healthy male volunteers. These results suggest that micafungin has clinical potential in deep-seated mycosis, and could be administered safely in patients.

This multicenter, open-label study was conducted to investigate the efficacy and safety of micafungin in patients with deep-seated mycosis caused by Aspergillus or Candida spp.

PATIENTS AND METHODS

Patients

Male and female patients with deep-seated mycosis caused by Aspergillus or Candida spp., aged 20–79 y, were included in this study. The diagnostic criteria for inclusion in this study consisted of both clinical findings (compatible symptoms and diagnostic image) and mycological evidence (1 or more positive results in culture, histopathological examination, serological tests, or polymerase chain reaction assay). Serological tests (detecting fungal antigen or β-D-glucan) were used as subsidiary tests, because their sensitivity and specificity are still insufficient. Positive fungal antibody was accepted only in chronic forms of aspergillosis. The diagnosis of invasive pulmonary aspergillosis (IPA) required infiltrates on chest X-ray or computed tomographic scan together with clinical features (refractory fever against broad-spectrum antibacterial agents, CRP increase) and mycological evidence. The results of culture or histopathological examination were accepted in pulmonary aspergillosis if the specimens were taken from a sterile lung lesion (or near the lesion) or in cases with sequential positive sputum cultures. Diagnostic criteria for chronic necrotizing pulmonary aspergillosis (CNPA) were defined as shown in Table I, and diagnosis of CNPA was made in patients who met all 5 criteria. Pulmonary aspergilloma required a fungus ball in a cavitary lesion on chest X-ray or computed tomographic scan, together with clinical symptoms (cough, sputum, fever) and positive antibody. For candidiasis, diagnosis of candidemia was defined as persistent fever (> 38°C) with a positive blood culture or positive serological tests. Esophageal candidiasis required the endoscopic findings of plaque on esophageal mucosa and mycological evidence. Diagnosis of disseminated mycosis was made if the patients had multiple lesions on radiological imaging with compatible clinical findings. In cases where the patients had received prior antifungal treatment, responding patients were excluded. The validity of inclusion and diagnosis of all patients was reviewed by an independent expert medical panel.

Table I. *Diagnostic criteria for chronic necrotizing pulmonary aspergillosis*

Criteria[a]
1. Chronic pulmonary symptoms or systemic symptoms with at least 1 of the following:fever, weight loss, productive cough, hemosputum
2. Cavitary pulmonary lesion with evidence of paracavitary infiltrates orcavitary change developing over a time-frame of several weeks to several months
3. Positive results in serological tests (antigen/antibody detected) or documented Aspergillus species from the pulmonary or pleural cavity by culture or by pathological examination
4. Elevated inflammatory markers (e.g. C-reactive protein)
5. Exclusion of other pulmonary pathogens by appropriate cultures and serological tests that are associated with a similar disease presentation including Mycobacteria and other bacterial infections

[a] Diagnosis of chronic necrotizing pulmonary aspergillosis was made in patients who met all 5 criteria above.

Study design

This study was conducted as an open-label trial at 41 sites in Japan. This clinical research was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines, and the protocol was approved by the institutional review boards at each participating center. Informed consent was obtained from all enrolled patients.

Patients received a once-daily intravenous infusion of micafungin at dosages between 12.5 and 150 mg/d, for a minimum of 7 d and a maximum of 56 d. If required, dose escalation was allowed after 7 d of dosing at the same level (4 d for esophageal candidiasis). No other antifungal drug was administered within the micafungin treatment period.

Efficacy evaluations

The primary endpoint was overall clinical response ('success', 'failure', or 'not evaluable') at the end of therapy. This assessment was made on the basis of clinical response (clinical findings and/or clinical symptoms), mycological response (culture or histopathological examination), serological response (serological tests detecting specific fungal antigen or β-D-glucan), and diagnostic imaging improvement (radiological or endoscopic examination). The improvement was judged regardless of partial or complete response for each factor. The independent expert medical panel reviewed the eligibility and the investigators' efficacy assessment of all patients. Treatment success in overall clinical response was defined for each diagnosis as follows: For IPA, radiological improvement without clinical deterioration was required. For CNPA, the treatment success was defined by radiological and clinical improvement. For pulmonary aspergilloma, it was defined by radiological improvement without any deterioration in clinical and mycological findings, or by clinical improvement without any deterioration in radiological and mycological findings. For candidemia, clinical improvement with mycological and/or serological improvement was required. For esophageal candidiasis, the treatment success was defined by clinical and endoscopic improvement. For disseminated mycosis, clinical and radiological improvement was required.

