樹状細胞や活性化リンパ球を用いた癌治療の開発も実施されている．表4には自家細胞を用いた研究の代表例をあげたが，同種他家細胞を用いた製品の開発も急速に進んでいる（表5）．また，図9にEUでの開発状況をまとめたが，癌免疫療法の臨床研究が最も多く，ついで心血管系治療が多くなっており，我が国の趨勢と異なる点があることが分かる．

このような細胞治療薬の急速な開発状況に対応するために，欧米でも既にいくつかの指針等が作成されている[3,5,27-29]．これらの指針等で最も重要視されている安全性上の課題は，ウイルス等の感染症伝播をいかに防止するかである．細胞治療に用いる細胞は滅菌や高度な精製といった処理ができないため，ウイルス等の感染因子が混入した場合に患者ばかりでなく患者の家族等へ感染が広がる危険性がある．すなわち個の安全性ばかりでなく公衆衛生の観点からも，製品の安全性を担保することが最も重視されている．

また，不適切な製造による不良品の製造，不適切な製品の取扱いや使用による問題の発生を防止することが目的とされている．これらの問題への対処を定めることにより，高品質で安全性の高い細胞組織利用医薬品等の開発を推進することができると考えられる．

2）原材料となる細胞・組織の由来とウイルス安全性

細胞組織利用医薬品では，原材料として用いられる細胞・組織が自己由来であるか非自己であるかを明確にし，細胞・組織の入手方法およびその生物学的特徴について説明し，当該細胞・組織を選択した理由を明らかにすることが必要である．特にウイルス安全性に関しては，原材料となる細胞・組織の適格性について，HBV，HCV，HIV，HTLV，ヒトパルボウイルスB19，さらに必要に応じてサイトメガロウイルスやEBウイルスについて，血清学的試験や核酸増幅法等の検査を行う必要がある[30]．さらに，ウイルス等の検査においては，ウインドウ期の存在を念頭において，適切な時期に再検査を行うことが推奨されている．

ただし，自己由来の細胞・組織を用いる場合は，感染因子に関して必ずしもドナースクリーニングを行う必要はないとの考え方もある．しかし，自己由来の細胞・組織を用いる場合においても，製造従事者への安全性や製造工程へウイルス陽性原料を持ち込む可能性について十分な配慮が必要であり，必要に応じて上記したウイルス否定試験や迷入ウイルス試験の実施や，培養工程で特定のウイルス増幅が起きないことを確認することが必要となる．

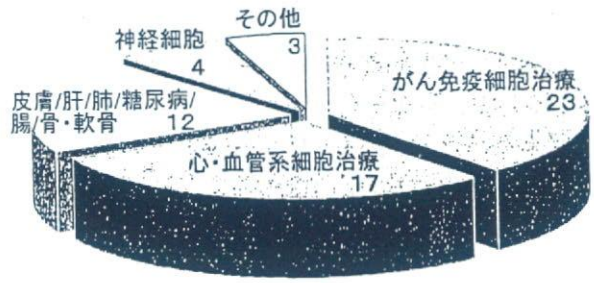

図9　EUにおける体細胞治療臨床研究申請件数
（2004.8 ～ 2006.10）

表5　同種細胞治薬等の開発状況

細　胞	供給源	対象疾患	方　法
培養皮膚（真皮）	割礼組織	難治性潰瘍，重症熱傷	ヒト線維芽細胞を培養
造血幹細胞	臍帯血	白血病治療	臍帯血造血幹細胞の増幅
リンパ球輸注	同種	移植片対宿主病(GVDH)抑制	同種造血幹細胞移植後にドナーリンパ球輸注
神経幹細胞	ヒト胎児脳細胞	セルロイド・リボフスチン症	ヒト胎児脳細胞増殖
神経幹細胞	ヒト胎児脳細胞	脳卒中	ヒト胎児神経幹細胞培養
間葉系幹細胞	ヒト骨髄由来	造血幹細胞移植 GVDH 抑制	ヒト間葉系幹細胞増幅
ヒト造血幹細胞	臍帯血	造血幹細胞移植	臍帯血造血幹細胞増幅

原材料となる細胞・組織について，安全性確保上必要な情報が確認できるように，ドナーに関する記録が整備，保管されていることが必要である．これらの記録の保管は，製造記録とともに製品の最終有効期限より少なくとも10年間とされている．この期間については，今後，遅発性感染症の情報によっては再検討が必要とされている．また同様の観点から，治療の成否の検証や患者等が感染症を発症した場合等の原因究明のために，採取した細胞・組織の一部等の適当な試料を，適切な期間保存することが推奨されている．

採取した細胞・組織について，細胞の採取収率，生存率や細胞・組織の特性解析と平行して，微生物汚染がないことを示す検査を行う必要がある．

3)　細胞培養方法

製造工程で細胞培養を行う場合は，培地の組成，培養条件，培養期間，収率等を具体的に記載することが求められている．使用する原材料は，医薬品又は医薬品原料に匹敵する基準で品質管理されているものを用いる必要がある．血清は，必須でなければ使用しないことが望ましく，使用が避けられない場合には，血清からの感染因子の混入・伝播の防止策を設ける必要がある．血清を使用する場合には，混入が想定されるウイルスについて否定試験を行ったものを使用する必要があり，さらに可能な限りγ線照射等の処理を実施し，潜在するウイルスの低減化・不活化を行う必要がある．

4)　高感度ウイルス検出法

輸血でのウイルス感染に関しては，数コピーから数十コピーのウイルスで感染が起きる場合があることが知られており，細胞治療薬のようにウイルスの不活化・除去工程が実質できない製品の場合には，可能な限り高感度なウイルス否定試験の開発が望まれている．現在最も高感度なウイルス検出法としては，PCR などの核酸増幅検査（NAT）があげられるが，NAT を用いても，ウイルス感染初期のウインドウ期や低濃度キャリアーではウイルスゲノムの検出が不可能な場合があることが知られている．従って，ウイルス濃縮法等を利用することによるウイルス検出の高感度化ができれば，細胞治療薬のウイルス安全性に大きく貢献することが期待出来る．我々は，新規ウイルス濃縮法としてポリエチレンイミン磁気ビーズ（PEI 磁気ビーズ）を用いた手法を開発し[15]（図10），PEI 磁気ビー

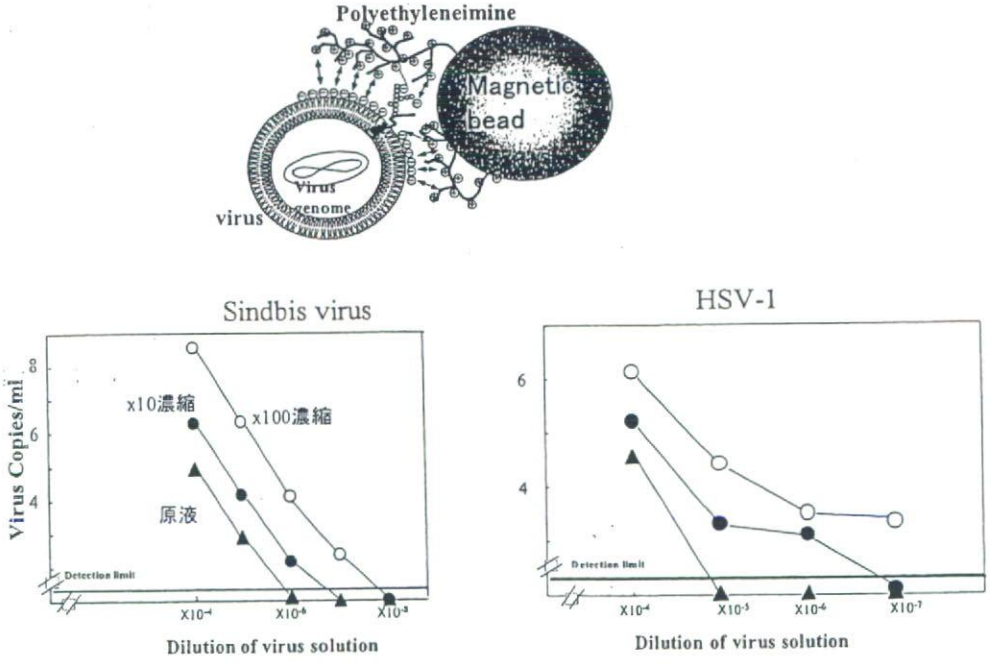

図10　PEI 磁気ビーズを用いたウイルス濃縮

表6　PEI磁気ビーズによるウイルスの濃縮結果

ウイルス	宿主	ウイルスゲノム	脂質膜	サイズ（nm）	PEI-磁気ビーズ濃縮
モデルウイルス					
サイトメガロウイルス	サル	DNA	＋	180-200	＋
ヘルペスウイルスⅠ型	ヒト	DNA	＋	150-200	＋
水疱性口内炎ウイルス	ウシ	RNA	＋	70-150	＋
同種指向性マウス白血病ウイルス	マウス	RNA	＋	80-110	＋
Sindbis ウイルス	ヒト	RNA	＋	60-70	＋
アデノウイルス5型（Ad-5）	ヒト	DNA	－	70-90	＋
SV-40ウイルス（SV-40）	サル	DNA	－	40-50	＋
ブタパルボウイルス（PPV）	ブタ	DNA	－	18-24	＋*
ポリオウイルス Sabin 1 型	ヒト	RNA	－	25-30	＋**
ヒト感染性ウイルス					
ヒト免疫不全ウイルス（HIV）	ヒト	RNA	＋	80-100	＋
B型肝炎ウイルス（HBV）	ヒト	DNA	＋	40-45	＋
C型肝炎ウイルス（HCV）	ヒト	RNA	＋	40-50	＋
A型肝炎ウイルス（HAV）	ヒト	RNA	－	25-30	＋*

