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Five Cases of Cytomegalovirus Infection Detected by *in situ* Hybridization and Antigenemia Assay

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We report five cases of cytomegalovirus infection in immunocompromised patients which were detected by either cytomegalovirus antigenemia assay or in situ hybridization. Four cases had leukemia and the other had chronic renal failure. All the three BMT recipients suffered from GvHD. Interestingly, there was an unique case of CMV disease without a history of BMT, which reminded us that CMV could attack immunocompromised patients who had not undergone transplantation, too. Four out of five cases died. We think that cytomegalovirus infection or disease should not be regarded as a minor problem in post-transplantation infection in Korea.

Key Words : *Cytomegalovirus, Bone marrow, Transplantation, In situ hybridization.*

INTRODUCTION

Cytomegalovirus(CMV) is known to be the major cause of morbidity and mortality after bone marrow transplantation(BMT) especially during the early postengraftment phase(Meyers et al., 1990 ; Sable and Donowitz, 1994). It attacks about 50% of marrow transplant recipients. In Korea, however, there have only been a few sporadic reports about CMV infection and diseases(Kim et al., 1992 ; Park et al., 1993) and no sophisticated data at all such as the overall incidence of CMV infection in transplant recipients despite a high prevalence of IgG anti-CMV antibodies. Because there have been few CMV-detection methods available and little attention has been paid to it, we cannot exclude CMV as a major causative pathogen of post-BMT infection and thus we believe that it may also rank as one of the main post-BMT infectious problems in Korea. Recently we have detected five cases of CMV infection in immunocompromised patients, between July and October 1993, using CMV antigenemia assay and *in situ* hybridization(ISH). Hence we report these five cases and would like to suggest the necessity of initiating a systematic investigation of the overall incidence and trend of CMV infection after BMT in Korea.

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MATERIALS AND METHODS

In situ Hybridization

All the procedures were performed with MicroProbe incubator(Biomeda corp., U.S.A.) using complementary DNA of CMV mRNA. A CMV-infected lung tissue was used as a positive control. The

whole procedure was done within 2 hours.

1) preparation

The 5 μm tissue section was attachd to specialized slide(ProbeOn plus microscopic slide, Fischer Scientific, U.S.A.). Paraffin was removed by the 1 : 3 mixture solution of xylene and Histoclear(National Diagnostics, Somerville, U.S.A.). Then dehydration was done by 100% alcohol. The tissue section was dipped into pepsin solution(Biomeda corp., U.S.A.) at 110℃ for 5 min.

2) hybridization

The biotin-labeled probe was applied to the tissue for 1 min followed by heating for 10 min. at 92℃. After 2 min. at room temperature, it was held at 40℃ for 30 min. Then serial washing procedure was done using 10% ethanol and 10×automation buffer (Biomeda corp., U.S.A.), and 5×SSC(sodium chloride 17.53 g, sodium citrate 8.82 g, distilled water 1,000ml) three times for 30 seconds each.

3) chromogenic detection

Detection procedure was done using alkaline phosphatase(Dako A/S, Denmark), naphthol AS-MX phosphate(Sigma Co., U.S.A.), dimethyl formamide(Amreco., U.S.A.), fast red TR salt(Amreco., U.S.A.), and 0.1M sodium carbonate. An red-colored intranuclear staining was regarded as a positive signal.

Antigenemia assay

Mononuclear cells were obtained from heparinized blood & separated by dextran sedimentation. The pellet was suspended in 0.2 ml phosphate buffer solution(PBS). Cytospin preparations were made with 100uL of a suspension of 1.0×10^6 cells/ml by centrifugation for 4min. at 800rpm. The preparations were fixed in acetone, air dried. The slides were placed in PBS and then incubated in duplicate with an monoclonal antibody mixture directed at pp65 antigen for 45 min. at room temperature. After using peroxidase-labeled rabbit antimouse immunoglobulin at room temperature for 45min, we applied freshly prepared 3-amino-9-ethylcarbazole solution for 10min. at room temperature. The slides were washed with acetone buffer(pH 4.9, 0.05 M) and the counterstaining with hematoxyln for 30 seconds and rinsing were done. The dark-brown nuclear staining was interpreted as a positive result. CMV infected fibroblasts were used as positive controls.

