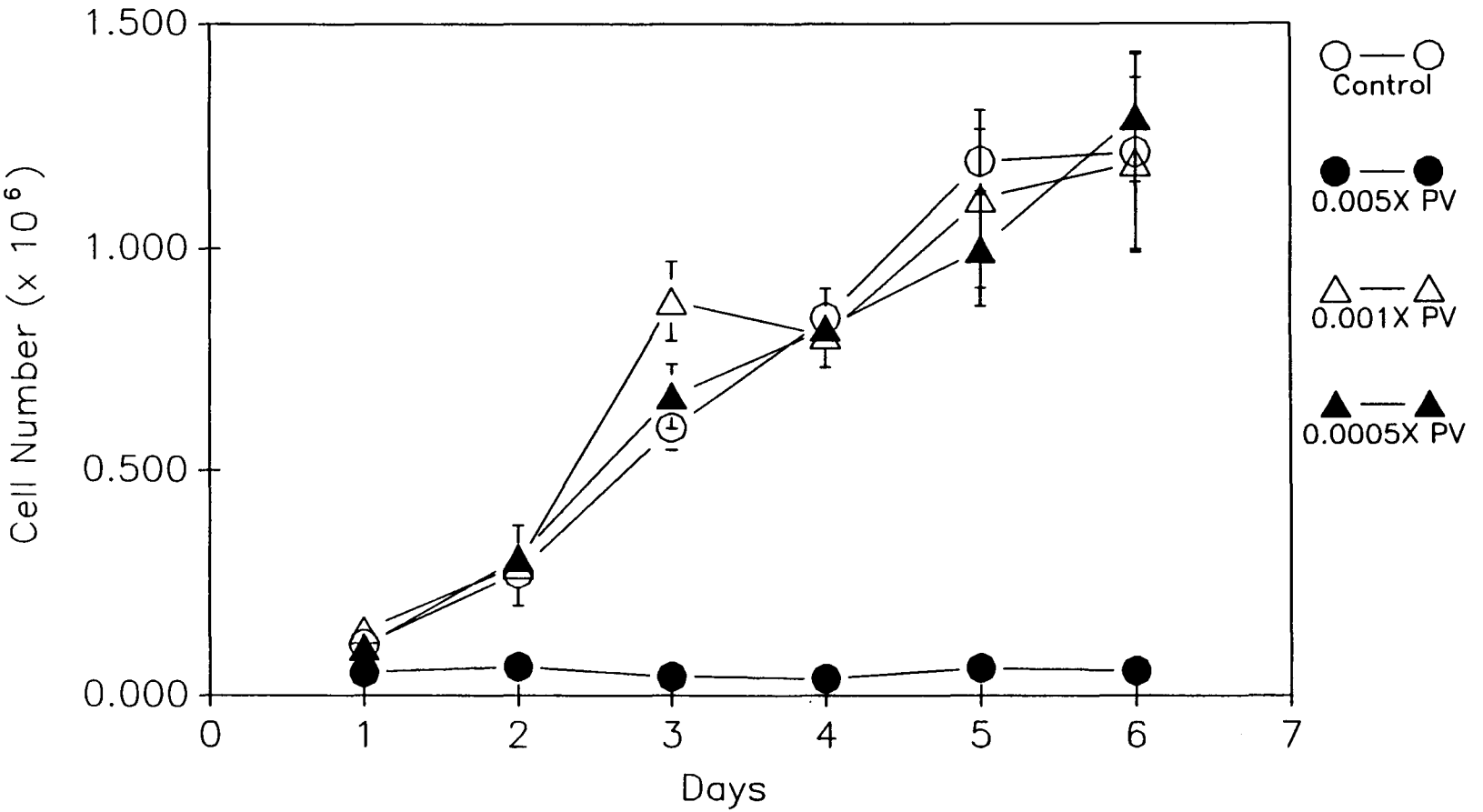
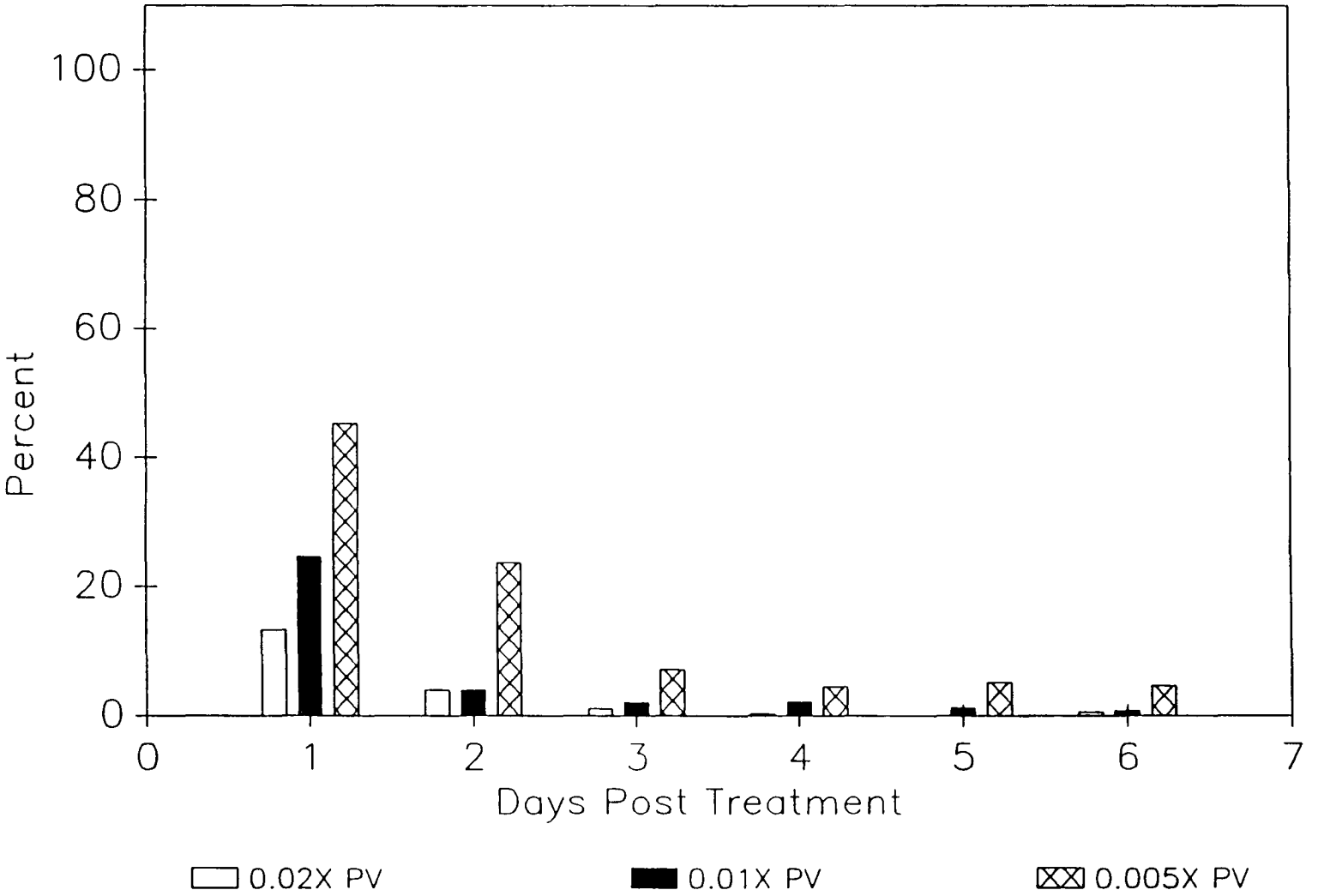

1.500
1.000
0.500
0.000
Cell Number (x 10⁶)
0
1
2
3
4
5
6
7
Days
Control
0.005X PV
0.001X PV
0.0005X PV

Figure 42. The significantly different continuously exposed proliferation assay results represented as percent of control cellular counts. The toxic PV levels were at 0.02X, 0.01X, and 0.005X.


100
80
60
40
20
0
Percent
0
1
2
3
4
5
6
7
Days Post Treatment
0.02X PV
0.01X PV
0.005X PV

the final continuous exposure experiment utilized 0.005X PV with glutaraldehyde, 0.001X PV with glutaraldehyde, 0.005X PV without glutaraldehyde, and 0.001X PV without glutaraldehyde. The PV concentration of 0.005X was more toxic to the fibroblasts in the presence of glutaraldehyde than in its absence (figures 43, 44, and 47). Therefore, the absence of glutaraldehyde attenuated the toxic effects of PV. In contrast, 0.001X PV was not toxic to the cells, irrespective of the presence or absence of glutaraldehyde (figures 45, 46, and 47). These results suggest that PV toxicity may actually be decreased in the absence of glutaraldehyde, i.e., glutaraldehyde may contribute to cytotoxicity.

F. Assessment of the cytotoxic effects of PV-treated demineralised bone matrix in culture

1. *In vitro* proliferation assay

Acid demineralized bone matrix (DMB) is an allograft material that is extensively used in clinical settings. DMB tends to absorb liquids; therefore, after PV treatment, DMB

Figure 43. Comparative growth characteristics of cells grown in the presence of 0.005X PV and glutaraldehyde and control cells.

