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Use of anionic polymer, poly(methyl vinyl ether-maleic anhydride)-coated beads for capture of respiratory syncytial virus

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ABSTRACT

Respiratory syncytial virus (RSV) is the single most important cause of severe lower respiratory tract infections in infants and young children, and a major public health concern in pediatrics. However, current diagnostic methods for RSV are not sufficiently sensitive. In addition, there is no simple method for enhancing RSV detection. Here, a method for capturing RSV from nasal fluid has been developed using magnetic beads coated with an anionic polymer, poly(methyl vinyl ether-maleic anhydrate). The beads were incubated with RSV-infected nasal fluid, then separated from the supernatant by applying a magnet field and washed. The absorption of RSV by the beads was confirmed by immunochromatography, reverse transcription-polymerase chain reaction, Western blotting and an enzyme-linked immunosorbent assay, which indicated the presence of nucleocapsid protein, fusion protein, and the viral genome of RSV on the incubated beads. Therefore, this capture method will contribute to the improvement of RSV detection.

Respiratory syncytial virus (RSV) causes severe lower respiratory tract infections (LRIs) in infants and young children with an estimated 64 million infections and 160,000 fatalities per year worldwide.[1] It is difficult to recognize RSV infections from clinical signs especially as other respiratory diseases such as influenza virus and adenovirus infections have similar symptoms. Several methods for detecting RSV have been developed using the enzyme-linked immunosorbent assay (ELISA), the polymerase chain reaction (PCR), immunochromatography.[2] Immunochromatography is useful for a rapid diagnostic assay of RSV infections. However, these methods are not as sensitive as cell culture assays.

There are two major limiting factors to the development of methods to concentrate a virus; compatibility with current methods of detection and convenience. Several approaches to the concentration of viruses have been attempted to enhance sensitivity.[3–5] The representatives are ultracentrifugation and polyethyleneglycol (PEG) precipitation. Both these methods are used for all types of viruses. Ultracentrifugation is well-known, but time-consuming and can increase the false-positive rate when combined with PCR.[6,7] Although PEG precipitation is simple and easy to perform, the PEG inhibits PCR. One alternative approach is to use magnetic beads coated with molecules, which efficiently binds viral particles. Here, we report that magnetic beads coated with an anionic polymer, poly(methyl vinyl ether-maleic anhydrate) [poly(MVE-MA)][8] are useful for the capture of RSV.

To examine the capacity of the beads to capture RSV from nasal aspirates,[9,10] immunochromatography, ELISA, reverse transcription (RT)-PCR, and Western blotting were performed. Immunochromatography for the RSV fusion protein (Binax Now kit; Eiken Chemical Co. Ltd, Tokyo, Japan) showed that the RSV fusion protein was predominantly detected in the beads fraction (BD) at similar levels to total sample containing the same quantity of nasal aspirate as BD (TL), but not in the supernatant (SP) (Fig. 1A). Furthermore, although the sample before concentration (BF) had undetectable levels of RSV fusion protein, the fusion protein could be detected in the beads fraction (BD), indicating that this method is useful for not only the capture but also the concentration of RSV. Taking into account the volume of sample and buffer, this method may enable at least 10-fold concentration.

We also used RT-PCR[11] to investigate the capacity of the magnetic beads by detecting RSV genomic RNA (Fig. 1B). RT-PCR analysis showed a single band of 243 bp in the beads fraction (BD) and sample containing the same quantity of nasal aspirate as BD (TL) but not in the supernatant after incubation (SP). The 243-bp band was confirmed to be a RSV fusion protein gene of subtype B (identical to Genebank accession number AB245477 except for C→T at 651) by DNA sequencing (ABI PRISM3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Therefore, these results suggest that the beads fraction where RSV was captured include RSV genomic RNA.