CASE REPORT

Case 1

A 22-year old man was admitted for a BMT from his HLA-identical brother for acute myelogenous leukemia, M_2. His pretransplant IgM CMV antibody was negative. Leukocyte count was 3,600/ μl(64% neutrophil) on the day of admission, and other laboratory findings were normal. Conditioning consisted of total body irradiation with 1320 Rad on day −6 through −3 followed by cyclophosphamide at 50 mg/kg given on day −2 to −1 with marrow infusion on day 0. Cyclosporine was given post-transplant. A bone marrow biopsy on day +21 showed 20% cellularity which indicated good engraftment. On day +23, a skin rash developed. A skin biopsy revealed acute graft versus host reaction(GvHD), so he received solumedrol pulse therapy. On day +38 he suffered from fever and watery diarrhea over ten times a day and fiberoptic

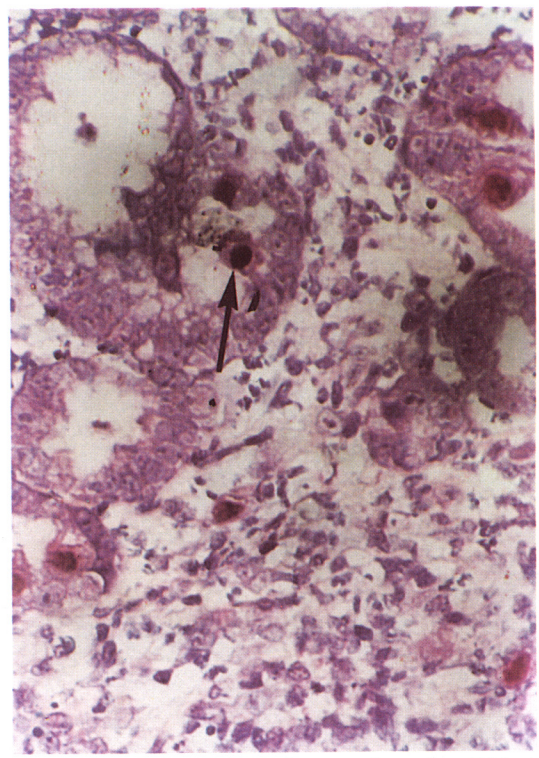

Fig. 1. *In situ* hybridization using complementary DNA of CMV mRNA as a specific probe showed a positive signal (arrow) on the gastric glandular cells of case 1.

examination of the colon & rectum revealed no definite finding. He also began to complain of epigastric pain on day +58 and a gastrofiberscopic biopsy showed ballooning degeneration and intranuclear inclusions in glandular cells strongly suggestive of CMV involvement. ISH using complementary DNA of CMV mRNA as a probe was done and revealed a positive signal(Fig. 1). CMV antigenemia assay also showed a positive result. Ganciclovir 5 mg/kg twice a day was given intravenously for 14 days simultaneously with intravenous immune globulin(I-VIG) 5 g per day. Although gastrointestinal symptoms and signs improved thereafter and a follow-up CMV antigenemia assay two weeks later showed negative conversion, he died of suddenly developed cerebral infarction.

Case 2

A 38-year old man who underwent an allogeneic BMT for chronic myelogenous leukemia three months previously was admitted due to diffuse abdominal pain and watery diarrhea. About one week before admission, he suffered from skin GvHD. His pretransplant IgM CMV antibody was negative. Leukocyte count was 8,900/ μ l(68% neutrophil) on the day of admission, and other laboratory findings were normal. His abdomen was soft but diffuse tenderness was noted. A sigmoidoscopic examination revealed an erythematous lesion with multiple shallow ulcerations on the mucosal surface. A biopsy showed clustering of ballooning cells on the base of the ulceration. ISH on the same tissue specimen showed a positive signal for CMV(Fig. 2) and a CMV antigenemia assay was positive. Ganciclovir 5 mg/kg twice a day was given intravenously for 14 days simultaneously with intravenous immune globulin(IVIG) 5 g per day. His gastrointestinal symptoms and signs improved and a follow up CMV antigenemia assay resulted in negative conversion. However, he suffered from impairment of liver function with jaundice and ascites. He was aggressively managed with the impression of veno-occlusive disease but several days later he died due to fulminant hepatic failure.