During the treatment period, clinical findings (parameters; fever, C-reactive protein level, erythrocyte sedimentation rate) were recorded for efficacy evaluation. In pulmonary aspergillosis, symptomatic parameters such as cough, sputum, hemosputum, hemoptysis, dyspnea and oxygen level in arterial blood were also recorded. In esophageal candidiasis, observation of plaque, odynophagia, and/or retrosternal pain were made additionally and recorded. Mycological examinations (culture, histopathological, and/or serological tests) and diagnostic imaging examinations (radiological imaging or endoscopy) were performed at scheduled intervals.

For efficacy analysis, the response rate was defined as the percentage of responding patients (evaluated as 'success') in the efficacy-evaluable population who received more than 7 doses of micafungin. Clinical response was defined as a significant improvement as judged by the investigator, based on changes of the parameters. Mycological response was judged by the eradication of the causative fungi isolated at entry to the study. Serological improvement was judged by the normalization or decrease in 30% or more of the β-D-glucan value, and/or disappearance of the fungal antigen. In pulmonary aspergillosis and esophageal candidiasis, radiological or endoscopic improvement was judged by a significant resolution on imaging or endoscopy.

Safety evaluations

All adverse events occurring during treatment were recorded, including abnormal laboratory profiles. Clinical laboratory tests (hematology, serum chemistry, and urinalysis) were performed at baseline, on d 4, d 8, and weekly thereafter. Ongoing adverse events at the end of therapy were followed up until the event resolved.

For safety analysis, the incidence of drug-related adverse events, including abnormal laboratory profiles, was assessed in the population of all patients who received at least 1 dose of micafungin.

RESULTS

A total of 70 patients were enrolled at 29 of 41 sites and received at least 1 dose of micafungin; 56 patients were evaluable for efficacy, 42 with aspergillosis and 14 with candidiasis. Patient numbers for each diagnosis are shown in Table II. The expert medical panel considered that 14 patients were not evaluable for efficacy, and these patients were excluded from efficacy analysis. Four patients were withdrawn from the study after receiving less than 7 doses of micafungin because of clinical deterioration or adverse events. After such a short treatment period the responses of these patients were not assessable, because there were insufficient findings for efficacy evaluation. One of these 4 patients, and 10 other patients, did not meet the diagnostic criteria, and were therefore excluded from efficacy evaluation–empirical entry was approved in the primary protocol and enrollment of these patients had been allowed at study entry. Seven of these 11 patients had no clear clinical features caused by fungal infection; their major clinical findings were affected by other illness such as bacterial infections or other underlying diseases, and these findings were not adequate for efficacy evaluation. One of the 7 patients, and 4 other patients, did not meet the inclusion criteria because there was no evidence of Aspergillus or Candida infection (causative fungi were not detected by any mycological examination, including serological tests and polymerase chain reaction assay).

Patient demographics and baseline characteristics are shown in Table III. Of the 56 patients involved in the efficacy analysis, 44 were male, and the mean age was 61 y (range 26 to 77 y). Hematopoietic disease was an important underlying disease in both aspergillosis (9/42 patients) and candidiasis (4/14 patients). Among the 10 patients with IPA, 8 patients had leukemia or lymphoma. Seven of these 8 patients had received chemotherapy prior to this study, and 2 of the 8 patients were neutropenic (cell count < 500) at enrollment. Two patients received bone marrow transplantation prior to the study, but these patients were not neutropenic at enrollment. Prior or current pulmonary tuberculosis was the most common underlying disease in aspergillosis (20/42 patients). Of these 20 patients, 2 had IPA, 5 had CNPA, and 13 had pulmonary aspergilloma. Diabetes mellitus was also common in aspergillosis (10/42