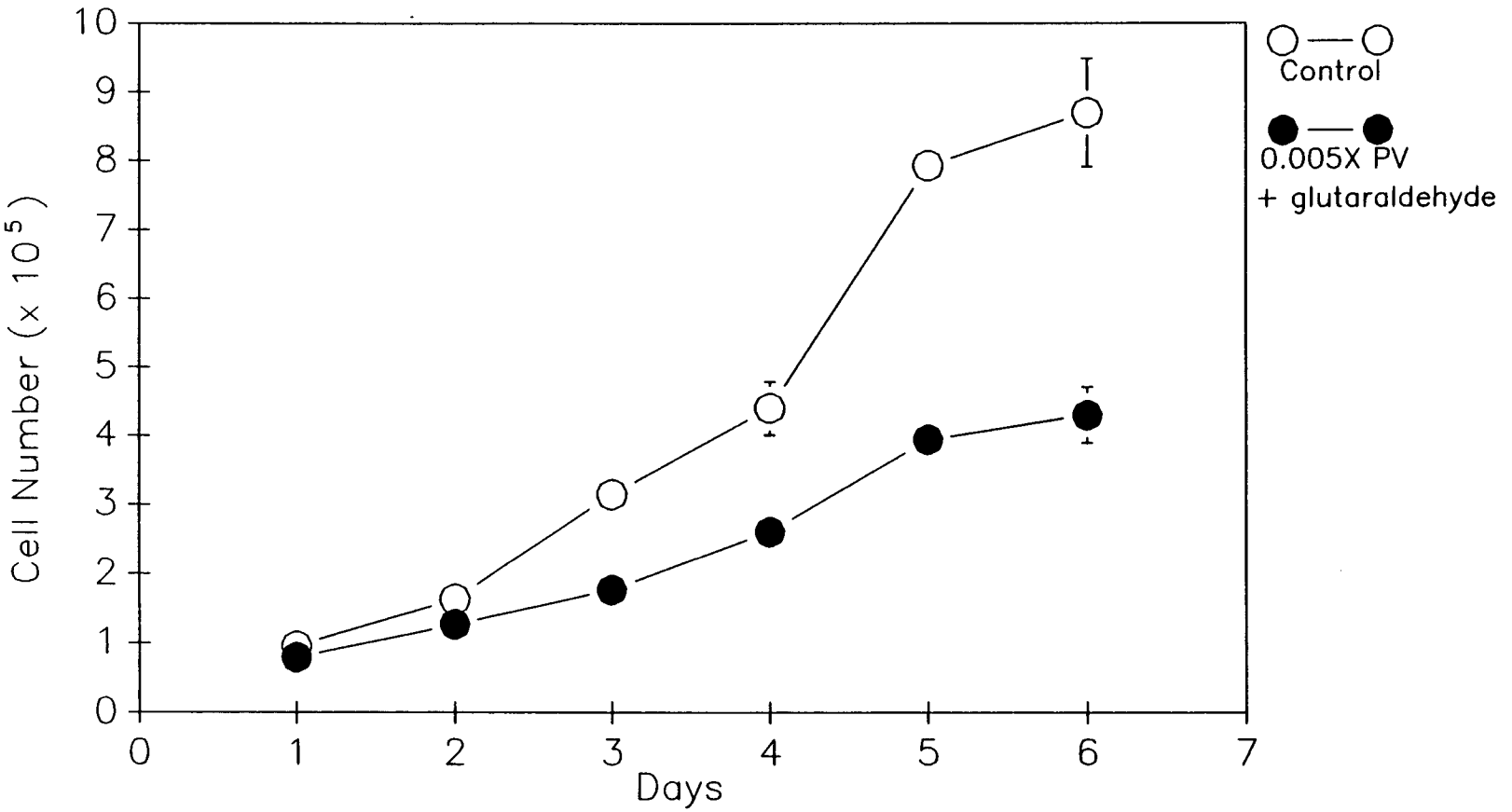

Cell Number (x 10^5)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Days
Control
0.005X PV
+ glutaraldehyde

Figure 44. Comparative growth characteristics of cells grown in the presence of 0.005X PV and no glutaraldehyde and control cells.

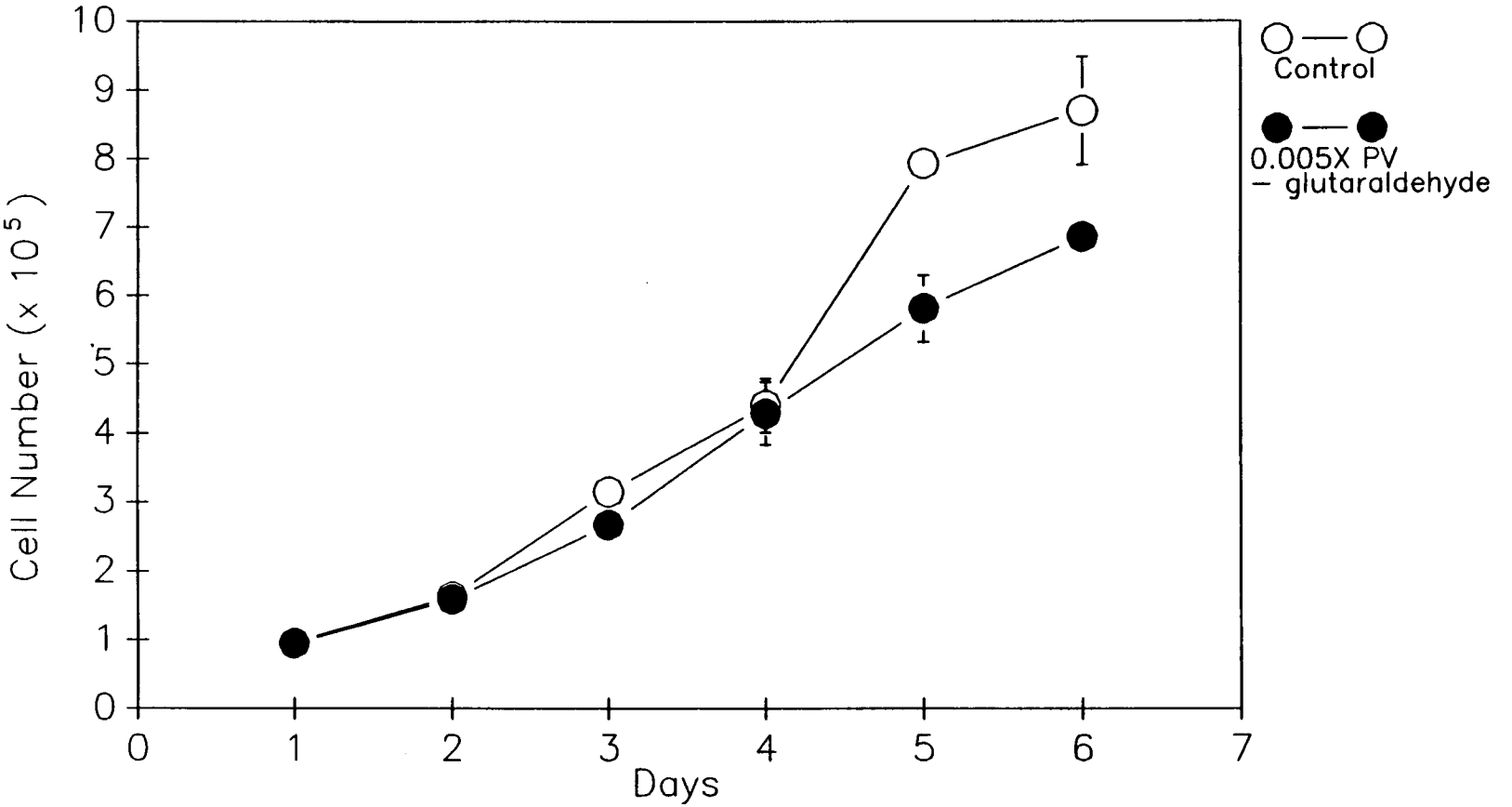

Cell Number (x 10^5)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Days
Control
0.005X PV
– glutaraldehyde

Figure 45. Comparative growth characteristics of cells grown in the presence of 0.001X PV and glutaraldehyde and control cells.

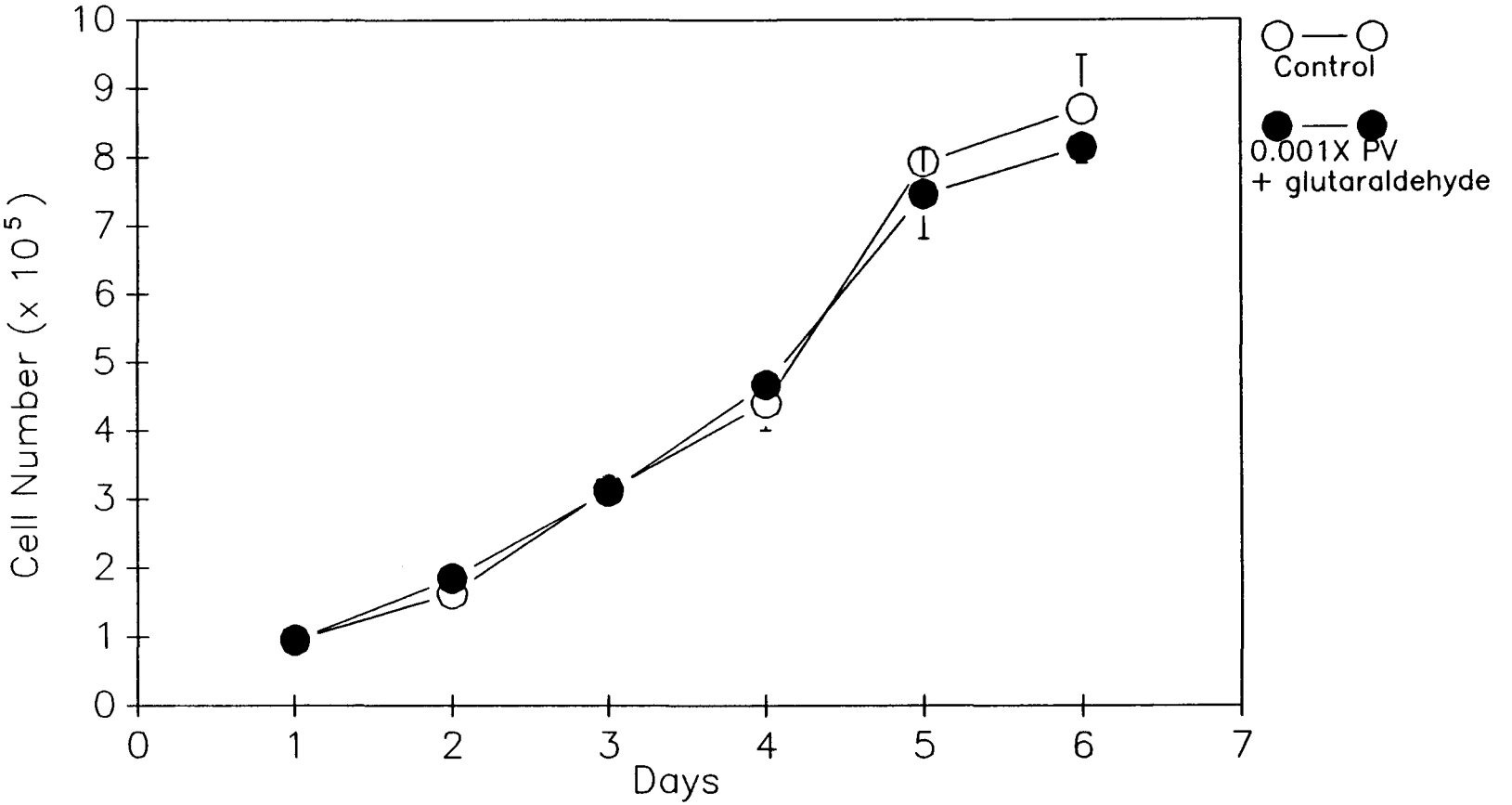

Cell Number (x 10^5)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Days
Control
0.001X PV
+ glutaraldehyde

Figure 46. Comparative growth characteristics of cells grown in the presence of 0.001X PV and no glutaraldehyde and control cells.