＊：条件により濃縮されない場合もある
＊＊：PEI磁気ビーズのみでは濃縮されないが，IgM 抗体や抗体と補体の添加により濃縮可能

ズを用いることにより，C型肝炎ウイルスやB型肝炎ウイルスをはじめとして多くのウイルスが濃縮可能であることを報告している(表6)．

4. 遺伝子治療薬や細胞治療薬のウイルス安全性確保を目指した将来的な課題

遺伝子治療薬や細胞治療薬などの先端技術医薬品のウイルス等の安全性確保に関しては，多くの検討すべき課題が残されている．また，これらの先端技術医薬品の開発はその周辺技術も含めて急速に進展しており，さらに腫瘍溶解性ウイルスベクターのようにこれまでの概念にない画期的な製品の開発も続いており，このような革新的技術を用いた製品については，その安全性を確保しつつ合理的な規制を行うことが，よりよい医療をできるだけ早く国民に届けることになる．このためにも，高感度・高精度のウイルス安全性検出技術等の基盤技術の開発を進めると共に，適切なリスク評価に基づいた行政施策の立案に資する研究が望まれている．

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参考文献

1) Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, Greenblatt JJ, Rosenberg SA, Klein H, Berger M, Mullen CA, Ramsey WJ, Muul L, Morgan RA, Anderson WF : T lymphocyte-directed gene therapy for ADA- SCID : initial trial results after 4 years. Science 270 : 475-480, 1995

2) 厚生省薬務局長通知：遺伝子治療用医薬品の品質及び安全性の確保に関する指針．薬発第1062号，医薬発第329004号，薬食発第1228004号，平成7年11月15日（平成14年3月29日，平成16年12月28日一部改正）

3) FDA/CBER : Guidance for industry : Guidance for human somatic cell therapy and gene therapy.

1998.3

4) EMEA : Note for guidance on the quality, pre-clinical and clinical aspects of gene transfer medicinal products. CPMP/BWP/3088/99, 2001.4

5) FDA : Guidance for reviewers : Instructions and template for chemistry, manufacturing, and control (CMC) reviewers of human somatic cell therapy investigational new drug applications (IND). 2003.8

6) 厚生省医薬安全局審査管理課長通知：ヒト又は動物細胞株を用いて製造されるバイオテクノロジー応用医薬品のウイルス安全性評価. 医薬審第329号, 平成12年2月22日

7) Marshall E : Gene therapy death prompts review of adenovirus vector. Science 286 : 2244-2245, 1999

8) Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML : Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adeno-viral gene transfer. Mol Genet Metab 80 : 148-158, 2003

9) Hutchins B : Development of a reference material for characterizing adenovirus vectors. BioProcessing Journal 1 : 25-28, 2002

10) Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, Fischer A, Davies EG, Kuis W, Leiva L, Cavazzana-Calvo M : Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. N Engl J Med 346 : 1185-1193, 2002

11) Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A : Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288 : 669-672, 2000

12) Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M : LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302 : 415-419, 2003

13) Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U, Glimm H, Kuhlcke K, Schilz A, Kunkel H, Naundorf S, Brinkmann A, Deichmann A, Fischer M, Ball C, Pilz I, Dunbar C, Du Y, Jenkins NA, Copeland NG, Luthi U, Hassan M, Thrasher AJ, Hoelzer D, von Kalle C, Seger R, Grez M : Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. Nat Med 12 : 401-409, 2006

14) FDA/CBER : Guidance for industry : Supplemental guidance on testing for replication-competent retro-virus in retroviral vector-based gene therapy products and during follow-up of patients in clinical trials using retroviral vectors. 2000.10

15) Satoh K, Iwata A, Murata M, Hikata M, Hayakawa T, Yamaguchi T : Virus concentration using polyethyleneimine-conjugated magnetic beads for improving the sensitivity of nucleic acid amplification tests. J Virol Methods 114 : 11-19, 2003

16) Uchida E, Sato K, Iwata A, Ishii-Watabe A, Mizuguchi H, Hikata M, Murata M, Yamaguchi T, Hayakawa T : An improved method for detection of replication-competent retrovirus in retrovirus vector products. Biologicals 32 : 139-146, 2004

17) Ishii-Watabe A, Uchida E, Iwata A, Nagata R, Satoh K, Fan K, Murata M, Mizuguchi H, Kawasaki N, Kawanishi T, Yamaguchi T, Hayakawa T : Detection of replication-competent adenoviruses spiked into recombinant adenovirus vector products by infectivity PCR. Mol Ther 8 : 1009-1016, 2003

18) Farson D, Tao L, Ko D, Li Q, Brignetti D, Segawa K, Mittelstaedt D, Harding T, Yu DC, Li Y : Development of novel E1-complementary cells for adenoviral production free of replication-competent adenovirus. Mol Ther 14 : 305-311, 2006

19) Murakami P, Pungor E, Files J, Do L, van Rijnsoever R, Vogels R, Bout A, McCaman M : A single short

stretch of homology between adenoviral vector and packaging cell line can give rise to cytopathic effect-inducing, helper-dependent E1-positive particles. Hum Gene Ther 13：909-920, 2002

20) Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, Auger C, Cramer SJ, van Ormondt H, van der Eb AJ, Valerio D, Hoeben RC：New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum Gene Ther 9：1909-1917, 1998

21) Aghi M, Martuza RL：Oncolytic viral therapies - the clinical experience. Oncogene 24：7802-7816, 2005

22) Lin E, Nemunaitis J：Oncolytic viral therapies. Cancer Gene Ther 11：643-664, 2004

23) Ries SJ, Brandts CH：Oncolytic viruses for the treatment of cancer：current strategies and clinical trials. Drug Discov Today 9：759-768, 2004

24) 厚生労働省医薬局長通知：細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方. 医薬発第266号, 平成13年3月28日

25) 厚生労働省医薬局長通知：ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針. 医薬発 第1314号, 平成12年12月26日

26) 厚生労働省医薬局長通知：ヒト幹細胞を用いる臨床研究に関する指針の施行等について ヒト幹細胞を用いる臨床研究. 健発第0703003号, 平成18年7月3日

27) EMEA：Point to consider on xenogenic cell therapy medicinal products. 2003.12.17

28) EMEA：Point-to-consider on the manufacture and quality control of human somatic cell therapy medicinal products. CPMP/BWP/41450/98, 2001.5.31

29) FDA/CBER：Suitability determination for donors of human cellular and tissue-based products. 97N-484S, 1999.9.30

30) 厚生労働省医薬局長通知：生物由来製品及び特定生物由来製品の指定並びに生物由来原料基準の制定等について. 医薬発第052001号, 平成15年5月20日

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Optimization of the virus concentration method using polyethyleneimine-conjugated magnetic beads and its application to the detection of human hepatitis A, B and C viruses

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Abstract

To enhance the sensitivity of virus detection by polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR), a novel virus concentration method using polyethyleneimine (PEI)-conjugated magnetic beads was developed in our previous study. However, several viruses could not be concentrated by this method. In this paper, the conditions of virus concentration were optimized to concentrate a wide range of viruses more efficiently. The PEI beads adsorbed viruses more efficiently than other cationic polymers, and the optimum virus concentration was obtained under weak acidic conditions. Mass spectrometric analysis revealed that several serum proteins, such as complement type 3, complement type 4 and immunoglobulin M (IgM), were co-adsorbed by the PEI beads, suggesting that the beads may adsorb viruses not only by direct adsorption, but also via immune complex formation. This hypothesis was confirmed by the result that poliovirus, which PEI beads could not adsorb directly, could be concentrated by the beads via immune complex formation. On the other hand, hepatitis A (HAV) and hepatitis C (HCV) viruses were adsorbed directly by PEI beads almost completely. Like poliovirus, hepatitis B virus (HBV) was concentrated efficiently by the addition of anti-HBV IgM. In conclusion, virus concentration using PEI beads is a useful method to concentrate a wide range of viruses and can be used to enhance the sensitivity of detection of HAV, HBV and HCV.

Keywords: Polyethyleneimine; Virus concentration; Immune complex; HAV; HBV; HCV

1. Introduction

Many useful biological/biotechnological medicinal products are produced from biological materials and by cell culture techniques. Recent progress in gene therapy and cell therapy products has provided new hope for the treatment of grave genetic diseases and lethal disorders. These innovative medicinal products, however, involve some risk in terms of the spread of transmissible agents and virus-mediated infectious diseases. To ensure the viral safety of biological/biotechnological products, it is important to confirm that the starting materials, intermediate products and final products are free from virus contamination. This is especially important for cell therapy products, since it is difficult to inactivate and/or remove contaminated viruses from these products.