Western blotting with blend of 4 monoclonal anti-RSV antibodies (2G12+1C3+5H5N+5A6) (Acris Antibodies GmbH, Hiddenhausen, Germany) demonstrated that the total sample fraction (TL)

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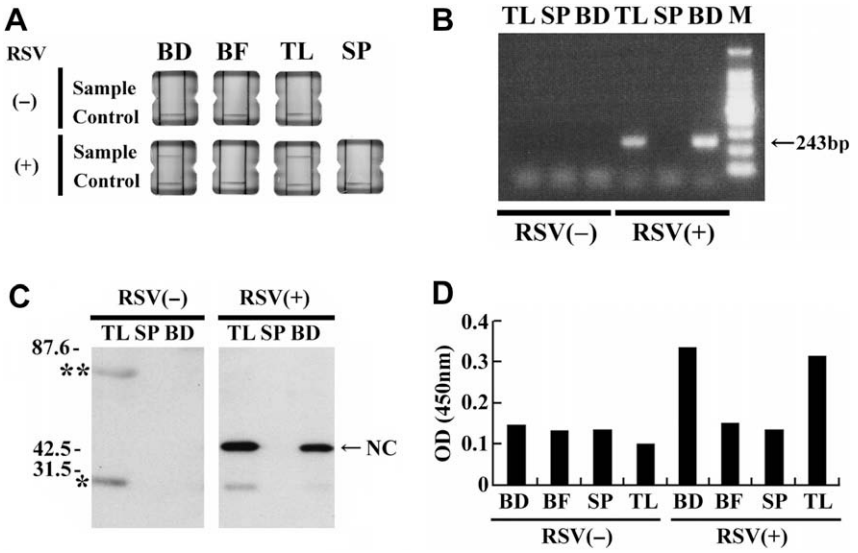

Figure 1. Detection of viral proteins and RNA genome in respiratory syncytial virus (RSV) absorbed on anionic magnetic beads. (A) Detection of fusion protein in respiratory syncytial virus (RSV) absorbed on anionic magnetic beads. Nasal aspirates of RSV-infected (+) (1 year old, female) and un-infected (−) (1 year old, male) children were diluted with PBS and subjected to incubation with anionic magnetic beads. Binax Now RSV test (Eiken Chemical Co. Ltd, Tokyo, Japan) was used for detection of RSV fusion protein by immunochromatography. Results of samples (Sample) were interpreted on the basis of the presence and absence of a line included in the kit as a positive control (Control). Samples were divided into four categories; a beads fraction (BD), sample before incubation with the beads (BF), supernatant after the incubation (SP), and total sample containing the same quantity of nasal aspirate as BD (TL). They were solubilized with lysis buffer and subjected to immunochromatography. (B) Detection of RNA genome in RSV absorbed on anionic magnetic beads. Viral genomic RNA was extracted from the above-mentioned fractions using a QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) and subjected to reverse transcription (RT)-reaction. The resultant cDNAs of RSV fusion protein gene (243-bp) were amplified by polymerase chain reaction (PCR). (C) Detection of RSV nucleocapsid protein in RSV absorbed on anionic magnetic beads. The RSV in 500 μl of diluted nasal aspirate (20 μl) was captured by anionic magnetic beads and subjected to Western blotting. Samples were solubilized with sodium dodecyl sulphate (SDS)-gel loading buffer and separated by SDS–polyacrylamide gel electrophoresis (PAGE), and analyzed by Western blotting with anti-RSV antibodies and horseradish peroxidase (HRP)-conjugated anti-mouse IgG. The arrow indicates RSV nucleocapsid protein (NC). The asterisk and double asterisks indicate serum proteins. (D) Quantitative analysis of RSV absorbed on anionic beads. RSV in nasal aspirates before and after absorption on anionic magnetic beads was quantitatively analyzed by enzyme-linked immunosorbent assay (ELISA) using a SERION ELISA antigen Quadrogen kit (Serion Immundiagnostica & Institut Virion/Serion GmbH, Würzburg, Germany). The quantity of RSV in each fraction was compared as index of absorbance at 450 nm.

and beads fraction (BD) but not supernatant fraction (SP) in RSV(+) had a major band of 43 kDa (Fig. 1C). A size of 43 kDa is consistent with that calculated from the deduced amino acid sequence of RSV nucleocapsid protein. These results support that RSV was detected not only in the total sample fraction (TL) but also in the beads fraction (BD) at a similar level but not in the supernatant fraction (SP). No bands for RSV nucleocapsid protein were detected in RSV(−) samples. These results support that RSV is efficiently captured by anionic magnetic beads. On the other hand, there were several non-specific bands in the total sample. Two bands (25 and 66 kDa) were observed in the total sample fraction of both RSV(−) and RSV(+) by Western blotting. These bands are possibly due to proteins from blood during the collection procedure, because they were decreased in the beads fraction (BD) and supernatant fraction (SP). Furthermore, these non-specific bands were not observed in nasal aspirate samples not containing blood (data not shown). Regretfully, Coomassie brilliant blue (CBB) staining showed that these bands remained in the beads fraction (data not shown). In addition, incubation of the beads with diluted serum showed binding of serum proteins to the beads.[12] The results suggest that the anionic magnetic beads mainly bind to RSV but non-specifically bind to other components of nasal fluid and blood.