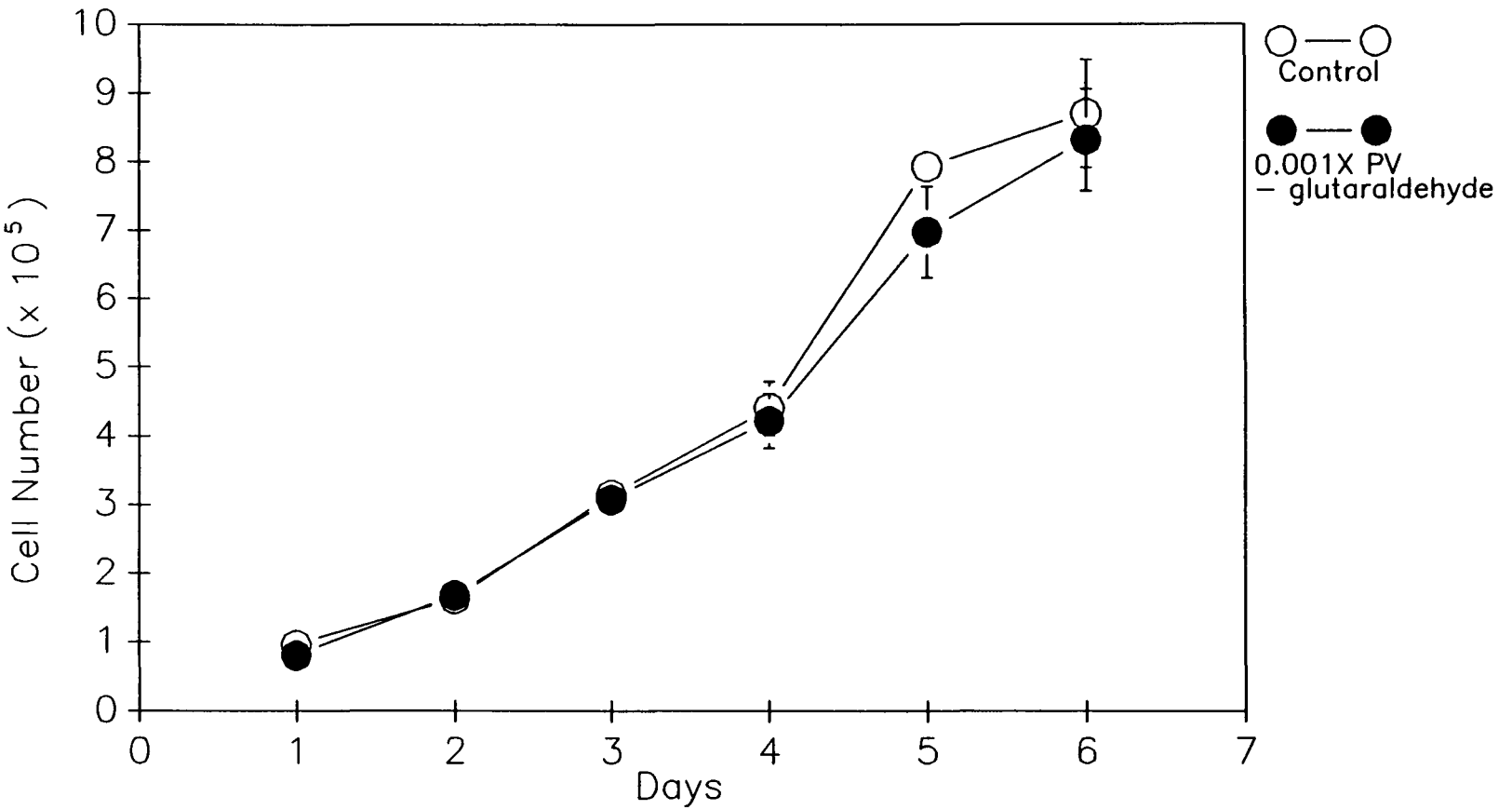
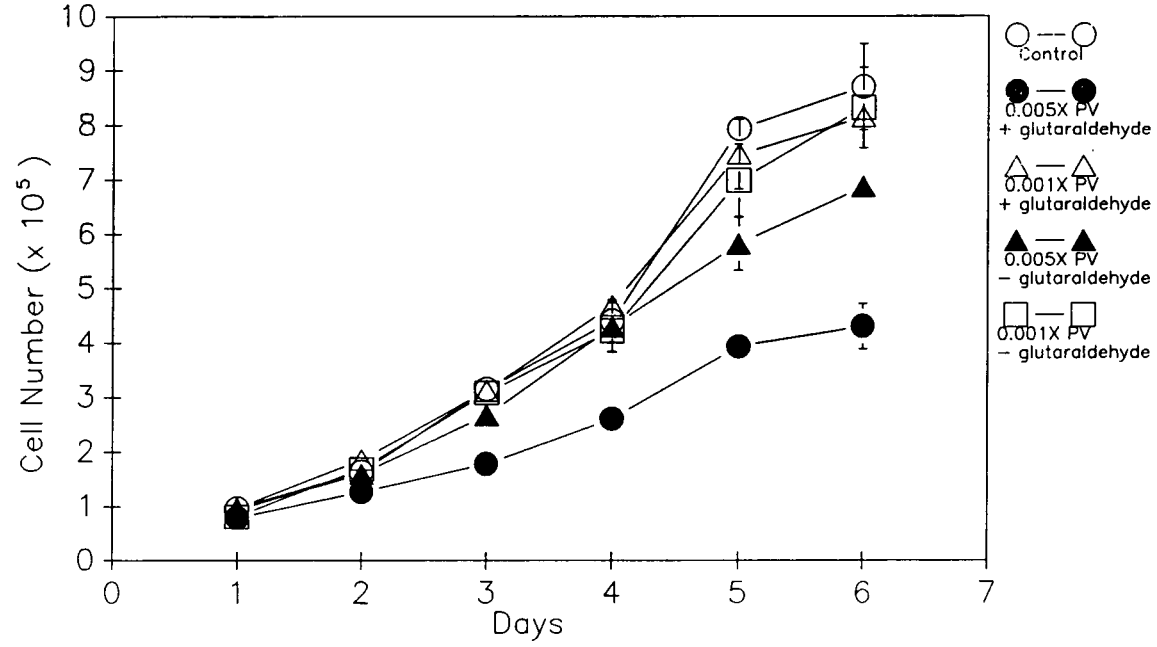
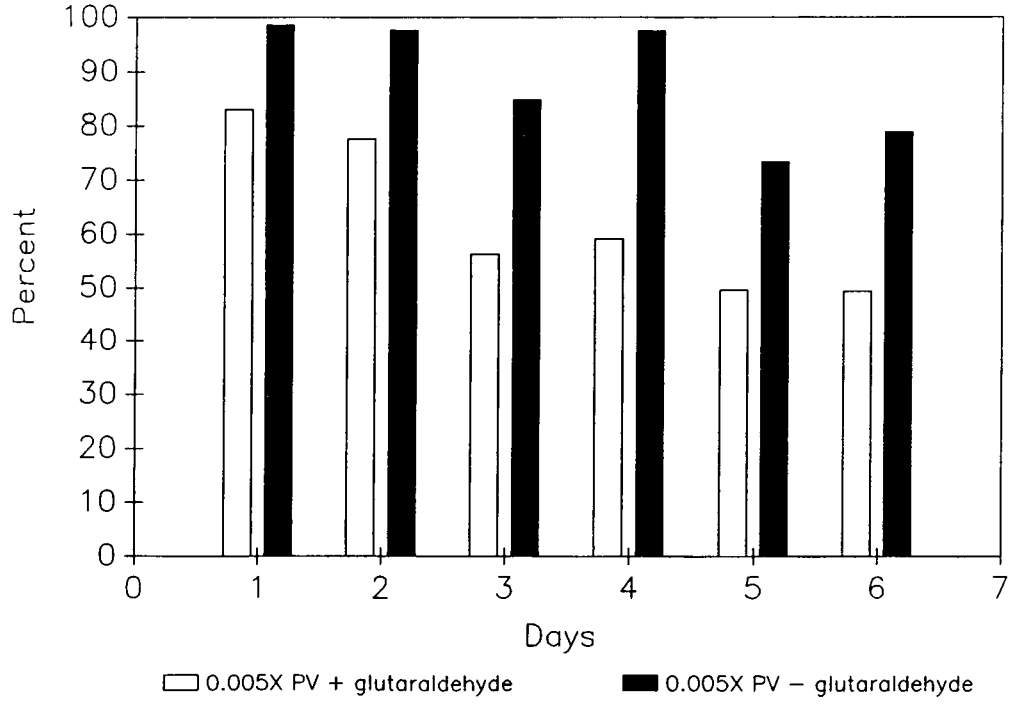

Cell Number (x 10^5)
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Days
Control
0.001X PV
– glutaraldehyde

Figure 47. Summaries of the glutaraldehyde inclusion experiments.
a) Comparative growth characteristics of all treatment groups. b) The significantly different results represented as percent of control cellular counts. The toxic PV levels were at 0.005X +/- glutaraldehyde.

a.


Cell Number (x 10^5)
Days
Control
0.005X PV + glutaraldehyde
0.001X PV + glutaraldehyde
0.005X PV − glutaraldehyde
0.001X PV − glutaraldehyde

b.