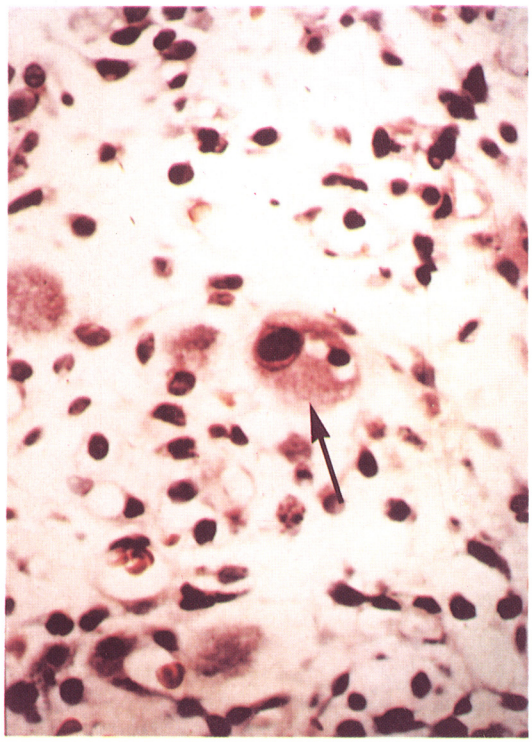

Fig. 2. *In situ* hybridization on the sigmoidoscopic biopsy specimen of case 2 showed a positive signal(arrow) for CMV.

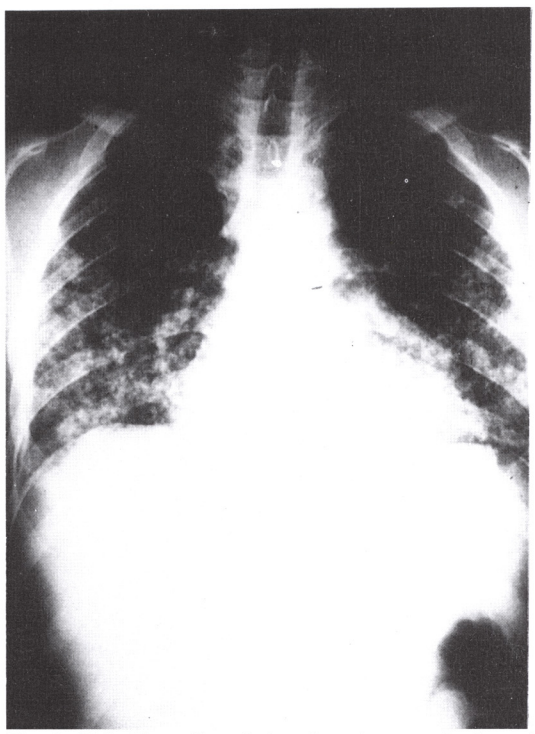

Fig. 3. Plain X-ray film of the chest in case 3 showed diffuse interstitial infiltration over the whole lung fields.