Polymerase chain reaction (PCR) is a highly sensitive method for the detection of virus genomes (Saiki et al., 1988). Several nucleic acid amplification test (NAT) methods other than PCR have also been developed (Alter et al., 1995; Kamisango et al., 1999; Kern et al., 1996; Notomi et al., 2000; Sarrazin et al., 2000). These tests are reported to be able to detect only some copies of virus genomes. Therefore, in many countries, NAT methods have been employed to detect specific viruses in the virus screening of blood-derived products (Willkommen et al.,

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1999). NAT methods are also thought to be useful in testing biotechnology products derived from cell lines and cell therapy products. However, since all NAT methods used for the detection of viruses have a detection limit, it is impossible to deny virus contamination completely. In order to reduce the virus risk of both biological/biotechnological products and cell therapy products, it is essential to develop more sensitive methods of virus detection. One way to improve the sensitivity of virus detection is to concentrate the target viruses before NAT testing.

Recently, a novel viral concentration method using polyethyleneimine (PEI)-conjugated magnetic beads was developed (Satoh et al., 2003). It was shown that PEI beads efficiently adsorbed many model viruses, such as simian virus 40 (SV-40), herpes simplex 1 virus (HSV-1), Sindbis virus and vesicular stomatitis virus (VSV), and that the method improved the sensitivity of NAT for the detection of virus genomes about 10 to 100 times. It has also been reported that PEI beads efficiently adsorb amphotropic murine leukemia virus, and that the virus concentration method provided sensitive detection of replication-competent retrovirus in retrovirus vector products (Uchida et al., 2004). However, several small non-enveloped viruses such as poliovirus could not be concentrated or were only partially concentrated by PEI beads (Satoh et al., 2003). In addition, the mechanism of virus adsorption by PEI beads remains to be elucidated.

In the present study, the viral concentration method using PEI beads was optimized in order to allow the efficient concentration of several viruses. It was demonstrated that poliovirus can be concentrated by PEI beads via the formation of immune complexes. In addition, it was shown that the virus concentration method using PEI beads is applicable to human infectious viruses such as the hepatitis A(HAV), hepatitis B (HBV) and hepatitis C (HCV) viruses, which are important viruses to test for in order to ensure the viral safety of biological products and cell therapy products.

2. Materials and methods

2.1. Viruses

SV-40 virus, HSV-1 (stain F), porcine parvovirus (PPV; strain 90HS) and poliovirus (strain Sabin 1) were obtained and amplified as described previously (Satoh et al., 2003). Briefly, the supernatants of Vero cells infected with HSV-1 or poliovirus were used as virus samples. CV-1 cells were infected with SV-40 virus, and 5 days after infection, the supernatant was saved as the SV-40 sample. The supernatant of ESK cells infected with PPV was used as the PPV sample. In order to remove cell debris from the collected virus suspension, each virus suspension was centrifuged at 3000 rpm for 10 min. After removing cell debris, the resulting stock viruses (SV40: 4×10^7 copies/ml; PPV: 1×10^6 copies/ml; HSV-1: 1×10^8 copies/ml; poliovirus: 2×10^7 copies/ml) were aliquoted and stored at $-80\,^{\circ}C$ until use. Human adenovirus type 5 reference material (ATCC VR-1516; 5.8×10^{11} particles/ml) was obtained from the American type culture collection (ATCC) and used without amplification. HAV was obtained from ATCC (strain HM175/18f), infected into FRhK-4 cells, and the supernatant of the cell was saved 9–11 days later as the HAV sample (1×10^8 PFU/ml). The first Japanese national standard for HBV DNA (Genotype C; potency: 4.4×10^5 IU/ml) and the first Japanese national standard for HCV RNA (Mizusawa et al., 2005); genotype HCV-1b; potency: 100,000 IU/ml) were directly used as the HBV sample and HCV sample, respectively.

2.2. Preparation of PEI beads

PEI beads were prepared by coupling PEI (MW 70,000; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) with magnetic beads (IMMUTEX-MAG™; mean diameter: 0.8 μm; JSR Inc., Tokyo, Japan) by the 1-ethylene-3-(3-dimethylaminopropyl) carbodiimide coupling method, as described previously (Satoh et al., 2003). The final concentration of the PEI beads was 50 mg/ml. Different molecular weights of PEI beads were prepared as described above but including the coupling of PEI with a molecular weight (MW) of 1800 or PEI (MW 10,000) to magnetic beads, instead of PEI (MW 70,000). Polyarylamine (PAA)-conjugated magnetic beads and poly-L-lysine (PLL)-conjugated magnetic beads were prepared in the same way as PEI beads, using PAA (MW 150,000) or PLL (MW > 300,000) instead of PEI, respectively.

2.3. Virus concentration using PEI beads

The essential adsorption procedure for each virus was as follows. Virus samples were diluted with virus dilution medium (Dulbecco's modified Eagle's medium (DMEM) or DMEM supplemented with 2% fetal calf serum (FCS)). The exact concentration of the virus suspension used for each experiment is described in the corresponding figure legends. Next, 1 ml of each virus suspension was incubated with 100 μl of PEI beads for 10 min at room temperature. The complexes of virus and PEI beads were trapped by a magnetic field (Magnetic Trapper™; Toyobo Co., Tokyo, Japan) for 5 min and separated from the supernatant fraction. The virus DNA or RNA was extracted from virus-bead complex or from untreated virus suspensions (100 μl) with an SMI-TEST EX R&D Kit (Medical & Biological Laboratories Co., Nagano, Japan) following the manufacturer's instructions. Extracted DNA or RNA was dissolved in 50 μl of TE buffer (10 mM Tris–HCl (pH 7.4)/0.1 mM EDTA), and 10 μl of the solution was used for real-time PCR or RT-PCR reaction.

2.4. Effect of pH on virus concentration by PEI beads

Good's buffers with pH 4–9 (1 M MES, pH 4.0; 1 M MES, pH 5.0; 1 M MES, pH 6.0; 1 M HEPES, pH 7.0; 1 M HEPES, pH 8.0; 1 M Tricine, pH 9.0) were prepared and added to the virus dilution medium at a final concentration of 20 mM. Virus samples were then diluted with the virus dilution media at different pH values, and concentrated with PEI beads as described. The exact concentration of the virus suspension used for each experiment is described in the corresponding figure legends.

2.5. Real-time PCR and RT-PCR

Real-time PCR and reverse transcription PCR (RT-PCR) were carried out in a 50-μl reaction mixture containing 10 μl of extracted DNA or RNA, 0.5 μM of each primer set with a fluorescence probe, 25 μl of PCR master mix and, in the case of RT-PCR, a reverse transcriptase mix prepared according to the kit manual. The following real-time PCR and RT-PCR master mix kits were used: a QuantiTect Probe PCR kit (Qiagen, Hilden, Germany) for HSV-1, SV-40, adenovirus and PPV; a QuantiTect Probe RT-PCR kit (Qiagen) for poliovirus, HAV and HCV; and a Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA, USA) for HBV. The 5′-primers, 3′-primers and fluorescence probes used for the real-time PCR and RT-PCR detection of viruses are shown in Table 1. The real-time PCR and RT-PCR were performed on an ABI PRISM 7000 Sequence-Detection System (Applied Biosystems, Foster City, CA, USA).

2.6. SDS-PAGE analysis of serum proteins adsorbed on PEI beads

The virus suspension (HSV-1) diluted with DMEM supplemented with 5% FCS was incubated with PEI beads for 10 min. The fraction of serum proteins adsorbed on the beads and the untreated virus suspension were then boiled with sodium dodecly sulfate (SDS) sample buffer and applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on a slab gel ($T = 7.5\%$) with a BE-120 system from Biocraft (Tokyo, Japan). Protein bands were visualized by Coomassie Brilliant Blue staining.