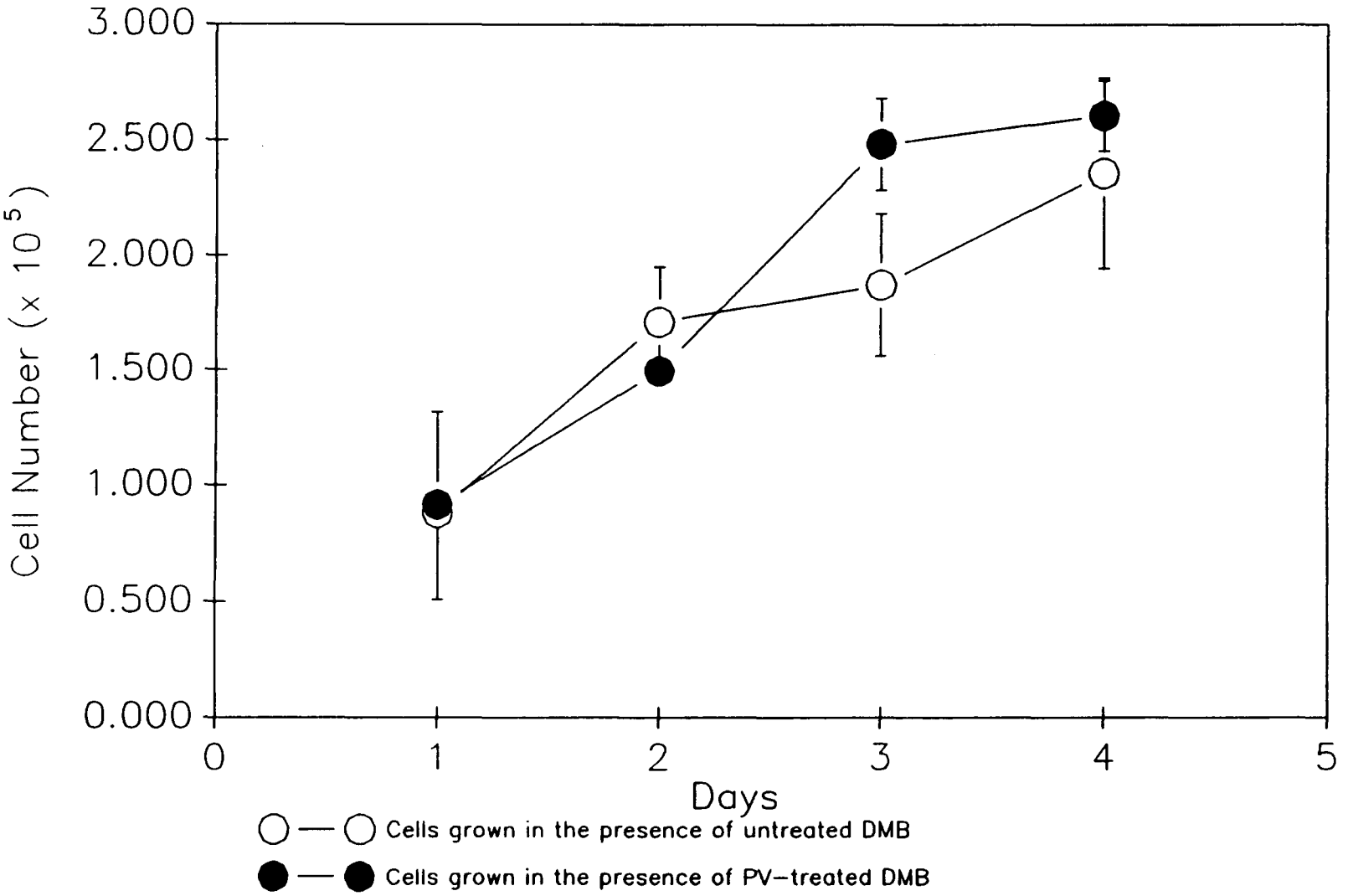

Percent
Days
0.005X PV + glutaraldehyde
0.005X PV − glutaraldehyde

must be rinsed sufficiently so that the residual PV concentration is below the defined toxicity level. In fact, residual PV within DMB may affect subsequent migration of host cells into the graft material following implantation into a patient. Therefore, control and PV-treated DMB were added to human dermal fibroblast cell cultures in order to assess the effects of residual PV. As noted in figure 48, the growth characteristics of fibroblasts exposed to PV-treated DMB were not greatly different from cells exposed to control DMB.

2. Microscopic studies

As determined using a light microscope, the morphology of fibroblasts exposed to PV-treated DMB was slightly different from that of fibroblasts exposed to control DMB. Human fibroblasts grown in the presence of DMB normally differentiate into osteoblast-like cells, exhibiting a diffuse morphological character and developing spindle processes (Wolfinbarger and Zheng, 1993). Cells grown in the presence of PV-treated DMB do not differentiate considerably and tend

Figure 48. Comparative growth characteristics of cells grown in the presence of untreated and PV-treated demineralized bone matrix.

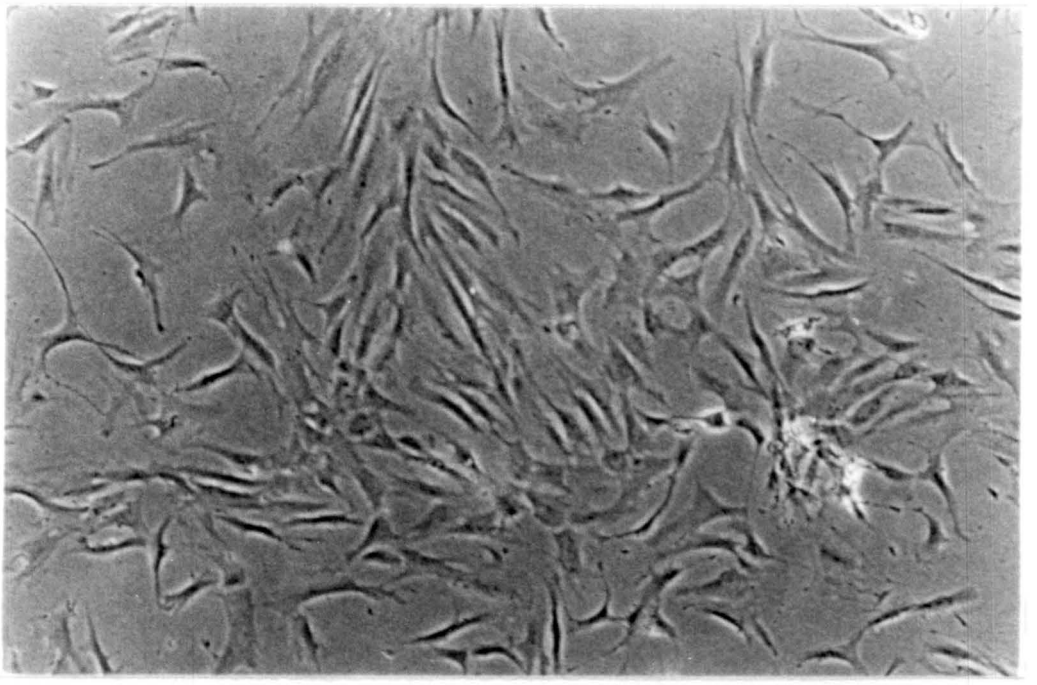

3.000
2.500
2.000
1.500
1.000
0.500
0.000
Cell Number (x 10^5)
0
1
2
3
4
5
Days
Cells grown in the presence of untreated DMB
Cells grown in the presence of PV-treated DMB

to retain the typical fibroblastic morphology relative to the DMB-induced differentiated cells (figures 49).

Scanning electron microscopic studies were performed to determine the ability of cells to grow over and into the PV-treated DMB matrix. The PV treatment did not appear to compromise the fibroblasts' ability to penetrate the matrix (figures 50 and 52). Furthermore, the fibroblasts were able to grow on top of the treated bone particles as well as they did on the nontreated bone particles (figures 51 and 53). These results suggest that PV treatment of DMB does not affect the proliferation of mammalian fibroblasts on and in the allograft matrix. However, the morphological characteristics were slightly different. When fibroblasts that were grown in the presence of DMB became confluent, the layer of cells possessed a smooth texture (figures 50a and 51). Fibroblasts grown in the presence of PV-treated DMB retained the characteristic confluence pattern of typical fibroblasts. They aligned in parallel and circular arrays that were distinct in scanning electron micrographs (figures 52a and 53).

Figure 49. Photomicrographs of subconfluent human dermal fibroblasts grown in the presence of untreated and PV-treated demineralized bone matrix. a) Fibroblasts with PV-treated bone, day 3 (magnification = 100X). b) Fibroblasts with untreated bone, day 4 (magnification = 100X). Amorphous shape in lower right hand corner represents demineralized bone particle.

a.



b.

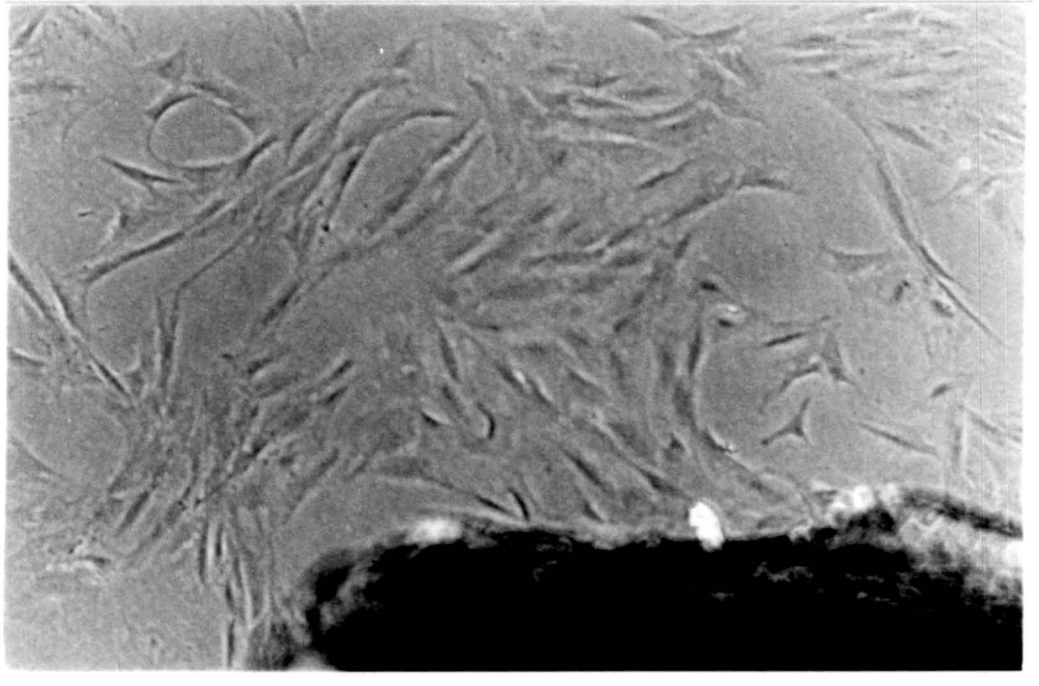


Figure 50. Scanning electron micrograph of human dermal fibroblasts growing in the presence of untreated demineralized bone particles. a) Note smooth texture of the cell monolayer around the bone particles. Arrow represents the area shown in (b) (bar = 1 mm, magnification = 31X). b) Higher magnification of fibroblasts showing the cells growing within the demineralized bone matrix (bar = 100 μm, magnification = 238X).