Finally, we examined the efficiency with which RSV was concentrated by this method by conducting a quantitative analysis using ELISA.[13] RSV recovered by the anionic magnetic beads (BD) was at a level similar to that in sample containing the same quantity of nasal aspirate as BD (TL), whereas RSV was under the detectable limit in sample before the incubation with beads (BF) and supernatant after the incubation (SP) (Fig. 1D), suggesting that most of the RSV was efficiently captured by the magnetic beads. In addition, ELISA combined with this concentrating method enabled the detection of RSV in samples negative by the conventional method (RS1 and RS2) (Fig. 2). Meanwhile, the co-presence of influenza A or B viruses with RSV interfered with the concentration of RSV by magnetic beads (RS3, RS3+A, and RS3+B).

There are several methods of concentrating viruses such as ultracentrifugation and PEG precipitation. The former requires large instruments, special techniques, and a relatively long time. The latter method is simple but is sometimes incompatible with conventional methods of detection. It should be emphasized that

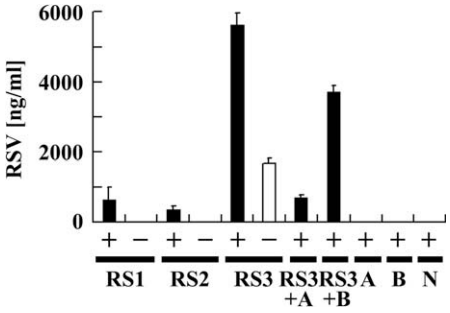

Figure 2. Quantitative analysis of RSV absorbed on anionic beads. RSV in concentrated samples with beads (+) (black columns) and non-treated samples (−) (white columns) from nasal aspirates was quantitatively analyzed by ELISA using a SERION ELISA antigen Quadrogen kit (Serion Immundiagnostica & Institut Virion/Serion GmbH, Würzburg, Germany). RSV-infected (RS1: 2 years old, female; RS2: 2 years old, female; RS3: 1 year old, male), influenza A virus-infected (A: 2 years old, male), influenza B virus-infected (B: 3 years old, male), and un-infected (N: 3 years old, male) patients were subjected to the analysis. Samples (RS3+A) or (RS3+B), mixed with RSV-infected sample (RS3) and influenza A virus-infected sample (A) or mixed with RSV-infected sample (RS3) and influenza B virus-infected sample (B), respectively, were also included. The quantity of RSV was compared with purified RSV as an index of absorbance at 450 nm.

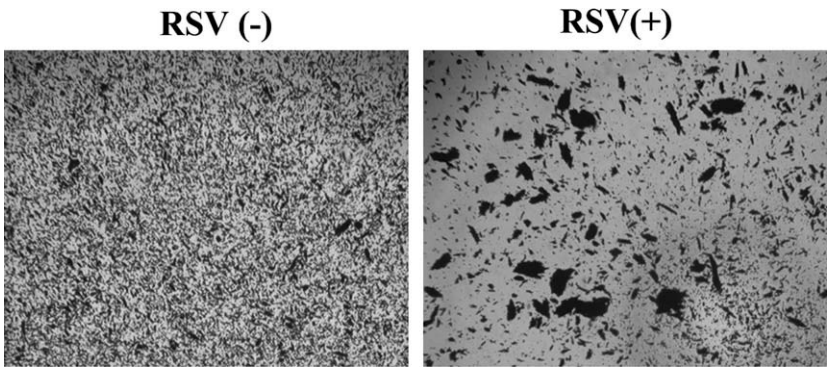

Figure 3. Aggregation of anionic magnetic beads incubated with RSV-infected nasal aspirates. The anionic magnetic beads were washed twice with binding buffer and PBS. Then, the magnetic beads were incubated for 20 min with RSV-infected (+) or non-infected (−) nasal aspirates diluted with PBS at room temperature. Light micrographs of the incubated beads are shown. Aggregation of the beads was observed after incubation with RSV-infected nasal aspirates, whereas no aggregation was observed after incubation with non-infected nasal aspirates.