2.7. In-gel digestion

Protein bands of interest were excised from the SDS-PAGE gel, destained three times in 50% acetonitrile and 25 mM ammonium bicarbonate for 10 min each time, and dehydrated in acetonitrile. The gel pieces were dried in a vacuum centrifugal concentrator and incubated with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate at 56 °C for 60 min. After cooling to room temperature, the DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide in 25 mM ammonium bicarbonate. After incubation for 45 min at room temperature in the dark, the gel pieces were washed in 25 mM ammonium bicarbonate for 5 min and dehydrated by the addition of 50% acetonitrile and 25 mM ammonium bicarbonate for 5 min. After this procedure was repeated twice, the gel pieces were dried in a centrifugal concentrator. The gel pieces were allowed to swell in 2 μl of a digestion buffer containing 25 mM ammonium bicarbonate, 0.1% octyl glucoside, and 25 ng/μl trypsin (sequence grade; Promega, Madison, WI, USA) in ice for 5 min, and then 15 μl of a digestion buffer without trypsin was added. After 30 min, the supernatant was discarded, and the gel pieces were incu-

Table 1
Primer and probe sets used for the real-time PCR and RT-PCR

Virus	Primer and probe set
HSV-1	Forward primer: 5′-GCGTCATGGTACTGGCAAG-3′ Reverse primer: 5′-TTGACTCTACGGAGCTGGCC-3′ Probe: 5′-FAM-TGGAGCTGATGCCGTAGTCGG-TAMRA-3′
SV-40	Forward primer: 5′-GACATTCCTAGGCTCACCTCACA-3′ Reverse primer: 5′-ACCTTGCCAAACTGTCCCTTAAA-3′ Probe: 5′-FAM-CTTGAAAGAAGAACCCAAAGA-TAMRA-3′
PPV	Forward primer: AACAACTACGCAGCAACTCCAATA-3′ Reverse primer: ACGGCTCCAAGGCTAAAGC-3′ Probe: 5′-FAM-AGGAGGACCTGGATTT-MGB-3′
Adenovirus[*1]	Forward primer: TCCGGTCCTTCTAACACACCTC-3′ Reverse primer: ACGGCAACTGGTTTAATGGG-3′ Probe: 5′-FAM-TGAGATACACCCGGTGGTCCCGC-TAMRA-3′
Poliovirus	Forward primer: 5′-CCCGAGAAATGGGACGACTA-3′ Reverse primer: 5′-TGGAGCTGTTCCGTAGGTGTAA-3′ Probe: 5′- FAM-ACATGGCAAACCTCATCAAATCCATCAATC-MGB-3′
HAV[*2]	Forward primer: 5′-GGTAGGCTACGGGTGAAAC-3′ Reverse primer: 5′-AACAACTCACCAATATCCGC-3′ Probe: 5′-FAM-CTTAGGCTAATACTTCTATGAAGAGATGC-TAMRA-3′
HBV[*3]	Forward primer: 5′-GGACCCCTGCTCGTGTTACA-3′ Reverse primer: 5′-GAGAGAAGTCCACCMCGAGTCTAGA-3′ Probe: 5′-FAM-TGTTGACAARAATCCTCACCATACCRCAGA-TAMRA-3′
HCV[*4]	Forward primer: 5′-TGCGGAACCGGTGAGTACA-3′ Reverse primer: 5′-CTTAAGGTTTAGGATTCGTGCTCAT-3′ probe: 5′-FAM-CACCCTATCAGGCAGTACCACAAGGCC-TAMRA-3′

Each primer set was prepared according to the original papers described below ([*1] to [*4]) or designed using Primer Express software (Applied Biosystems). [*1] Adenovirus (Ishii-Watabe et al., 2003), [*2] HAV (Jothikumar et al., 2005), [*3] HBV (Pas et al., 2000), [*4] HCV (Martell et al., 1999).

bated overnight at 37 °C. To extract tryptic fragments, the gel pieces were shaken in 50% acetonitrile and 5% trifluoroacetic acid (TFA) for 30 min. After this procedure was repeated twice, the extraction solutions were pooled, dried in a centrifugal evaporator, and dissolved in 20 μl of 0.1% TFA. The samples were then absorbed onto reverse-phase ZipTipC18 (Millipore, Bedford, MA, USA). The resin was washed with 0.1% TFA and the peptides were eluted with 3 μl of 75% acetonitrile/0.1% TFA. The eluate was analyzed by mass spectrometry (MS) as described below.

2.8. MS and database searching

The peptide mixture (0.5 μl volume) elution was deposited onto a matrix assisted laser desorption/ionization (MALDI) target plate, and this was closely followed by the deposition of 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA. MS and tandem MS (MS/MS) analysis of the peptide mixtures was performed using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Peptide mass fingerprinting and MS/MS ion searches were performed for protein identification by a Mascot search based on the MSDB protein database.

2.9. Preparation of anti-mouse IgG-rabbit IgM antibody

Anti-mouse immunogloblulin G (IgG) rabbit antiserum was obtained from rabbits immunized with highly purified mouse IgG (11 mg/ml; Jackson ImmunoResearch, West Grove, PA, USA) at 11 days after immunization, when IgM titer was increased. The antiserum (3 ml) was then diluted with an equal volume of phosphate buffered saline (PBS) (−), and applied to a mouse-IgG agarose affinity column (Invitrogen). After washing with 10 ml of PBS (−), the bound fraction was eluted with 0.1 M glycine–HCl (pH 3.0) and neutralized with 1 M Tris–HCl (pH 8.0). A PEI-sepharose-6MB column was prepared by coupling PEI to CNBr-activated sepharose-6MB (GE Healthcare Bioscience, Piscataway, NJ, USA). Anti-mouse IgG rabbit antiserum purified with a mouse IgG-agarose column was applied to a PEI-sepharose-6MB column and washed with PBS (−), and the bound fraction was eluted with 1.4 M NaCl/50 mM HEPES (pH 7.6). The eluted fraction was concentrated and used as anti-mouse IgG rabbit IgM antibody (final concentration, 4 μg/ml).

2.10. Poliovirus concentration via immune complexes

When poliovirus suspension was concentrated by PEI beads via immune complex formation, anti-poliovirus 1 mouse monoclonal antibody (IgG1: 5 μl; Chemicon International, Temecula, CA, USA) and purified anti-mouse IgG rabbit IgM antibody (20 μl), or anti-poliovirus 1 mouse monoclonal antibody and human complement C1 (5 μl; Merck Biosciences/Calbiochem, Darmstadt, Germany) and C4 (3 μl; Calbiochem) were added to the virus suspension before incubation with PEI beads.

2.11. Preparation of anti-HBV IgM antibody

Anti-hepatitis B surface antigen (HBsAg) IgM antibody was prepared as follows. Rabbits were immunized with a mixture of the adw and adr subtypes of recombinant HBsAg (Advanced ImmunoChemical, Long Beach, CA, USA). Anti-HBsAg rabbit antiserum was obtained at 10 days after immunization, when IgM titer was increased. The antiserum (3 ml) was diluted with an equal volume of PBS (−), applied to a PEI-sepharose-6MB column, washed with 20 ml PBS (−), and eluted with 1.4 M NaCl/100 mM HEPES (pH 7.0). PEI-sepharose-6MB-bound fractions were pooled, desalted with a PD-10 column equilibrated with 1.2 M NaCl/50 mM HEPES buffer (pH 7.4), and purified with an ImmunoPure IgM purification kit (Pierce Biotechnology, Rockford, IL, USA). IgM fractions were concentrated and used as anti-HBsAg IgM antibody.

3. Results

3.1. Optimization of the virus concentration method using PEI beads

In order to optimize the virus concentration method using PEI beads, the relationship between the MW of PEI coupled with magnetic beads and the efficiency of the virus concentration was examined. When PEIs with average molecular masses of 1800, 10,000 and 70,000 Da were compared, the PEI of MW 70,000 Da efficiently concentrated HSV-1, while magnetic beads with the PEI of MWs 1800 and 10,000 Da could not adsorb HSV-1 (Fig. 1). Therefore, the PEI beads with MW 70,000 Da were used in the following experiments.

Next, the virus adsorption ability of PEI was compared to that of other cationic polymers. As shown in Fig. 2, PEI beads exhibited a markedly higher virus adsorption ability than PAA- or PLL-conjugated magnetic beads for all model viruses tested.

The effect of pH on the efficiency of virus concentration was then examined. HSV-1 and SV-40 virus suspensions at different

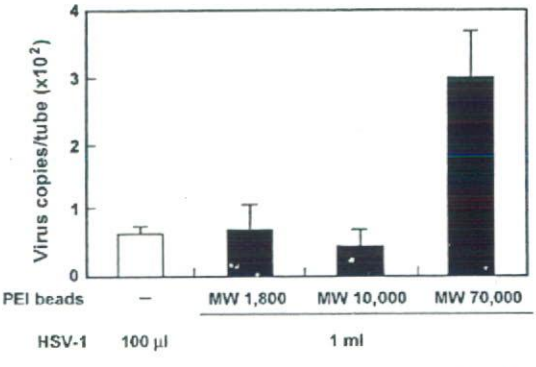


Fig. 1. Comparison of virus concentrations by magnetic beads coupled with PEIs of three different molecular weights. HSV-1 suspension (1×10^3 copies/ml, 1 ml/tube) was incubated with PEI beads whose PEI had a molecular weight of 1800, 10,000 or 70,000 Da. Viral genome DNA was extracted from the PEI bead fraction and from untreated HSV-1 suspension (100 μl). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