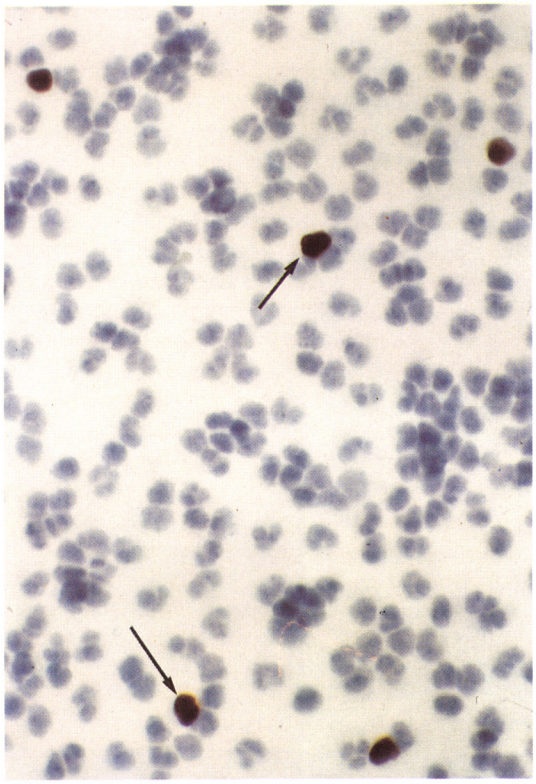

Fig. 4. A CMV antigenemia assay of case 3 showed a positive result(arrows).

Case 3

A 15-year old boy was re-admitted due to suddenly developed fever, dyspnea and productive cough. 40 days before admission, he had undergone an allogeneic BMT for hybrid leukemia. Until the second admission, he had received a maintenance treatment for acute GvHD. A plain chest film showed diffuse interstitial pneumonitis(Fig. 3) and a CMV antigenemia showed a positive result(Fig. 4). Ganciclovir was instituted but he died due to respiratory failure on the admission day.

Case 4

A 39-year old man was admitted due to fever and pain in his right ankle. Physical examination on admission revealed no remarkable findings except for erythematous swelling in his right ankle. The white blood cell count was 15,000/mm^3 with 80% of blast cells. The blood chemistry was normal. An electrocardiography and a chest roentgenogram were normal. A bone marrow biopsy revealed acute lymphoblastic leukemia, L_2. Induction chemotherapy including methotrexate(2 g/m^2 for 1 day) with leukovorin rescue(20% dosage of methotrexate four times a day for 3 days), vincristine(1 mg/m^2 for 1 day), and prednisolone(20 mg three times a day for 7 days) was given. After induction chemotherapy, the pain in the right ankle was relieved but pancytopenia persisted and a high fever with dyspnea developed. However, physical examination revealed no definite abnormalities and a plain chest X-ray was normal. Ceftazidime 2.0 g/day and amikacin 750 mg/day were instituted empirically, followed by teicoplanin 400 mg/day and ornidazole 1.0 g/day 2 days after. However, his symptoms and signs never improved. An antifungal agent, amphotericin B 1 mg/kg, was added but his condition deteriorated day after day. A follow-up chest X-ray showed diffuse interstitial infiltration over the whole lung field. A bone marrow biopsy on postchemotherapy day 21 showed extremely hypocellular marrow. Despite aggressive management, he eventually died of sepsis and respiratory failure eventually. A percutaneous biopsy of the lung after death unexpectedly revealed swelling and intranuclear inclusions of alveolar macrophage strongly suggestive of CMV infection. ISH on the same specimen showed positive signals for CMV.

Case 5

A 53-year old man with end stage renal disease received an allogeneic kidney transplantation. He suffered from acute rejection immediately after transplantation, but improved successfully with cyclosporine and prednisolone. He was well until he noticed severe epigastric discomfort 2 weeks later and a gastrofiberscopic examination was done. On gastrofiberscopy, a gastric ulcer was found. A biopsy revealed giant ballooning glandular cells with intranuclear inclusions and ISH for CMV gave a positive result. Because his general condition was very fine despite evidence of a CMV gastric ulcer, no specific treatment aimed at CMV was given to him. The epigastric discomfort relieved soon after administration of an anti-ulcer regimen. He was discharged in an improved condition.

The characteristics and clinical course of these five patients are summarized in table 1.

Table 1. Case Summary of CMV Infection

	Case 1	Case 2	Case 3	Case 4	Case 5
Age/Sex	22/male	38/male	16/male	39/male	53/male
Underlying disease	AML	CML	AML/ALL	ALL	CRF
Transplantation	BMT	BMT	BMT	—	KT
Complication after transplantation	GVHD	GVHD	GVHD	—	acute rejection
Clinical manifestation	gastritis	enterocolitis	pneumonitis	pneumonitis	gastric ulcer
Detection method	ISH, antigenemia	ISH, antigenemia	antigenemia	ISH	ISH
Treatment	GCV+IVIG	GCV+IVIG	GCV+IVIG	—	symptomatic treatment
Final outcome	death	death	death	death	alive

AML : Acute Myelogenous Leukemia, ALL : Acute Lymphocytic Leukemia, BMT : Bone Marrow Transplantation, CML : Chronic Myelogenous Leukemia, CRF : Chronic Renal Failure, GCV : ganciclovir, GVHD : Graft versus Host Reaction, ISH : *in situ* Hybridization, IVIG : intravenous immune globulin, KT : Kidney Transplantation.