Table II. *Study population*

	Aspergillosis	Candidiasis	Unknown	Total
	No. of patients	No. of patients	No. of patients	No. of patients
All patients enrolled	46	19	5	70
Received at least 1 dose of study drug	46	19	5	70
Evaluable for efficacy	42	14	0	56
Diagnosis				
Invasive pulmonary aspergillosis	10			10
Disseminated aspergillosis	1			1
Chronic necrotizing pulmonary aspergillosis	9			9
Pulmonary aspergilloma	22			22
Candidemia		6		6
Disseminated candidiasis		1		1
Esophageal candidiasis		7		7
Demographics and baseline characteristics				
Gender				
Male	33	11		44
Female	9	3		12
Age				
Mean	62	57		61
SD	11	14		12
Range	26–77	30–73		26–77
Common underlying disease				
Hematopoietic disease	9	4		13
Neutropenia	2			2
Bone marrow transplantation	1	1		2
Pulmonary tuberculosis (prior or current)	20	1		21
Diabetes mellitus	10	4		14
AIDS		4		4

Table III. *Overall clinical response (Aspergillosis)*

No. (%) of patients responding	Maximum dose (mg/d)	No. (%) of patients responding by maximum dose	Mean treatment period (range)
Invasive pulmonary aspergillosis ($n = 10$)			
6 (60)	50 ($n = 2$)	1 (50)	23 (17–28)
	75 ($n = 3$)	1 (33)	15 (8–28)
	150 ($n = 5$)	4 (80)	42 (21–56)
Disseminated aspergillosis ($n = 1$)			
0 (0)	150 ($n = 1$)	0 (0)	19 (19)
Chronic necrotizing pulmonary aspergillosis ($n = 9$)			
6 (67)	25 ($n = 1$)	0 (0)	13 (13)
	50 ($n = 1$)	1 (100)	56 (56)
	75 ($n = 3$)	2 (67)	47 (29–57)
	150 ($n = 4$)	3 (75)	43 (11–56)
Pulmonary aspergilloma ($n = 22$)			
12 (55)	25 ($n = 6$)	2 (33)	25 (18–29)
	50 ($n = 4$)	3 (75)	47 (28–56)
	75 ($n = 8$)	5 (63)	40 (28–56)
	100 ($n = 1$)	0 (0)	25 (25)
	150 ($n = 3$)	2 (67)	42 (28–56)
Total ($n = 42$)			
24 (57)			36 (8–57)

patients). Four of these 10 patients had CNPA, and 6 had pulmonary aspergilloma. All AIDS patients had candidiasis.

The overall clinical response rates by the maximum dose are shown in Table IV for aspergillosis, and in Table V for candidiasis. The maximum doses of micafungin ranged from 25 to 150 mg/d. The mean duration of treatment was 36 d (range 8–57 d) for aspergillosis and 16 d (range 7–29 d) for candidiasis.

The overall response rates were 57% (24/42) in aspergillosis, and 79% (11/14) in candidiasis. The overall response rates by diagnosis were 60% (6/10) in IPA, 67% (6/9) in CNPA, 55% (12/22) in pulmonary aspergilloma, 100% (6/6) in candidemia, and 71% (5/7) in esophageal candidiasis. Two patients with disseminated fungal infection (1 with aspergillosis, 1 with candidiasis) did not respond. Prior antifungal treatment had no influence on efficacy. The overall response rates were similar for patients with prior antifungal treatment (20/33, 61%), which was considered ineffective or toxic, and patients without prior treatment (15/23, 65%). The response rate was 58% (18/31) in the refractory or intolerant patients who had received antifungal treatment in adequate doses for > 7 d. Clinical response rates were 55% (22/40) in aspergillosis, and 85% (11/13) in candidiasis. Response rates by diagnosis were 60% (6/10) in IPA, 63% (5/8) in CNPA, 52% (11/21) in pulmonary aspergilloma, 100% (6/6) in candidemia, and 83% (5/6) in esophageal candidiasis. Mycological response was assessed in patients in whom mycological examination could be performed both pre- and