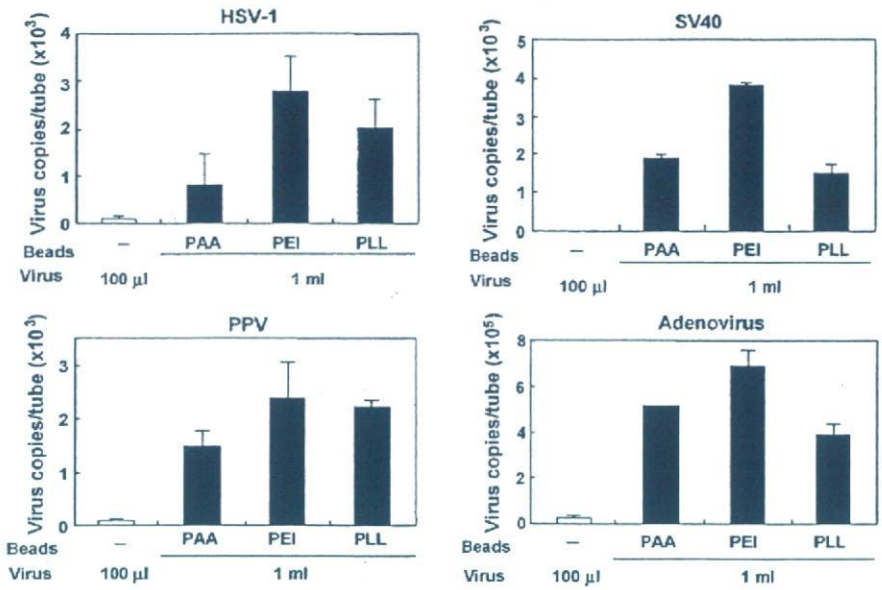


Fig. 2. Comparison of virus concentration by magnetic beads coupled with three different cationic polymers. HSV-1 (5×10^3 copies/ml), SV-40 (5×10^3 copies/ml), PPV (5×10^3 copies/ml) and adenovirus suspensions (1×10^6 copies/ml) (1 ml each) were incubated with PAA-, PEI- or PLL-conjugated magnetic beads. Viral genome DNA was extracted from each magnetic bead fraction and from untreated virus suspensions (100 µl each). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

pH levels (pH 5–9) were concentrated by PEI beads following the standard method. A pH levels of 6 was found to be optimal for the concentration of these viruses (Fig. 3).

3.2. Analysis of serum proteins adsorbed on PEI beads

To improve the virus concentration method using PEI beads, the serum components co-adsorbed by the beads during virus concentration were analyzed. When a virus suspension containing 5% FCS was concentrated by PEI beads and analyzed by SDS-PAGE, several proteins were specifically adsorbed by the beads (Fig. 4). Using MS and MS/MS analyses of these protein bands, complement type 3, complement type 4 and IgM heavy chain were identified as serum components concentrated by PEI beads. Since complement components and IgM were adsorbed by the beads, it is hypothesized that PEI beads may adsorb viruses not only by direct adsorption, but also via the formation of immune complexes that involve IgM antibody and/or complements.

3.3. Concentration of poliovirus by PEI beads via immune complexes

To confirm this hypothesis, concentrations of poliovirus, which PEI beads could not adsorb directly, via the formation of immune complexes were examined. Instead of anti-poliovirus IgM antibody, anti-poliovirus mouse monoclonal antibody (IgG) was used in combination with anti-mouse IgG rabbit IgM anti-

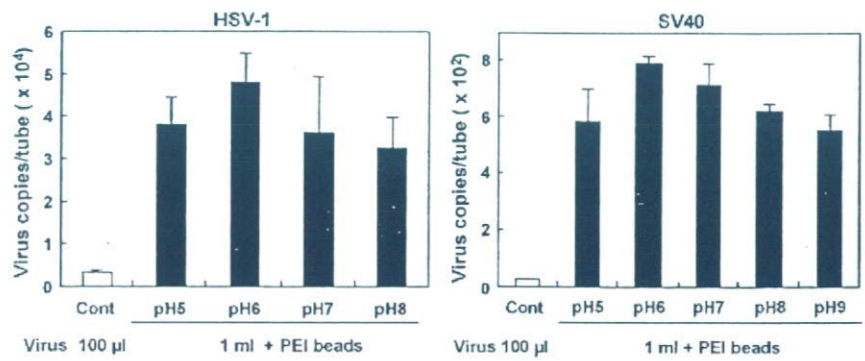


Fig. 3. Effect of pH on the efficiency of virus concentration by PEI beads. HSV-1 (5×10^4 copies/ml) and SV-40 (1×10^3 copies/ml) suspensions diluted with virus dilution medium at different pH levels (HSV-1: pH 5, 6, 7 and 8; SV-40: pH 5, 6, 7, 8 and 9) (1 ml each) were incubated with PEI beads. Viral genome DNA was then extracted from PEI bead fraction and from untreated virus suspensions (100 µl each). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

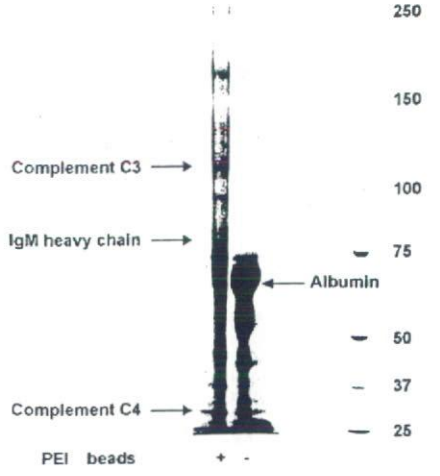
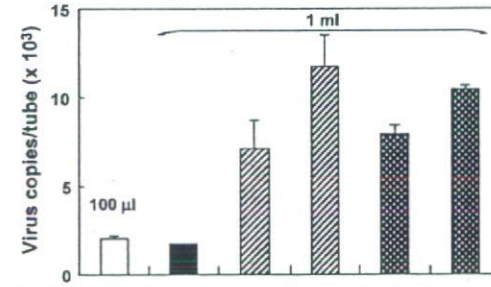


Fig. 4. Serum proteins adsorbed on PEI beads during virus concentration. HSV-1 suspension diluted with DMEM supplemented with 5% FCS was incubated with PEI beads. PEI bead fraction (+) and untreated virus suspension (−) were then boiled with SDS sample buffer and applied to SDS-PAGE. Serum protein bands concentrated by PEI beads were identified by MS/MS analysis, as shown in Fig. 5.



PEI beads	-	+	+	+	+	+
Anti-poliovirus mouse IgG MoAb	-	-	+	+	+	+
Anti-mouse IgG-rabbit IgM	-	-	-	+	-	-
C1 + C4	-	-	-	-	+ (r.t.)	+ (37°C)

Fig. 6. Concentration of poliovirus by PEI beads via the formation of immune complexes. Poliovirus suspension (2×10^4 copies/ml, 1 ml each) was incubated with PEI beads at room temperature or 37 °C in the absence or presence of anti-poliovirus mouse IgG monoclonal antibody, anti-mouse IgG-rabbit IgM, or a combination of complements C1 and C4. Viral genome RNA was extracted from the PEI bead fraction and from the untreated virus suspension (100 µl). Virus copy numbers were determined by real-time RT-PCR. Data are expressed as the mean ± S.D. ($n = 3$).

body to induce the formation of immune complexes. Anti-mouse IgG rabbit IgM antibody was prepared from rabbit anti-mouse IgG antiserum and purified by a mouse-IgG affinity column followed by a PEI-sepharose-6MB column. Since the PEI-sepharose-6MB column adsorbed IgM (Fig. 5) but not IgG (data not shown), the PEI-sepharose-6MB adsorbed fraction was used as the anti-mouse IgG rabbit IgM antibody. When poliovirus alone was incubated with the PEI beads, it was not adsorbed, but poliovirus was adsorbed when coincubated with anti-poliovirus IgG antibody, and a further significant improvement in the efficiency of virus concentration was achieved by the addition of anti-mouse IgG rabbit IgM along with the anti-poliovirus IgG (Fig. 6). The addition of the combination of complement C1, complement C4 and anti-poliovirus IgG to the reaction mixture of virus and PEI beads also increased the efficiency of virus concentration by PEI beads, but only when the complement system was activated by [incubation at] 37 °C (Fig. 6).

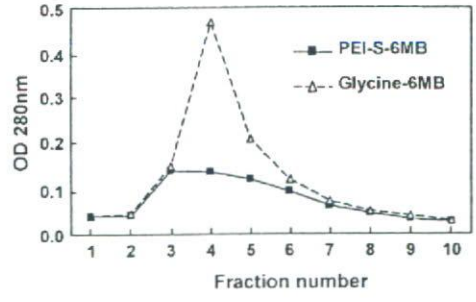


Fig. 5. Adsorption of IgM to a PEI-sepharose column. One ml of human IgM solution (1 mg/ml) was applied to a PEI-sepharose 6MB (PEI-S-6MB) column or to a control column without PEI (Glycine-6 MB) and washed with PBS (−). The eluates were fractionated into ten 1 ml fractions, and the OD280 of each fraction was determined using a spectrophotometer.

3.4. Application of the virus concentration method using PEI beads to human hepatitis viruses

The virus concentration method using PEI beads was applied to human HAV, HBV and HCV. Fig. 7 shows the effect of pH on the virus concentration efficiency. HAV was efficiently adsorbed by the PEI beads (Fig. 7A). The number of viral copies obtained in the PEI bead fraction when using 1 ml of virus suspension was about 10-fold the number extracted from untreated virus suspension (100 µl), suggesting that the concentration of HAV almost reached the predicted level. Neither the presence or absence of serum nor the pH condition affected the efficiency of the HAV concentration. HCV was also efficiently adsorbed by PEI beads, even in the presence of 2% FCS, and the optimum pH was found to be 5 (Fig. 7C). On the other hand, the efficiency of HBV concentration by PEI beads was lower than the efficiencies of HAV and HCV concentrations. The number of viral copies obtained in the PEI bead fraction under the optimum condition of pH 5 without serum was about six-fold the number extracted from untreated virus suspension (Fig. 7B). The presence of FCS significantly reduced the adsorption of HBV by PEI beads.