any method should be simple, because the possibility of loss and cross-contamination among samples is increased with multiple steps. Therefore, capture by magnetic beads is a promising approach. There are several methods for concentrating a virus using magnetic beads coated with an antibody for a specific virus[6,14,15] and polymers such as polyethyleneinime (PEI) for simian virus 40 (SV40),[16] herpes simplex virus type 1 (HSV-1),[16] Sindbis virus,[16] vesicular stomatitis virus (VSV),[16] amphotropic murine leukemia virus,[17] poliovirus,[18] hepatitis A virus (HAV),[18] hepatitis B virus (HBV),[18] hepatitis C virus (HCV),[18] and cytomegalovirus[19] or sulfonated (SO) magnetic beads in the presence of divalent cations for cytomegalovirus,[19] Sindbis virus,[19] poliovirus,[19] and porcine parvovirus.[19] Our recent studies have shown that poly(MVE-MA)-coated magnetic beads can be used for efficient capture of avian and human influenza viruses.[12,20]

Previous studies have shown that antibody-conjugated, PEI-conjugated, poly(MVE-MA)-coated, or SO magnetic beads can be used to concentrate several viruses. However, the concentration of RSV has not been described previously. The present study clearly showed that poly(MVE-MA)-coated magnetic beads can be used to capture RSV. Until now, the mechanism by which the magnetic beads bind to RSV has remained unknown. As HBV with an envelope protein could not be captured by these anionic magnetic beads (Sakudo, unpublished result), the binding ability does not depend on the presence of an envelope protein. One possible explanation is that electrostatic, hydrophilic, and hydrophobic interactions, along with steric phenomena, are involved in this binding. The anionic magnetic beads are negatively charged, and modification of the spatial organization of the beads could decrease the binding capacity.[21] In addition, the pH affects the binding capacity of the beads to RSV (Supplementary Fig. 1). The charge density and steric spatial organization may provide some information on the binding mechanism. Interestingly, in contrast to HBV, the envelope proteins of influenza virus and RSV, which are captured by the anionic magnetic beads, recognize receptor structures with a negative charge, for example, sialic acid[22] and heparan sulfate,[23] respectively. Moreover, the pre-incubation of anti-RSV fusion protein (F) or G glycoprotein (G) decreased the levels of RSV recovered by the beads (Supplementary Fig. 2). This suggests that the surface structure and charge of anionic beads might imitate the virus receptors, while RSV binds to the beads via F and G. In addition, aggregation of the magnetic beads was observed after incubation with RSV-infected nasal aspirates, whereas no aggregation was noted after incubation with non-infected nasal aspirates (Fig. 3). This aggregation may be a result of virus binding, but also may contribute to the mechanism by which RSV binds to the magnetic beads. Supporting these observations, although both neutral and acidic conditions (pH 7.4–5.6) were required for the binding of RSV to the beads (Supplementary Fig. 1), the levels of RSV bound to the beads were correlated with those of bead aggregation (Supplementary Fig. 3). More interestingly, influenza A and B viruses, which bind to magnetic beads, also cause the aggregation of the beads.[12] Furthermore, the binding of RSV to beads is abrogated in a competitive manner by the presence of influenza A and B virus, suggesting that the binding mechanism is shared with these viruses. Unfortunately, the anionic magnetic beads appear to bind blood components. Moreover, under the current experimental conditions, we could not recover more than 2–3 μg of RSV using 50 μl of magnetic beads from samples containing an even higher concentration of RSV (data not shown). Thus, the limit of the binding capacity is estimated to be as 2–3 μg of RSV per 50 μl of magnetic beads. Therefore, this method is appropriate for the detection of RSV in samples containing a low concentration of RSV but not for purification or obtaining a high yield of RSV. However, modifications affecting the charge density, surface chemistry, and binding capacity may reduce the non-specific binding and increase the yield of RSV.