A

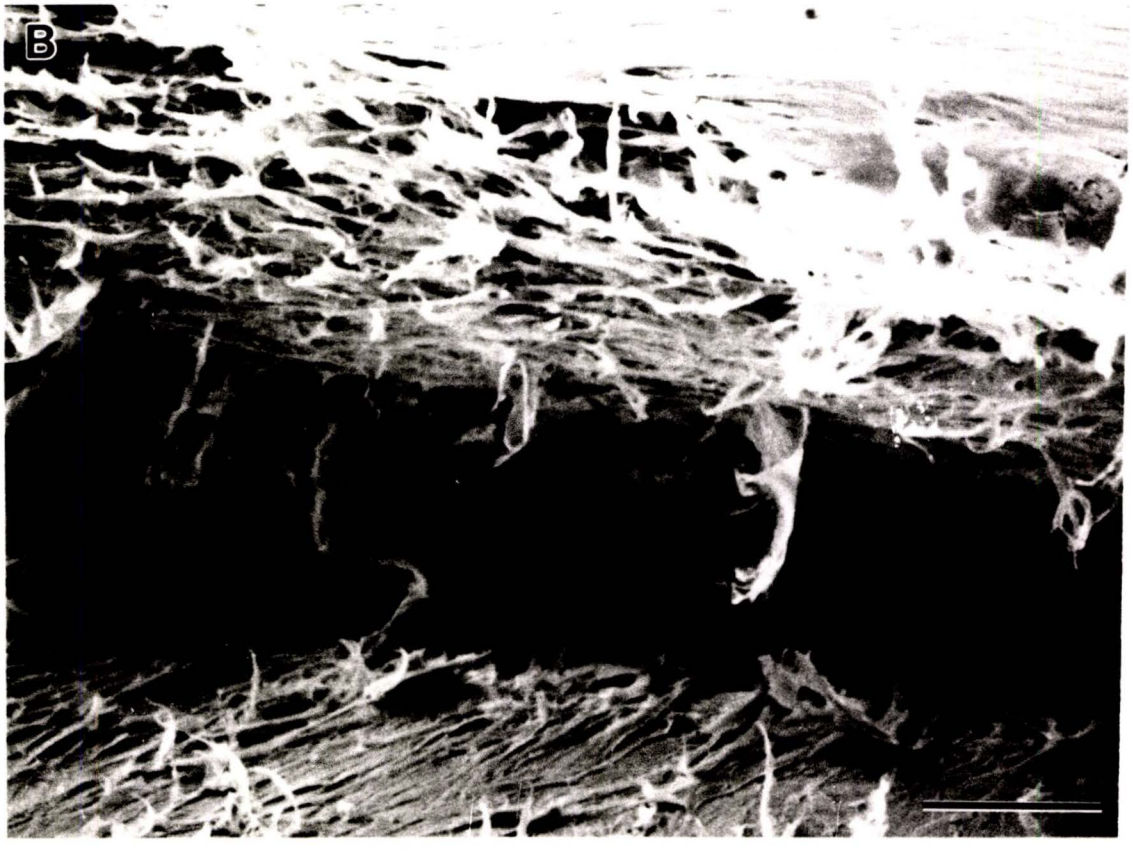
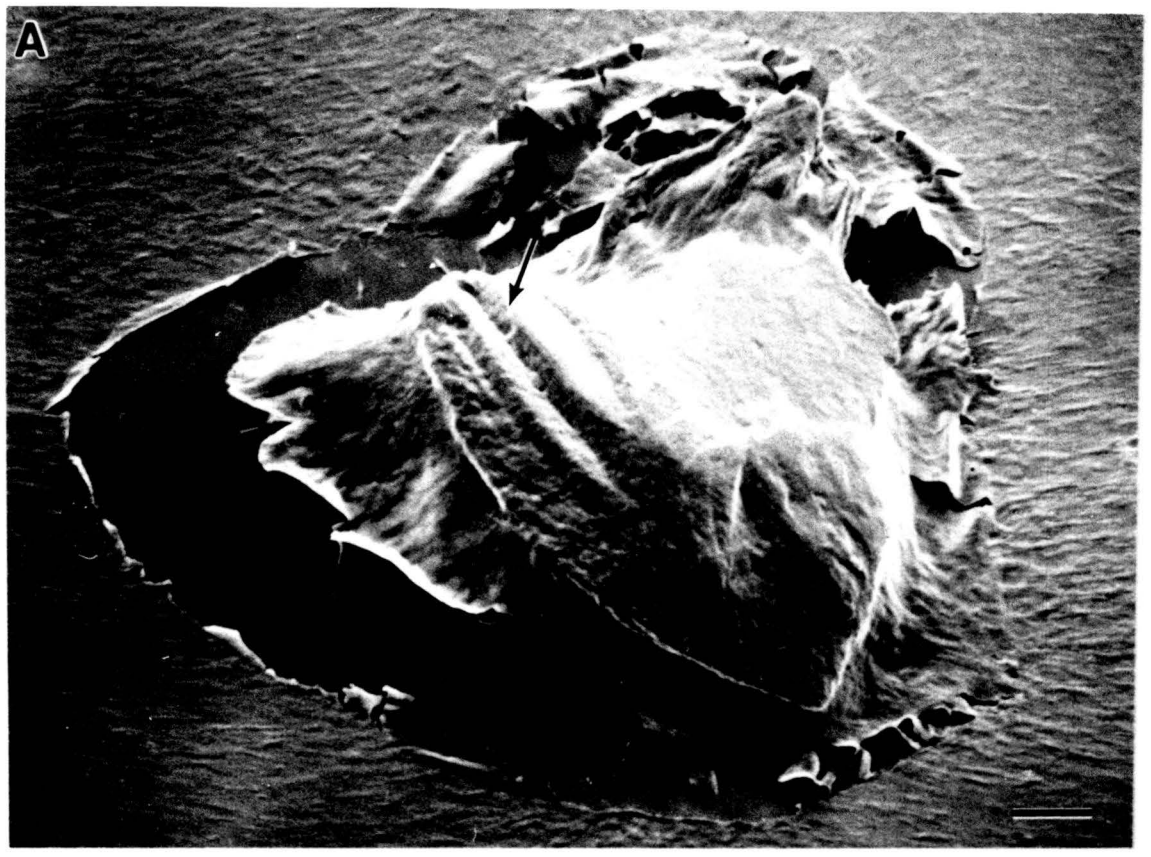

B

Figure 51. Scanning electron micrograph of human dermal fibroblasts growing in the presence of an untreated demineralized bone particle. a) Note smooth texture of the cell monolayer growing over bone particle and on the surrounding surface. Arrow represents the area shown in (b) (bar = 100 μm, magnification = 100X). b) Higher magnification of cell layers growing over a demineralized bone particle (bar = 10μm, magnification = 864X).