In order to improve the concentration of HBV obtained by PEI beads, anti-HBV IgM antibody was prepared and the concentration of HBV via immune complex formation was examined. As shown in Fig. 8, the concentration of HBV by PEI beads was improved by the addition of anti-HBV IgM antibody. Under the optimum condition, the number of viral copies obtained in the PEI bead fraction was more than seven-fold the number extracted from the untreated virus suspension even in the

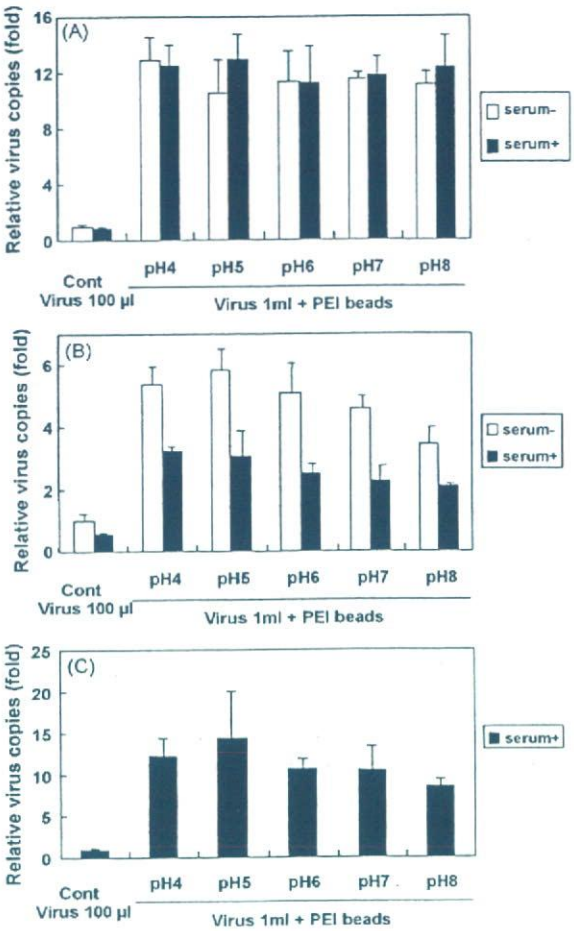


Fig. 7. Effect of pH on the concentration of HAV, HBV and HCV by PEI beads. HAV (A), HBV (B), and HCV (C) were diluted with virus dilution media of different pH levels supplemented with or without 2% FCS. Virus suspensions (HAV: 5×10^4 PFU/ml; HBV: 8.8×10^3 IU/ml; HCV: 1×10^3 IU/ml; 1 ml/tube) with different pH levels were incubated with PEI beads. Viral genome DNA and RNA were then extracted from PEI bead fraction and analyzed by real-time PCR and RT-PCR. Data are expressed as the mean ± S.D. ($n = 3$).

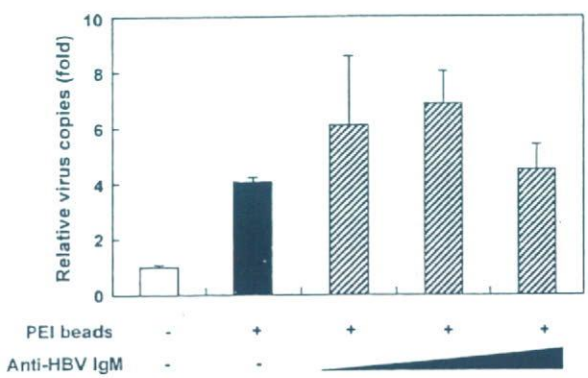


Fig. 8. Effect of anti-HBV IgM antibody for HBV concentration by PEI-beads. HBV suspensions (8.8×10^3 IU/ml; 1 ml/tube) were incubated with PEI beads in the absence or presence of 5, 15 or 50 µl of anti-HBV IgM antibody. Viral genome DNA was then extracted from the PEI bead fraction and analyzed by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

presence of serum. Therefore, the virus concentration achieved by PEI beads was shown to be enhanced by the formation of immune complexes.

Table 2 shows a summary of virus concentrations by PEI beads for all of the viruses examined. A wide range of viruses, including small non-enveloped viruses and human hepatitis viruses (HAV, HBV and HCV), were efficiently concentrated by PEI beads under the optimum condition, either directly or by the formation of immune complexes.

4. Discussion

In the present study, the virus concentration method using PEI beads (Satoh et al., 2003) was optimized, and was applied to human hepatitis A, B and C viruses.

First, the effects of various cationic polymers, PEI molecular weights, and pH values were examined in order to determine the optimal conditions for virus concentration. Among PEI beads with three different molecular weights (1800, 10,000 and 70,000 Da), only the PEI whose MW was 70,000 Da was able to adsorb viruses (Fig. 1). With respect to the cationic polymers, PEI magnetic beads showed a higher virus adsorption ability than PAA- or PLL-conjugated magnetic beads (Fig. 2). The optimum pH for the concentration of model viruses by PEI beads was subacidic (Fig. 3). The virus adsorption mechanism of PEI beads remains unclear. However, it is hypothesized that the positively charged field of the PEI molecule may interact tightly with the negative charge of surface lipids or the negatively charged surface proteins on viruses (Satoh et al., 2003). PEI is a polycationic polyamine with the highest cationic charge density among existing polymers (Futami et al., 2005). PEI has a branched backbone containing primary, secondary and tertiary amine groups. In contrast, PAA is a linear polycation having only primary amine groups, and PLL is a linear polycation with primary and secondary amine groups. Therefore, it is suggested that the high-density cationic charge of PEI and its branched structure on the surface of the magnetic beads may be important for efficient virus adsorption. According to the analysis of Owada et al. (1999), the interaction between PEI-coated membranes and human immunodeficiency virus type 1 (HIV-1) or plasma protein may be dependent on the surface area of each particle, and this fundamental principle was consistent with their observation that PEIs with higher MWs bound more intensely to HIV-1. This is also consistent with the data that PEI with a MW of 70,000 Da was able to adsorb viruses more efficiently than PEIs of 1800 Da or 10,000 Da.

In order to improve the efficiency of virus concentration by PEI beads, the serum components co-adsorbed by the beads were analyzed. MS analysis revealed that several proteins, including complement type 3, complement type 4 and IgM, were specifically co-adsorbed by PEI beads during virus concentration (Fig. 4), suggesting that the beads were able to adsorb immune complexes that involved IgM antibody and/or complements. Therefore, it is hypothesized that in addition to direct adsorption, PEI beads may adsorb viruses via the formation of immune complexes. This hypothesis was confirmed by the fact that PEI beads were able to adsorb poliovirus under con-

Table 2
Summary of concentration of viruses by PEI beads

Viruses	Natural host	Virus genome	Envelope	Size (nm)	PEI beads concentration
Model viruses cytomegalovirus (CMV)	Simian	DNA	+	180–200	+
Herpes symplex virus type 1 (HSV-1)	Human	DNA	+	150–200	+
Vesicular stomatitis virus (VSV)	Bovine	RNA	+	70–150	+
Amphotropic murine leukemia virus	Murine	RNA	+	80–110	+
Sindbis virus	Human	RNA	+	60–70	+
Adenovirus type 5	Human	DNA	–	70–90	+
Simian virus 40 (SV40)	Simian	DNA	–	40–50	+
Porcine parvovirus (PPV)	Porcine	DNA	–	18–24	+
Poliovirus sabin 1	Human	RNA	–	25–30	+[a]
Human hepatitis viruses hepatitis B virus (HBV)	Human	DNA	+	40–45	+[a]
Hepatitis C virus (HCV)	Human	RNA	+	40–50	+
Hepatitis A virus (HAV)	Human	RNA	–	25–30	+

[a] Concentrated by the addition of antibodies.

ditions which fostered immune complex formation, such as the addition of anti-poliovirus mouse IgG antibody with anti-mouse IgG rabbit IgM, or the addition of anti-poliovirus IgG antibody with activated complements (Fig. 6). Poliovirus is a very small (25–30 nm) non-enveloped virus, and could not be concentrated by PEI beads in our previous study (Satoh et al., 2003). Another possible explanation is that the increase in the surface area of virus particles due to the formation of immune complexes enhances the interaction between the poliovirus and the PEI beads, as hypothesized by Owada et al. (1999).