Table IV. *Overall clinical response (Candidiasis)*

No. (%) of patients responding	Maximum dose (mg/d)	No. (%) of patients responding by maximum dose	Mean treatment period (range)
Candidemia ($n = 6$)			
6 (100)	25 ($n = 3$)	3 (100)	16 (7–28)
	50 ($n = 1$)	1 (100)	28 (28)
	75 ($n = 2$)	2 (100)	16 (14–17)
Disseminated candidiasis ($n = 1$)			
0 (0)	75 ($n = 1$)	0 (0)	16 (16)
Esophageal candidiasis ($n = 7$)			
5 (71)	25 ($n = 2$)	0 (0)	11 (7–14)
	50 ($n = 3$)	3 (100)	7 (7–8)
	75 ($n = 2$)	2 (100)	29 (28–29)
Total ($n = 14$)			
11 (79)			16 (7–29)

post-treatment using an adequate specimen (from a lung lesion or sputum in pulmonary aspergillosis, or blood in candidemia, or from esophageal mucosa in esophageal candidiasis). Fungal eradication was observed in 63% (12/19) of the aspergillosis patients and 50% (4/8) of the candidiasis patients. A variety of organisms were eradicated after micafungin treatment, including Aspergillus fumigatus (eradication rate: 50%, 7/14 isolates), Aspergillus flavus (100%, 1/1 isolate), Aspergillus terreus (100%, 1/1 isolate), Aspergillus niger (100%, 2/2 isolates), Candida albicans (43%, 3/7 isolates), Candida glabrata (100%, 1/1 isolate), and Candida krusei (100%, 1/1 isolate). The serological response rates were 47% (14/30) in aspergillosis, and 56% (5/9) in candidiasis. All 42 patients with aspergillosis had pulmonary lesions, and were assessed by chest X-ray or by computed tomographic scan as having radiological improvement. The improvement rates were 70% (7/10) in IPA, 56% (5/9) in CNPA, and 41% (9/22) in pulmonary aspergilloma. One patient with disseminated aspergillosis also showed an improvement in the chest radiograph. The endoscopic improvement rate was 71% (5/7) in patients with esophageal candidiasis. A follow-up assessment was made 2 weeks after the end of micafungin treatment. IPA flared up again in 1 patient (1/6 IPA cases) who had shown a partial response to micafungin. Except for this case, there were no other recurrent cases of fungal infection in the responders. Among 21 micafungin non-responders, 14 patients were treated with other antifungals. Three patients (3/14) responded to the other antifungals after 2 weeks.

Among the 70 patients who received at least 1 dose of micafungin, only 1 patient discontinued treatment because of a drug-related adverse event (serious neutropenia). Nine other patients required discontinuation of treatment because of adverse events which were not considered to be drug-related. Adverse events related to micafungin occurred in 21 of 70 patients (30%) (Table V). Most events occurred in only 1 patient, and events which occurred in 2 or 3 patients included phlebitis, elevations in alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, blood urea nitrogen, and serum creatinine. Most adverse events were mild to moderate in severity except for 1 case of serious neutropenia. There was no dose-related occurrence of any adverse event.

DISCUSSION

The incidence of systemic fungal infections has recently increased. The most common causative fungi are Candida spp. (18), but the frequency of occurrence and the relative importance of aspergillosis are on the increase worldwide (19). However, active antifungal agents are still limited, especially against Aspergillus spp. Furthermore, the emergence of azole-resistant Candida spp. has become more problematic recently (7, 20, 21). Increased use of FLCZ is considered to be linked to increased rates of blood-stream infection with non-albicans Candida spp. (7, 22, 23). Therefore, a new broad-spectrum antifungal agent has been needed. Recently, several new agents with improved formulations or improved antifungal spectrum have been developed, such as lipid formulations of AMPH-B (24), and an intravenous formulation of ITCZ (25) and voriconazole. Voriconazole has shown efficacy against invasive aspergillosis and esophageal candidiasis (10, 26–28).

Micafungin has broad-spectrum activity against Aspergillus spp. and Candida spp. Because its mechanism of action inhibiting fungal cell wall synthesis differs from other antifungal agents, it is also active against fungi resistant to other agents. Therefore, micafungin is expected to provide a new type of antifungal treatment. In this study, the efficacy of micafungin was indicated against various forms of aspergillosis (IPA, CNPA, and pulmonary aspergilloma) and candidiasis (candidemia and esophageal candidiasis).

IPA is the most acute, aggressive type of aspergillosis. Of the 10 IPA patients in this study, 7 patients developed IPA after receiving chemotherapy for leukemia or lymphoma. Two patients were neutropenic (cell count < 500) at enrollment. Six of the 10 IPA patients, including 2 neutropenic patients, responded to micafungin at the maximum dosage of 50–150 mg/d. Improvements of radiologic invasive shadows and clinical symptoms (fever, C-reactive protein level, chest pain, cough, etc.) were observed in all responding patients.