A

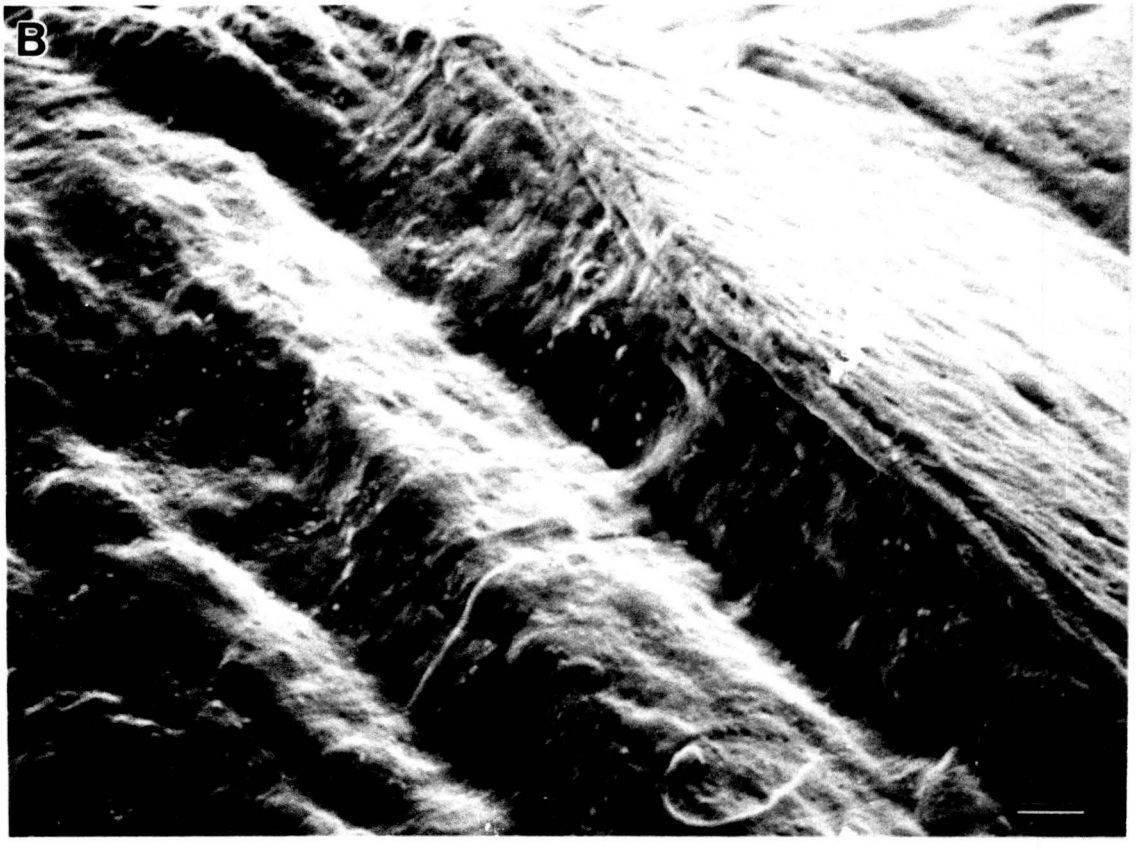
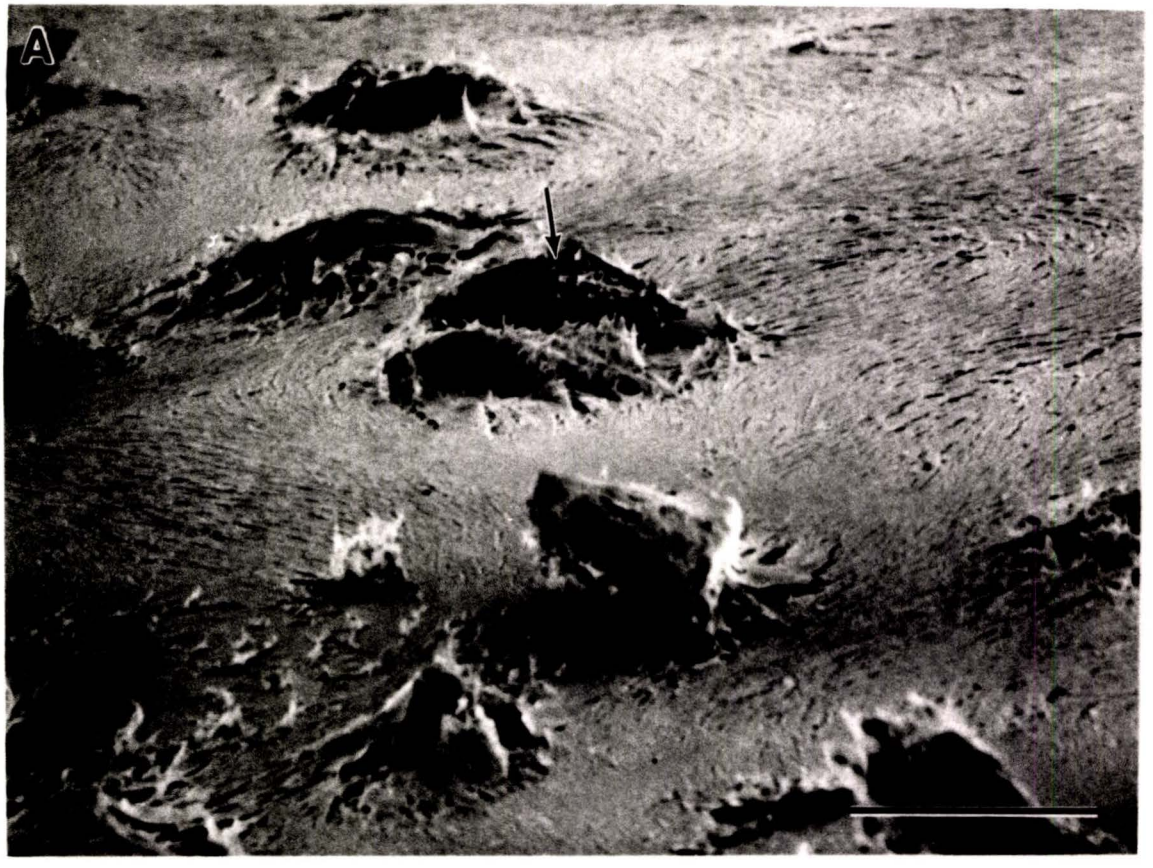
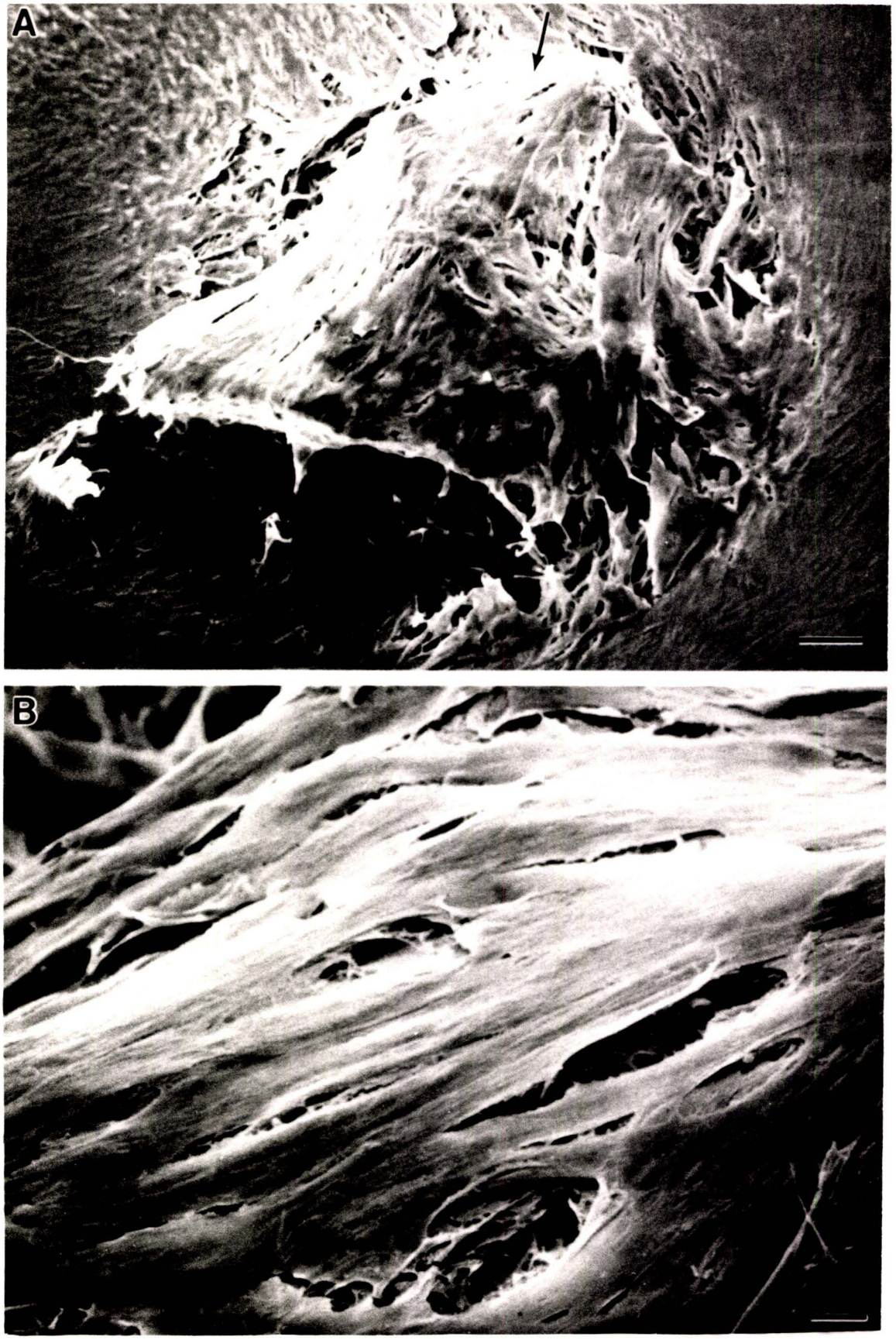

B

Figure 52. Scanning electron micrograph of human dermal fibroblasts growing in the presence of PV-treated demineralized bone particles. a) Note the characteristic circular and parallel arrays of the confluent cells surrounding the bone particles. Arrow represents the area shown in (b) (bar = 1 mm, magnification = 32X). b) Higher magnification of cells growing within the bone matrix (bar = 100 μm, magnification = 191X).


A


B

Figure 53. Scanning electron micrograph of human dermal fibroblasts growing in the presence of a PV-treated demineralized bone particle. a) The cells retain the typical confluent fibroblastic morphology and do not assume the smooth morphology of confluent cultures grown in the presence of untreated demineralized bone matrix. Arrow represents the area shown in (b) (bar = 100 μm, magnification = 100X). b) Higher magnification of cell layers growing over a demineralized bone particle. Note irregular texture (bar = 100 μm, magnification = 810X).


A
B

DISCUSSION

Detergents are known to solubilize membranes and thereby disrupt cellular integrity and function. This fact is the foundation for the concept of inactivating viruses via the use of surfactants. Furthermore, Nonoxynol 9, an operative spermicide by virtue of its solubilizing properties, may be able to interfere with the proper functioning of the HIV reverse transcriptase.

This unique solubilizing characteristic of detergents has led to the development of a liquid panoply of surfactants called Panavirocide (PV) that is capable of rendering viruses noninfectious (Sharma *et al.*, 1990). PV was originally designed to inactivate viruses in biological fluids, including blood, and tissues from patients. Because nonionic surfactants are milder in action than ionic surfactants, three nonionic detergents -- Nonoxynol 9, Brij 35, and Nonidet P40 -- are the active ingredients in Panavirocide. An optimal ratio of the three detergents preferentially lyses free virus

and the leukocyte vectors which harbor HIV. Red blood cells are theoretically not affected (Sharma *et al.*, 1990). To further stabilize the membranes of red blood cells, glutaraldehyde or sucrose is included in the formulation.

As in all potentially infectious material recovered from donors, allografts must be sterilized in a particular fashion to inactivate all pathogens. The requirements for treating allografts differ from those for blood and blood products. Specifically, any flushing of allografts need not require the deliberate and preferential solubilization of only one type of cell. Because PV was initially intended for use with fluids, the formulation may have to be modified for flushing allografts, a process which entails universal destruction of all cell types.

A preliminary step leading to the putative modification of the PV formula involves the determination of critical micelle concentration (CMC) values of the constituent detergents -- Nonidet P40, Nonoxynol 9, and Brij 35. The method devised by Vulliez-Le Normand and Eiselé (1993) is a

simple and efficient technique that can be implemented as a standard protocol for determining CMC values of various detergents. Published results state that the CMC for Nonidet P40 is 0.29 mM (Helenius and Simons, 1975) or 0.11 mM (Neugebauer, 1994). The discrepancy may result from a number of possibilities, including different pH and ionic strengths of the buffers that were used in determining the two values; however, ionic strength should not significantly impact on the CMC values of nonionic detergents (Neugebauer, 1994). The method described in this study was able to determine the value for Nonidet P40 to be 0.234 mM +/- 0.005 mM, which is closer to the reported value of 0.29 mM. Similarly, the CMC values for Brij 35 and Nonoxynol 9 at 0.120 mM +/- 0.026 mM and 0.062 mM +/- 0.008 mM, respectively, were close to the published values of 0.092 mM for Brij 35 (Calbiochem, personal communication, 1994) and 0.0812 mM for Nonoxynol 9 (Rhone-Poulenc, personal communication, 1994).