The results obtained from model viruses suggest that the virus concentration method using PEI beads may be applicable to a wide range of viruses. Therefore, this method was applied to human hepatitis viruses. A recent study reported that in some HAV patients, the duration of the viremic phase persisted for more than 1 year with low viral load levels (10^3–10^4 HAV genome equivalents/ml) (Normann et al., 2004). In the case of HBV, the presence of occult HBV infection (HBV DNA positivity in the setting of negative serum hepatitis B surface antigen) has been documented, and the majority of these infections were associated with low viral loads (<10^5 copies/ml) (Minuk et al., 2004). Several studies have demonstrated high rates of transmission of HCV through transfusions with extremely low viral loads (Operskalski et al., 2003). HCV is particularly infectious during the early window period, with levels as low as 1 viral copy in 20 ml plasma able to transmit infection by transfusion (Busch et al., 2003), though intermittent low-level HCV viremia can occur as long as 2 months before the periods of exponential increase in viral load (Glynn et al., 2005). Therefore, it is extremely important to develop a highly sensitive detection method for these viruses. In the present study, it was possible to concentrate HAV and HCV by PEI-beads to almost the predicted levels (Fig. 7). In contrast, HBV was not fully concentrated even under optimum conditions around pH 5. Therefore, the concentration of HBV via the formation of immune complexes was tested. As expected, the concentration of HBV was improved by the addition of anti-HBV IgM antibody (Fig. 8), indicating that the virus concentration method using PEI beads is applicable for the concentration and sensitive detection of HAV, HBV and HCV by PCR and RT-PCR reaction.

To enhance/establish the utility of this virus concentration method using PEI meads for viral safety of biological products and cell therapy products, examination using actual patient sera and different genotypes/subtypes of each virus may be required. In a preliminary experiment, it is confirmed that this PEI beads method can be used for hepatitis virus samples spiked in human plasma. Applicability to different genotypes will be examined using a Japanese genotype panel of HBV and HCV, which will be available soon.

PEI beads may be applicable not only for virus concentration but also for the efficient infection of viruses. Scherer et al. (2002) report that superparamagnetic nanoparticles coated with PEI enhanced the infection of adenovirus and retrovirus vectors under a magnetic field. This infection method (magnetofection) also enhanced the infection of measles virus (Kadota et al., 2005). In a preliminary experiment, the PEI beads used in the present study also enhanced the infectivity of several viruses under a magnetic field. Therefore, it is suggested that PEI beads may be useful for the sensitive detection of both virus genomes and virus infectivity.

In conclusion, the present study demonstrates that the virus concentration method using PEI beads is effective for the concentration and sensitive detection of a wide range of viruses, including HAV, HBV and HCV.

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References

Alter, H.J., Sanchez-Pescador, R., Urdea, M.S., Wilber, J.C., Lagier, R.J., Di Bisceglie, A.M., Shih, J.W., Neuwald, P.D., 1995. Evaluation of branched DNA signal amplification for the detection of hepatitis C virus RNA. J. Viral Hepat. 2, 121–132.

Busch, M.P., Hirschkorn, D.F., Herring, B.L., Delwart, E.L., McAuley, J., Murthy, K.K., Alter, H.J., 2003. Defining the minimal infectious dose and infectious wind[o]w period for HCV using plasma donor panels and the chimpanzee transfusion model. Transfusion 43, S107–S140.

Futami, J., Kitazoe, M., Maeda, T., Nukui, E., Sakaguchi, M., Kosaka, J., Miyazaki, M., Kosaka, M., Tada, H., Seno, M., Sasaki, J., Huh, N.H., Namba, M., Yamada, H., 2005. Intracellular delivery of proteins into mammalian living cells by polyethylen[e]imine-cationization. J. Biosci. Bioeng. 99, 95–103.

Glynn, S.A., Wright, D.J., Kleinman, S.H., Hirschkorn, D., Tu, Y., Heldebrant, C., Smith, R., Giachetti, C., Gallarda, J., Busch, M.P., 2005. Dynamics of viremia in early hepatitis C virus infection. Transfusion 45, 994–1002.

Ishii-Watabe, A., Uchida, E., Iwata, A., Nagata, R., Satoh, K., Fan, K., Murata, M., Mizuguchi, H., Kawasaki, N., Kawanishi, T., Yamaguchi, T., Hayakawa, T., 2003. Detection of replication-competent adenoviruses spiked into recombinant adenovirus vector products by infectivity PCR. Mol. Ther. 8, 1009–1016.

Jothikumar, N., Cromeans, T.L., Sobsey, M.D., Robertson, B.H., 2005. Development and evaluation of a broadly reactive TaqMan assay for rapid detection of hepatitis A virus. Appl. Environ. Microbiol. 71, 3359–3363.

Kadota, S., Kanayama, T., Miyajima, N., Takeuchi, K., Nagata, K., 2005. Enhancing of measles virus infection by magnetofection. J. Virol. Meth. 128, 61–66.

Kamisango, K., Kamogawa, C., Sumi, M., Goto, S., Hirao, A., Gonzales, F., Yasuda, K., Iino, S., 1999. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J. Clin. Microbiol. 37, 310–314.

Kern, D., Collins, M., Fultz, T., Detmer, J., Hamren, S., Peterkin, J.J., Sheridan, P., Urdea, M., White, R., Yeghiazarian, T., Todd, J., 1996. An enhanced-sensitivity branched-DNA assay for quantification of human immunodeficiency virus type 1 RNA in plasma. J. Clin. Microbiol. 34, 3196–3202.

Martell, M., Gomez, J., Esteban, J.I., Sauleda, S., Quer, J., Cabot, B., Esteban, R., Guardia, J., 1999. High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. J. Clin. Microbiol. 37, 327–332.

Minuk, G.Y., Sun, D.F., Greenberg, R., Zhang, M., Hawkins, K., Uhanova, J., Gutkin, A., Bernstein, K., Giulivi, A., Osiowy, C., 2004. Occult hepatitis B virus infection in a North American adult hemodialysis patient population. Hepatology 40, 1072–1077.

Mizusawa, S., Okada, Y., Horiuchi, Y., Tanaka, T., Sato, K., Kaneko, K., Sasaki, Y., Tanaka, T., Tomono, T., Tomomizu, T., Hayami, S., Hijikata, M., Hirako, I., Mayumi, M., Mikami, K., Mishiro, S., Miyamoto, S., Muta, K., Weimer, T., Gierman, T., Komuro, K., Yamaguchi, T., 2005. Establishment of the first national standard for nucleic acid amplification technology assay for HCV RNA. Jpn. J. Transfus. Med. 51, 515–519.

Normann, A., Jung, C., Vallbracht, A., Flehmig, B., 2004. Time course of hepatitis A viremia and viral load in the blood of human hepatitis A patients. J. Med. Virol. 72, 10–16.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucl. Acids Res. 28, E63.

Operskalski, E.A., Mosley, J.W., Tobler, L.H., Fiebig, E.W., Nowicki, M.J., Mimms, L.T., Gallarda, J., Phelps, B.H., Busch, M.P., 2003. HCV viral load in anti-HCV-reactive donors and infectivity for their recipients. Transfusion 43, 1433–1441.

Owada, T., Miyashita, Y., Motomura, T., Onishi, M., Yamashita, S., Yamamoto, N., 1999. Anti-HIV-1 activity of an ionically modified porous polypropylene membrane determined by filtration of a viral suspension. Microbiol. Immunol. 43, 141–151.

Pas, S.D., Fries, E., De Man, R.A., Osterhaus, A.D., Niesters, H.G., 2000. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J. Clin. Microbiol. 38, 2897–2901.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487–491.

Sarrazin, C., Teuber, G., Kokka, R., Rabenau, H., Zeuzem, S., 2000. Detection of residual hepatitis C virus RNA by transcription-mediated amplification in patients with complete virologic response according to polymerase chain reaction-based assays. Hepatology 32, 818–823.

Satoh, K., Iwata, A., Murata, M., Hikata, M., Hayakawa, T., Yamaguchi, T., 2003. Virus concentration using polyethyleneimine-conjugated magnetic beads for improving the sensitivity of nucleic acid amplification tests. J. Virol. Meth. 114, 11–19.

Scherer, F., Anton, M., Schillinger, U., Henke, J., Bergemann, C., Kruger, A., Gansbacher, B., Plank, C., 2002. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. Gene Ther. 9, 102–109.

Uchida, E., Sato, K., Iwata, A., Ishii-Watabe, A., Mizuguchi, H., Hikata, M., Murata, M., Yamaguchi, T., Hayakawa, T., 2004. An improved method for detection of replication-competent retrovirus in retrovirus vector products. Biologicals 32, 139–146.

Willkommen, H., Schmidt, I., Lower, J., 1999. Safety issues for plasma derivatives and benefit from NAT testing. Biologicals 27, 325–331.