In conclusion, we demonstrated that magnetic beads coated with an anionic polymer are useful for the capture and concentration of RSV. In the captured RSV, the presence of a viral genome, nucleocapsid protein, and fusion protein were confirmed by RT-PCR, ELISA, immunochromatography, and Western blotting. This method can be used in combination with conventional means of detection. The applicability of this method to different types of virus is now being studied.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.127.

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8. Monozide 300 nm-diameter magnetic particles (reducing sedimentation and offering a broad binding surface) with a high ferrite content (allowing face separation under a magnetic field) were prepared by grafting of poly(MVE-MA) (CAS No. 9011-16-9) in dimethyl sulfoxide/phosphate buffer 5/95 solution for 3 h at 37 °C (Flavigny, E.; Gaboyard, M.; Merel, P.; Fleury, H. *Abstract of Papers*, 104th General Meeting, American Society for Microbiology: New Orleans, LA, 2004; Abstract 166). The anionic magnetic beads, Viro-adembeads, available commercially (Ademtech, Pessac, France) were used in the present study.
9. Clinical nasal aspirates were collected from pediatric patients at the Baba pediatric clinic. The method of collection was described previously (Baba, K. Japan Patent 2008-119552A, 2008). Briefly, saline was introduced into the nasal cavity, and fluid was collected using a nasal aspirator, Belvital (Melisana, Nogent-sur-Marne, France). In order to remove cell debris, the nasal fluid was filtered using a stainless steel mesh [200 grids per inch (25.4 mm)]. Nasal aspirates similarly collected from healthy donors were also used. The research project for the development of diagnostic methods for respiratory infectious diseases was approved by the Ethics Committee of the Research Institute for Microbial Diseases in Osaka University and written informed consent was obtained from the patients and healthy donors.
10. Viral capture of RSV from nasal aspirates using poly(MVE-MA) was performed as follows. Briefly, after two washes with binding buffer, anionic magnetic beads (50 μl) were further washed twice with phosphate-buffered saline (PBS). Then, 20 μl of nasal aspirate diluted with 500 μl of PBS was added to the washed beads and incubated for 20 min at room temperature. Tubes containing the magnetic beads were set under a magnet field. Then, the beads were subjected to magnet separation by discarding the supernatant and washed three times with PBS. The washed beads were resuspended with PBS (20 μl) and subjected to further analyses. After the separation, four fractions were obtained as follows: a beads fraction (20 μl of anionic magnetic beads after incubation with nasal aspirate) (BD), the sample before incubation with the beads [20 μl of nasal aspirate (20 μl) diluted with PBS (500 μl)] (BF), supernatant after the incubation and wash (20 μl of supernatant) (SP), and total sample containing the same quantity (20 μl) of nasal aspirate as BD (20 μl of nasal aspirate without any treatment) (TL) (Sakudo, A.; Baba, K.; Tsukamoto, M.; Sugimoto, A.; Okada, T.; Kobayashi, T.; Kawashita, N.; Takagi, T.; Ikuta, K. *Bioorg. Med. Chem.* **2009**, *17*, 752).
11. RT-PCR. Firstly, viral RNA from beads or aliquots of samples was extracted with the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To extract RNA from the magnetic beads, lysis buffer was added. Then the beads were removed after the lysis step. RNA was eluted in 60 μl of nuclease-free water. For the RT-reaction, random primers were used. After incubation at 25 °C for 10 min, RNA was reverse-transcribed at 65 °C for 50 min, followed by denaturation of the enzyme at 85 °C for 5 min. The diluted cDNA was amplified in a reaction mixture containing primers, Ex Taq (Takara bio Inc., Otsu, Japan), and Ex Taq buffer under conditions of 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. PCR was carried out using the following primers for the RSV fusion protein gene: RSVfusion-F: 5′-TTA ACC AGC AAA GTG TAA GA-3′; RSVfusion-R: 5′-TTT GTT ATA GGC ATA TCA TTG-3′.
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13. ELISA for the RSV antigen was performed using a SERION ELISA antigen Quadrogen kit for RSV (Serion Immundiagnostica & Institut Virion/Serion GmbH, Würzburg, Germany). RSV concentration was estimated by comparison with purified RSV (Hytest Ltd, Turku, Finland).
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