DISCUSSION

We have presented five cases of CMV infection which was previously regarded as a trivial problem after transplantation in Korea. There were various organ involvements-lung, stomach, and colon. Four cases had hematological malignancy(case 1-4) and the other(case 5) had chronic renal failure. All the three cases(case 1, 2, and 3) who received BMT suffered from GvHD, which suggested that CMV infection might be closely associated with GvHD. All the patients who received ganciclovir(cases 1-3) died. In case 1 and 2, however, CMV antigenemia disappeared in two weeks after initiation of ganciclovir and the causes of death such as cerebral infarction and veno-occlusive disease seemed to be independent of CMV infection. In case 3, it was too late to alter the clinical course by giving ganciclovir, therefore the efficacy of ganciclovir on these three patients could not be underestimated. We think that the efficacy of ganciclovir with or without intravenous immune globulin should be prospectively assessed further with an adequate number of cases.

Case 4(CMV pneumonitis) was unique in that CMV disease developed without a history of BMT, which indicated that CMV could attack immunocompromised patients who had not undergone transplantation, too.

One may argue that, in case 5, gastric ulcer after kidney transplantation was independent of CMV and the detected CMV may not be a causative agent but only represent a nonproductive latency. However, the detected signal by ISH was not from DNA but from mRNA of CMV which reflected active ongoing replication of this virus in the gastric tissue. Although CMV mRNA may be detected exceptionally in rare cases of inactive latency(Schrier et al., 1985), we believe that CMV caused the gastric ulcer in this patient on these grounds.

We have used CMV antigenemia assay and/or ISH to detect CMV infection. Because the diagnosis of CMV infections cannot be made solely on a clinical basis alone, laboratory confirmation is needed. Isolation of CMV from blood leukocytes(CMV viremia) is a reliable marker of disseminated CMV infection and predicts invasive CMV disease(Boeckh et al., 1992 ; Mazzulli et al., 1993). Therefore, rapid and sensitive methods for the detection of CMV viremia are important to identify patients at risk for developing invasive CMV disease, who may benefit from early antiviral therapy. The most specific method for detection of CMV infection is isolation of the virus in culture. However, it takes too much time to detect CMV infection and has a poor correlation to the clinical course(Landry and Ferguson, 1993). Recently, a new method—CMV antigenemia assay—has been proved to be both a

sensitive and a rapid technique for the detection of CMV infection. It has been reported to be more sensitive than shell vial as well as conventional culture(Erice et al., 1992). The antigenemia assay is based on direct detection of structural proteins of the viral matrix(pp65) in cytospin preparations of peripheral leukocytes using monoclonal antibody mixture directed against these proteins. It can also be used as an indicator to monitor the course and effectiveness of ganciclovir therapy. There are many reports that early detection and pre-emptive treatment of asymptomatic CMV infection on the basis of CMV antigenemia assay reduces overt CMV infection rate and improves the prognosis(Vlieger et al., 1992 ; *von* Bueltzingsloewen et al., 1992). A pre-emptive regimen included ganciclovir alone or in combination with immune globulin may be promising in the prevention of CMV infection after allogeneic bone marrow transplantation(Winston et al., 1993).

With the above results, we stand firm in our belief that CMV infection should not be overlooked after transplantation in Korea. Hence, a prospective investigation of the incidence of CMV infection after BMT has been underway in our center since June 1994 using CMV antigenemia assay and PCR as guidelines. On completion of this study, we will initiate a prospective trial of ganciclovir as a pre-emptive therapeutic modality on CMV antigen- or PCR-positive patients.

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