The determined toxicity level of PV towards human dermal fibroblasts was between 0.005X PV and 0.001X PV. At 1X PV, the individual concentrations of Brij 35, Nonoxynol 9, and

Nonidet P40 are 55 mM, 32 mM, and 33 mM, respectively. These concentrations are decisively higher than the CMC values. At 0.005X PV, the constituent detergent concentrations are still above their respective CMC values with the exception of Nonidet P40 if its CMC is distinguished as 0.29 mM or 0.234 mM. However, at the noncytotoxic level of 0.001X PV, the individual detergent concentrations fall below the CMCs (table 1). These results suggest that the concentration of PV must be above the CMC of the collective detergents in order to effect solubilization. Correspondingly, residual levels must be below the CMC of PV in order to be noncytotoxic.

The initial shelf-life studies using the modified method of Vulliez-Le Normand and Eiselé (1993) suggested that the CMCs of the detergents, and consequently of PV, remain stable with time. This stability is implied by the consistent optical densities of the individual detergents even after six months. However, due to certain quirks inherent in nonionic surfactants, detergent solutions should be made fresh and used within 48 hours.

One quirk derives from the fact that the ratio of ethylene oxide units in nonionic detergents to hydrocarbon chain length affects CMC (Helenius and Simons, 1975). The formation of micelles is driven by hydrophobic interactions; therefore, increasing hydrophobicity, i.e., decreasing ethylene oxide units per unit hydrocarbon, induces micelle formation, causing a subsequent reduction in the CMC value. The polymerization of ethylene oxide units in nonionic detergents is a purely statistical event (Helenius and Simons, 1975). Therefore, unless the detergents are fractionated following manufacture, the ethylene oxide head groups are polydisperse. In fact, the hydrophobic tail of synthetic surfactants may also be heterogeneous due to the manner in which they are synthesized (Helenius and Simons, 1975).

In addition to the inconstant ratio of ethylene oxide units to hydrocarbon units, some polyoxyethylene amphiphiles, by nature, undergo autooxidation in the presence of oxygen (Neugebauer, 1994; Ashani *et al.*, 1980; Lever, 1977). Transition-metal ions and light may expedite this process (Neugebauer, 1994; Lever, 1977). Alcohols can suppress

micelle formation (Neugebauer, 1994); therefore, the aldehyde and peroxide products that are formed from the autooxidation process may also influence micellization. In fact, these by-products may affect a vast array of detergent properties.

One question that must be addressed is the change of CMC when individual detergents are combined. The CMC of a multiple detergent solution is contingent upon the CMC and concentration of the constituent detergents (Helenius and Simons, 1975). The determination of CMC values becomes more complicated in the presence of membrane proteins and lipids (Neugebauer, 1994; Helenius and Simons, 1975). Any calculations of Panavirocide CMC in the absence of membranes must not be relied upon as the sole indicator of the amount of PV with which to flush bone allografts. In an ideal system, the individual detergent or PV concentration must be normalized to some quantity of membrane, i.e., a stoichiometric relation must exist between moles (or molecules) of detergent and moles (or molecules) of membrane solubilized. Table 2 indicates the calculated number of detergent molecules per cell at the initiation of the

Table 2. Number of individual and combined detergent molecules present in the initiation of the *in vitro* proliferation assays for the various PV concentrations used in the studies. Total cells seeded per flask equaled 75,000. Volumes of media were 5 ml for cultures counted on days 1 and 2, 10 ml for cultures counted on days 3 and 4, and 15 ml for cultures counted on days 5 and 6 (see Materials and Methods).

PV concentration	volume of media (ml)	Number of detergent molecules per cell			
		Brij 35	Nonoxynol 9	Nonidet P40	TOTAL
0.02X PV	5	4.4161 E13	2.5694 E13	2.6497 E13	9.6352 E13
	10	8.8323 E13	5.1388 E13	5.2994 E13	1.9271 E14
	15	1.3248 E14	7.7082 E13	7.9490 E13	2.8905 E14
0.01X PV	5	2.2081 E13	1.2847 E13	1.3248 E13	4.8176 E13
	10	4.4161 E13	2.5694 E13	2.6497 E13	9.6352 E13
	15	6.6242 E13	3.8541 E13	3.9745 E13	1.4453 E14
0.005X PV	5	1.1040 E13	6.4235 E12	6.6242 E12	2.4088 E13
	10	2.2081 E13	1.2847 E13	1.3248 E13	4.8176 E13
	15	3.3121 E13	1.9270 E13	1.9873 E13	7.2264 E13
0.001X PV	5	2.2081 E12	1.2847 E12	1.3248 E12	4.8176 E12
	10	4.4161 E12	2.5694 E12	2.6497 E12	9.6352 E12
	15	6.6242 E12	3.8541 E12	3.9745 E12	1.4453 E13
0.0005X PV	5	1.1040 E12	6.4235 E11	6.6242 E11	2.4088 E12
	10	2.2081 E12	1.2847 E12	1.3248 E12	4.8176 E12
	15	3.3121 E12	1.9270 E12	1.9873 E12	7.2264 E12

cultures. According to these determinations, the total number of detergent molecules per cell must be below 1.4453×10^{13} for an unequivocally nontoxic PV concentration.

PV is normally dissolved in Dulbecco's phosphate buffered saline (DPBS). The assay described in this paper is performed in a Tris buffer with added sodium chloride. Because ionic strength may affect the micellization of some surfactants, the assay should also be performed on detergents dissolved in DPBS. Furthermore, the pH of both buffers and the ambient temperature should be regulated and recorded. In short, due to the fact that many external factors may affect CMC values, the protocol must be described completely and every experimental detail must be documented.

After the allografts are flushed with PV, they must be rinsed thoroughly so that the residual levels of the detergents do not affect the host cells upon implantation into a recipient. According to the results of the *in vitro* proliferation assays, the toxicity level of PV to the mammalian fibroblasts used in the study was between 0.005X PV

and 0.001X PV. Therefore, the residual concentration of PV should be approximately 0.001X PV after rinsing. The ambiguous and contradictory results obtained with the commercial PV were most likely due to negligent attention associated with the presence and concentration of the various constituents during manufacture. Different stocks of PV prepared by Medicine and Applied Sciences, Inc. are composed of varying formulations, a fact which was not relayed until after the assays were performed. Furthermore, information regarding the diverse formulations was not forthcoming. When the PV that was manufactured in-house with stringent regulation of the concentrations of the various components was used in the assays, the results demonstrated that the toxicity level of PV towards the cells used in the studies was indeed between 0.005X PV and 0.001X PV.

Glutaraldehyde acts as a membrane stabilizer by covalently linking proteins to adjacent molecules (Alberts *et al.*, 1983). Because PV was originally specified to inactivate infectious material in blood and blood products, the presence of glutaraldehyde was believed to prevent the solubilization

of erythrocytes by preferentially crosslinking and thus strengthening the membranes of these cells. The results from the current study suggest that glutaraldehyde actually increases the toxicity of PV towards the human dermal fibroblasts used in the assays and/or is toxic itself. Evidently, glutaraldehyde does not stabilize the membranes of these fibroblasts. From one viewpoint, because flushing of the allografts requires an inclusive elimination of all membranous structures, including red blood cells and free virus, glutaraldehyde should be deleted from the formulation. In contrast, glutaraldehyde may actually heighten the solubilizing potentials of the nonionic detergents present in PV. A high concentration of glutaraldehyde in PV could even affect the membranes of red blood cells and their viability, thus enabling complete bone marrow solubilization. Glutaraldehyde, however, is inherently unstable and may affect cells in unforeseen ways. It is customarily used for the fixation of biological specimens in microscopy and, in addition to crosslinking the macromolecules within the membranes, causes cells to become permeable to stains (Alberts *et al.*, 1983). Its ultimate impact on the membranes of viable

cells in the presence of detergents is uncertain. It should therefore be excluded from the PV formulation used to flush allografts. Indeed, in view of the fact that glutaraldehyde affects cellular membrane permeability via covalent linkages, the membranes, and consequently certain functions, of erythrocytes may become permanently altered. Hence, the incorporation of glutaraldehyde in PV formulations used for biological fluids should also be carefully evaluated.

One of the main difficulties with membrane stabilizers, and indeed with any additive, is that the synergistic actions of the additives with the nonionic detergents are generally unknown or, at best, ambiguous. In PV lots (1) and (2), glutaraldehyde presumably served as a membrane stabilizer. As mentioned previously, the effect of glutaraldehyde in the presence of nonionic detergents is indistinct. Other formulations contain sucrose as a membrane stabilizer. However, sucrose may actually increase the effective solubilization capabilities of certain nonionic detergents (Helenius and Simons, 1975; Thompson, 1970). Thus, the justification for including sucrose in certain PV formulations

to stabilize the membranes of erythrocytes becomes moot.

Divalent cations can also stabilize the membrane structure (Helenius and Simons, 1975; Schnaitman, 1971a; Schnaitman, 1971b). The most effective way to deliver the cations would be in their salt form. However, ionic strength affects the solubilization proficiency of detergents and may also affect detergent characteristics, i.e., the micellar properties and the CMC values. Ultimately, any PV formulation that is utilized either in allograft flushing or for biological fluids must submit to a rigorous analysis of effectiveness, as determined by CMC value calculation, and cytotoxicity.

Many cytotoxicity tests are performed *in vivo* using animal models such as rodents and rabbits (Draize *et al.*, 1944; Faccini *et al.*, 1992; Merrick *et al.*, 1992). The Draize eye irritation test scores from rabbits calculate the irritant and lesion-inducing properties of various products (Draize *et al.*, 1944; Merrick *et al.*, 1992). Extrapolation of the results obtained from the rabbit system to predict human

responses is generally acceptable; however, the test itself is imperfect. It requires subjective analysis of the data, resulting in major variations among individual tests and individual laboratories (Merrick *et al.*, 1992; Weil and Scala, 1971). Furthermore, rabbits tend to be more sensitive to irritants, and the circumstances in which exposure occurs differ between rabbits and humans (Merrick *et al.*, 1992). These problems, in correlation with the inhumane aspects of the procedure, have led to the evolution of alternative *in vitro* testing (Goldberg and Frazier, 1989; Goldberg *et al.*, 1993).