医薬発第１３１４号
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ヒト又は動物由来成分を原料として製造される医薬品等の品質及び安全性確保について

ヒト又は動物由来成分を原料として製造される医薬品、医療用具、医薬部外品、化粧品（以下「医薬品等」という。）については、製造業者、輸入販売業者及び外国製造業者の国内管理人（以下「製造業者等」という。）において現時点の科学的水準に基づいた品質及び安全性確保対策を講ずることが必要と考えられるが、今般、中央薬事審議会バイオテクノロジー特別部会において「細胞・組織利用医薬品等の取扱い及び使用に関する考え方」（以下「基本的考え方」という。）が別添１のとおり、「ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」（以下「指針」という。）が別添２のとおり取りまとめられたことから、下記のとおり製造業者等による自主点検及び承認書の整備等を行うこととしたので、貴管下関係業者に対して指導方お願いする。

記

１　対象範囲

ヒト又は動物由来医薬品等の範囲は以下のとおりとする。ただし、生物学的製剤基準に収載されている血液製剤及び専ら人体に直接使用されないもの（体外診断用医薬品）等を除く。

(1)　ヒト又は動物の細胞・組織から構成される医薬品等

(2)　ヒト又は動物の細胞・組織からの抽出物又は分泌物に由来する成分を含有する医薬品等

(3)　ヒト又は動物の尿等からの抽出物に由来する成分を含有する医薬品等
(4)　ヒト又は動物由来細胞に対して細胞培養、遺伝子組換え技術を応用して製造される医薬品等
(5)　添加剤（製造過程の培地を含む。）として（1）～（4）の成分を用いて製造される医薬品等

2　ヒト又は動物由来医薬品等の取扱い及び使用について

1の（1）に該当する医薬品、医療用具については、今後、「基本的考え方」に基づき省令の改正や新たな基準の制定等を行うこととしているが、（2）～（5）のいずれかに該当する医薬品等についても、「基本的考え方」の第2章第1、第4から第6、第3章、第4章に準じて原材料の取扱い、製造管理を実施すべきこと。

3　自主点検

(1)　医薬品等については、製造業者等の責任において、その品質及び安全性を担保し、科学技術の進歩に応じて製造工程、品質規格等を見直すべきものであるが、今般、「基本的考え方」及び「指針」等に沿った自主的な点検を実施するものであること。
(2)　自主点検にあたっては、品質及び安全性確保の観点から、原材料を提供するヒト又は動物に対して実施されるドナースクリーニングの内容（検査項目や検査方法を含む。）、製造工程中の細菌、真菌、ウイルス等の不活化／除去処理等が、現在の科学技術水準に照らして感染症の伝播防止の観点から適切に行われていることを、製造業者等の責任において確認すること。
(3) 自主点検に際しては、製品の投与経路や適用部位等も勘案し、原料を含めた適切な製造管理及び品質管理が行われていることを確認すること。

4　承認書等の取扱い

(1) ヒト又は動物由来成分を原料として製造される医薬品等の品質及び安全性確保については、原料等を含む適切な製造管理及び品質管理により行われるものであるが、次に掲げる事項について、承認書に明確に記載することが必要であるので、必要に応じて承認書の整備として一部変更承認申請を行うこと。

ア　原料となるヒト又は動物由来成分の由来
イ　ドナースクリーニングの内容（検査項目や検査方法を含む。）
ウ　製造工程中の細菌、真菌、ウイルス等の不活化／除去処理の方法

エ　品質、安全性確保の観点から重要と考えられる製造工程

(2) 自主点検の結果、ドナースクリーニング、細菌、真菌、ウイルス等の不活化／除去処理の追加、変更等を行う場合には、承認事項の一部変更承認申請を行うこと。

また、必要なドナースクリーニング、細菌、真菌、ウイルス等の不活化／除去処理の追加、変更等が実施できないものについては、承認整理届の提出等必要な手続きを行うこと。

(3) これらの承認書の整備、自主点検にかかる一部変更承認申請等について迅速に審査を実施する方針であることから、平成13年3月末日までに自主点検を行い、実施の結果をまとめること。また、一部変更承認申請等が必要なものについては、平成14年３月末日までに行うこと。

（別添１）

細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方

第１章　総則

第１　目的

細胞・組織利用医薬品等については、細胞・組織に由来する感染症の伝播の危険性が懸念されるため、細菌、真菌、ウイルス等に汚染されていない原料の使用、製造工程中における汚染の防止等を図ることが不可欠である。また、不適切な製造等による不良製品の製造、不適切な製品の取扱いや使用による問題の発生を防止する必要がある。従って、このような観点に立ち、細胞・組織の採取から、製造、使用まで一貫した方策が必要である。

本文書は、細胞・組織を取り扱う際の基本的要件を示すとともに、細胞・組織利用医薬品等の品質及び安全性、並びに細胞・組織の取扱いに関する科学的及び倫理的妥当性を確保することを目的とする。

なお、本文書に示した方法以外の方法を採る場合には、品質及び安全性確保の観点からその必要性及び妥当性を説明し、その根拠を示すことが必要である。

本文書に示した事項は、細胞・組織利用医薬品等の承認後のみならず、治験時においても適用される。

第２　基本原則

細胞・組織利用医薬品等については、細胞・組織に由来する感染症の伝播等の危険性を完全には排除し得ないおそれがあることから、他の治療薬や治療法と比較して有用性が同程度以上であるときに使用すること。

第３　定義

この基本的考え方における用語の定義は次に掲げるとおりとする。

１　「細胞・組織利用医薬品等」とは、生物由来医薬品又は生物由来医療用具のうち、ヒト又は動物の細胞・組織から構成されたものをいい、自己の細胞･組織を原材料とする医薬品及び医療用具が含まれる。ただし、血液製剤は含まれない。

２　「ドナー」とは、細胞・組織利用医薬品等の原料となる細胞・組織を提供するヒトをいう。なお、臓器の移植に関する法律（平成9年法律第104号）に基づいて脳死と判定された人からの提供は想定していない。

3　「ドナー動物」とは、細胞・組織利用医薬品等の原料となる細胞・組織を提供する動物をいう。

4　「代諾者」とは、本人が説明を受け同意を与えることが困難な場合又は単独で完全な同意を与える能力を欠いている場合において、本人に代わって説明を受け同意を与える者で、本人が生存している場合にあっては本人に対して親権を行う者、配偶者、後見人その他これに準じる者等をいう。

5　「ドナースクリーニング」とは、ドナー又はドナー動物が細胞・組織利用医薬品等の原材料となる細胞・組織を提供するための適格性を満たしているかどうかを決定するための診断及び検査を行い、適格性を判断することをいう。

6　「ウインドウ・ピリオド」とは、感染初期に細菌、真菌、ウイルス等又は細菌、真菌、ウイルス等に対する抗体が検出できない期間をいう。

7　「作業区域」とは、細胞・組織利用医薬品等を直接取り扱い、製造作業を行う区域をいう。

第２章　細胞・組織採取について

第１　採取医療機関等

細胞・組織については、次に掲げる要件又はこれと同等以上の要件を満たす医療機関等で採取されていること。

1　細胞・組織の採取及び保存に必要な衛生上の管理がされており、採取に関して十分な知識、技術を持つ人員を有していること。

2　ヒトの細胞・組織を採取する場合には、採取を行うことの適否に関する調査審議を行うための倫理委員会が設置されていること。

3　２に定める倫理委員会については、次に掲げる要件を満たすこと。

（１）細胞・組織の採取について倫理的及び科学的観点から十分に審議を行う体制が確保されていること。

（２）運営方法に関する規則が定められており、それが公開されていること。

（３）委員には、倫理・法律面の有識者、科学面の有識者、市民の立場の人が参画していること。

（４）外部の人及び倫理･法律面の有識者又は市民の立場の人の参画に関しては、全体の委員の人数を勘案し、委員構成を適正な割合に保つことが必要であること。

（５）施設の長、細胞・組織を採取する者、細胞・組織の採取を依頼する者と密接な関係を有する者等が審議及び採決に参加していないこと。

（６）倫理委員会は、倫理・法律面の有識者または市民の立場の人が１名

以上出席しなければ、審議又は裁決のための会議を開くことができないこと。

4　採取されたヒトの細胞・組織を利用する製造業者、輸入販売業者又は国内管理人（以下「製造業者等」という）にあっても、2に準じた委員会を設置し、細胞・組織利用について倫理的及び科学的観点から調査審議を受けることを考慮すること。

第2　細胞・組織採取に関する説明、同意等

1　文書による説明と同意の取得

細胞・組織の採取を行う者はドナーとなる者に対して、ドナースクリーニングの実施前に細胞・組織の利用目的、個人情報の保護、その他採取に関する事項について当該者の理解を得るよう、文書を用いて十分に説明し、自由意思による同意を文書により得なければならない。

なお、説明に当たっては、同意の拒否及び撤回の権利があり、拒否又は撤回することにより当該者が不利益な扱いを受けないことを明らかにすること。

2　代諾について

ドナー本人が説明を受け同意を与えることが困難な場合又は単独で完全な同意を与える能力を欠いている場合において、下記の要件を満たす場合に限り、代諾者の同意により細胞･組織の採取を行うことができること。

(1) 当該ドナーからの細胞･組織採取が細胞･組織利用医薬品等の品質、安全性の確保の観点等から必要とされる合理的理由があること。

(2) 代諾者はドナーの意思や利益を最もよく代弁できると判断される者でなければならず、代諾者の同意に際しては、ドナーと代諾者の関係についての記録が作成され、同意書とともに保存されていること。

(3) この場合においても、細胞･組織を採取する者は可能な限りドナーにその理解力に応じた説明を行うとともにドナー本人からも同意を得るよう努めること。

(4) 採取を行う医療機関の倫理委員会において、当該ドナーからの細胞･組織の採取の科学的、倫理的妥当性が審査され、了承されていること。

3　ドナーが死亡している場合

死体から細胞･組織の提供を受ける場合には、遺族に対して1に従って説明し同意を得ること。なお、細胞･組織の採取は、当該ドナーが細胞･組織の提供を生前に拒否していない場合に限ること。

4　手術等で摘出された細胞･組織を利用する場合

手術等で摘出された細胞･組織を利用する場合においても、1及び2に