One of the major paradigms for *in vitro* toxicity testing involves the use of cultured cells. Exposure to a substance leading to changes in cellular viability (Barile *et al.*, 1993; Borenfreund and Puerner, 1984; Catchpoole and Stewart, 1993; Ciapetti *et al.*, 1993; Hansen *et al.*, 1989; Husoy *et al.*, 1993; van de Sandt *et al.*, 1993; Yamashoji *et al.*, 1992), metabolic activities (Ciapetti *et al.*, 1993; Galli *et al.*, 1993; Gulden, 1993; Husoy *et al.*, 1993), morphology (Catchpoole and Stewart, 1993; Gueniche and Ponec, 1993;

Gulden, 1993; Kndryk *et al.*, 1992;), proliferation (Boyce and Holder, 1993; Gueniche and Ponec, 1993; Hansen *et al.*, 1989; Hopkinson *et al.*, 1993; Kndryk *et al.*, 1992; Rao and Otto, 1992), and other cellular attributes (Barile *et al.*, 1993; Catchpoole and Stewart, 1993; Galli *et al.*, 1993; Gueniche and Ponec, 1993; Gulden, 1993; Hopkinson *et al.*, 1993; Husoy *et al.*, 1993; van de Sandt *et al.*, 1993; Yamashoji *et al.*, 1992) is indicative of the toxicity of the compound. Proliferative and morphological alterations were followed to ascertain the toxicity of PV. The results thus obtained are not comprehensive. Because the lipid and protein properties of cellular membranes differ according to cell type (Chapman, 1976; Merrick *et al.*, 1992), the extent of PV-induced cytotoxicity in other cells may not be analogous to the toxic effects observed in the human dermal fibroblasts used in these studies. Indeed, Merrick *et al.* (1992) determined that human keratinocytes were better suited for their toxicity screening experiments than rat hepatocytes and suggested that the cultured cells used for *in vitro* cytotoxicity testing should be specific to the target tissue that would be affected *in vivo*. Because fibroblasts may be the primary cell type to

migrate to and differentiate within a transplanted allograft, these cells are best suited for the *in vitro* cytotoxicity studies.

The delineated toxicity level of 0.001X PV cannot be extended to encompass all individuals. The cells that were used in the investigations were derived from only one organ donor. Although all human fibroblasts originating from the dermis possess conforming qualities, the generalization that the response from every such cell is unequivocally identical cannot be made. Fibroblasts stemming from various organ donors may react to surfactants in distinct manners. In addition, *in vitro* models cannot duplicate the numerous intricate relationships that occur *in vivo*. However, any variation from the determined PV toxicity level among individuals can reasonably be assumed to be slight.

Another weakness associated with *in vitro* cytotoxicity testing is that the allograft milieu is much larger than the environment of cultured cells. This problem becomes particularly complex when dealing with surfactants and their

ability to integrate into the plasma membrane or to extract elements out of it. One of the ultimate purposes of these studies is to define the concentration of PV and of the individual components with which to flush bone allografts in order to solubilize all potentially infectious materials and their vectors. Therefore, the surfactant-to-membrane ratio becomes crucial. A low surfactant-to-membrane ratio results in incomplete or no solubilization of the critical factors. In contrast, a high ratio would be cytotoxic. A PV concentration that solubilizes all membranes in a culture flask may not be able to do so in the allograft. Therefore, as mentioned previously, instead of defining PV amounts by final concentration in liquid, they may have to be specified by the concentration of PV per unit membrane, where the unit membrane is some predetermined quantity.

Additionally, the defined toxicity level towards the cells used in the study may not be the measure of toxicity *in vivo*. The second goal of these studies is to ascertain the residual quantity of PV that may safely remain within the allograft. Even if the concentration is above the determined

toxicity level of 0.001X PV, residual amounts of PV in the flushed allograft may not be cytotoxic when the bone is transplanted into a recipient. The rationale is the same as above, i.e., the actual system is much larger than the experimental system, and any cytotoxic effects of the residual PV become diluted in the context of the host system. However, because higher residual concentrations of PV within the allograft may not be toxic to the recipient, correspondingly higher concentrations of PV may be used to solubilize membranous structures in the allograft completely. The problem of potentially incomplete solubilization of bone marrow thus becomes amended.

Because the *in vitro* models have not yet been perfected, the experiments performed in these studies must be followed by the implantation of PV-treated materials or by the injection of PV into an animal model, preferably rodents. The animal studies would not only aid in the designation of a physiological toxicity level of PV but also provide the opportunity to study any immunological/inflammatory responses to the detergent formulation.

Bone allografts can evoke immunological responses that are cell-mediated, rather than humorally mediated, events. Surface transplantation antigens that are generated from the bone donor's major histocompatibility complex provoke the activation and proliferation of killer-suppressor T-cells (Stevenson and Horowitz, 1992). Techniques designed to preserve the allograft, such as freeze-drying and deep-freezing, may decrease its ability to stimulate alloreactivity (Friedlaender, 1987; Friedlaender, 1991; Guo *et al.*, 1991; Solomon, 1991; Stevenson *et al.*, 1991; Virolainen *et al.*, 1993). Demineralization, autolysis, and chemical extraction may further reduce its antigenicity (Guo *et al.*, 1991). Flushing allografts with PV may serve a dual function by inactivating all potentially infectious materials within the grafts and by decreasing or abolishing their antigenic properties. However, the PV-flushed graft itself may be immunogenic by virtue of the residual PV. The optimal manner in which to determine the immunogenicity of PV would be to implant PV-treated bone or to inject PV directly into an animal system, specifically mice. Any putative immune response must be qualified as well as quantified by

differentiating between a specific immunological reaction and a non-specific inflammation and by ascertaining the magnitude of the effect. The response must be further described to consider its potentially beneficial nature, i.e., not all immune reactions are indicative of graft rejection. The immune system actually plays a pivotal role in the successful assimilation of a bone allograft (Virolainen *et al.*, 1993). Furthermore, products of these immunocompetent cells help in the repair of fractures and in the regulation of bone homeostasis (Friedlaender, 1991).

A study by Horowitz and Friedlaender (1991) may be invoked as an argument against the feasible role of PV in reducing or destroying the alloreactivity stimulated by a bone allograft. In a division of their experiment, removal of the bone marrow did not affect the antigenicity of bone. However, the method by which they removed the bone marrow, "extensive flushing" with presumably saline, was not appropriate for a comprehensive extraction. This technique does not remove the adherent cells, including osteoclasts, that line the endosteal bone surface. These cells may express class-I and class-II

determinants on their surface which stimulate the immune system. Detergent flushing, followed by a succession of rinses, would serve to extract bone marrow more thoroughly by solubilizing all cells, including the adherent ones.

A major problem associated with the universal use of PV for bone allografts is potentially compromising the osteoinductive capabilities of the grafts, particularly of demineralized bone matrix (DMB). Osteoinduction can be defined as the induced ability of connective tissue to transform into osseous tissue (Glowacki and Mulliken, 1985). The inducers either mediate the effects of other factors or exert their own effects to modulate or control bone remodeling. The various compounds involved in osteoinduction and their individual functions have not yet been completely elucidated. However, bone morphogenetic protein (BMP), a term given to a group of acid-insoluble glycoproteins that can be extracted from acid-demineralized bone matrix, is thought to play an arguably critical role (Aspenberg *et al.*, 1988; Schwarz *et al.*, 1991).

DMB is an osteoinductive, calcium-free allograft material that is used extensively in clinical settings. It has increasingly comprehensive application in the repair of osseous defects. When grown in the presence of DMB, human dermal fibroblasts assume the properties of osteoblasts or chondroblasts (Wolfinbarger and Zheng, 1993). They lose the characteristic spindle-shaped morphology of fibroblasts and become more diffuse in nature. Furthermore, alkaline phosphatase can operate as a surrogate marker of cellular differentiation.

The results obtained from the present study suggested that PV did not adversely affect the ability of fibroblasts to grow in the matrix and on top of PV-treated DMB. Indeed, as determined by light and electron microscopy, the morphology of fibroblasts grown in the presence of PV-treated DMB appeared to resemble the original, nondifferentiated fibroblastic shape. Confluent cultures also retained the typical parallel and circular array pattern that is absent in differentiated cultures, i.e., in cultures that are grown in the presence of untreated DMB. In addition, fibroblasts tend to grow more

quickly in the absence of DMB than in its presence. These observations indicate that PV treatment may extract the inducers, including BMP, from the DMB and thus compromise osteoinductivity. PV application will be effected at the whole, intact allograft level, immediately after harvesting; however, even at this stage, PV could putatively extract the critical osteoinducing factors. Ethylene oxide sterilization was shown to destroy the bone induction compounds dose-dependently (Aspenberg, 1990; Solomon, 1991). Ethylene oxide is the starting material for the production of nonionic detergents, the active ingredients in PV. Therefore, PV may jeopardize bone osteoinduction properties by degrading the factors and/or by extracting them. Moore *et al.* (1990) determined, however, that the judicial application of ethylene oxide for the sterilization of bone did not inactivate osteoinductive capabilities. Furthermore, in the present research, DMB was soaked in a 1X stock solution of PV for 30 - 40 hours. PV flushing of intact allografts will not involve such an extended exposure period or, indeed, such a high PV concentration. Accordingly, an optimal PV concentration will balance the complete solubilization of the membranous

components and the retention of osteoinducing elements.

Initial studies have been performed in our laboratory assessing unknown concentrations of the three detergents using high performance liquid chromatography (HPLC). According to the results, the detectability of the detergents diminish with time. This result may be explained by the oxidizing impurities, i.e., the peroxides and aldehydes, that invariably form in polyether detergents; furthermore, alcohols suppress micellar formation. The detergents were placed in methanol for evaluation. If the HPLC analysis is based on micellization of the detergents, then the results may be misleading depending on the age of the detergents. As a precautionary measure, PV and its constituents should be made fresh and used within 48 hours for analyses and application.

As in all areas of research, the resolution of specific problems invariably leads to the inception of new ones. The work described in this paper is a mere prologue to the exhaustive characterization of PV that must be performed in order to understand fully its solubilization properties and

its effects on bone. Future research on PV should include, but not be limited to, i) the effects of glutaraldehyde in the formulations used for blood and blood products, ii) calculation of CMC values for PV and its detergents with varying experimental conditions, including buffers, pH, and ionic strength, iii) *in vitro* PV toxicity screening on erythrocytes and leukocytes, the target cells, iv) PV toxicity testing on cells derived from different individuals, v) the identification of the physiological response to PV using an animal model, and vi) determination of the PV concentration with which to flush bone allografts in order to maintain their osteoinductive capabilities. The answers to these and other questions will ultimately result in the effective and routine use of PV in allograft processing protocols.

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