CNPA is defined by Binder et al. (29) as an indolent, cavitating process in the lungs due to invasion of lung tissue by Aspergillus spp. CNPA is distinguished from aspergilloma by the aggressiveness of the inflammatory process and also by the absence of a pre-existing cavitary space (30). However, the concept of CNPA has not been universally accepted. Therefore, we defined new diagnostic criteria for CNPA in this study based on that used in overseas clinical studies at that time. The diagnostic criteria referred to were published by Denning later (31). Common risk factors for CNPA include underlying pulmonary disease, mild systemic immunosuppression by corticosteroid therapy, or underlying conditions such as diabetes mellitus (30). Of the 9 patients with CNPA in this study, 5 had prior tuberculosis, and 4 had diabetes mellitus. The treatment response was observed in 6 of the 9 patients with micafungin 50–150 mg/d (the maximum dose). In these responding patients, infiltrates or pleural thickening on chest X-ray or computed tomographic scan were improved, accompanying the improvements of clinical symptoms such as fever, C-reactive protein level, cough, sputum, and dyspnea.

13 of the 22 patients with pulmonary aspergilloma in this study had prior or current tuberculosis. The optimal treatment strategy for pulmonary aspergilloma remains uncertain (19). The definitive treatment for pulmonary aspergilloma is surgical resection, but surgery has been associated with high morbidity and mortality. Antifungal therapy is therefore considered to be a useful treatment for

Table V. *Adverse events related to study drug*

Adverse event	No. of patients					
	Dose at onset (mg/d)					
	25	50	75	150	Total	
Clinical adverse events						
Chill		1			1	
Headache				1	1	
Hypertension		1			1	
Palpitation				1	1	
Vascular pain		1			1	
Phlebitis	2				2	
Rash or eruption			1	1	2	
Polyarthritis	1				1	
Diarrhea or loose stools			1	1	2	
Laboratory adverse events						
Neutropenia			1		1	
Eosinophilia				1	1	
AST or ALT increase	1		2		3	
Al-p increase	2		1		3	
γ-GTP increase	2				2	
LDH increase	1				1	
BUN or creatinine increase		2	2	1	5	
Creatinine clearance decrease			1		1	
K^+ increase		1			1	
K^+ decrease	1				1	
CPK increase			1		1	
Myoglobin increase			1		1	
Total	10	6	11	6	33	
Overall incidence[a] (%)						21/70 (30)

[a] No. of patients with adverse event(s) related to micafungin/total no. of patients received at least 1 dose.

pulmonary aspergilloma to calm symptoms or to improve QOL in patients who are intolerant to surgery, although the use of such drugs is still controversial. In this study, 12 of the 22 patients responded to micafungin treatment, showing clinical improvement (in fever, sputum, cough, C-reactive protein level, etc.) and/or radiological improvement (in the findings of fungus ball, cavity, or pleura) without major safety issues. Of 12 patients who were overall responders with pulmonary aspergilloma, 6 patients (50%) showed both a clinical response and radiological improvement, and 9 patients (75%) showed either a clinical response or radiological improvement. It is possible that bacterial infections or a variation in the course of underlying tuberculosis, fibrocystic sarcoidosis or bullous emphysema may have affected the efficacy evaluation. It is unlikely, however, that bacterial infection affected interpretation of the radiological examination. More than a half of the patients had not responded to intensive previous treatment with antibiotics, and no suspected case of bacterial superinfection was found during the course of the study. Since pulmonary aspergilloma and underlying diseases were analyzed differentially in the radiological assessment, such difficulty did not occur in this study. These results suggest that micafungin may provide a useful treatment option for pulmonary aspergilloma.

With regard to candidiasis in this study, all 6 patients with candidemia and 5 of 7 with esophageal candidiasis showed responses with relatively low doses of micafungin (candidemia: 25–75 mg/d; esophageal candidiasis: 50–75 mg/d, the maximum dose). In candidemia the response was observed as improvements in clinical symptoms (fever, C-reactive protein level) with mycological improvement (fungal eradication in culture) and/or serological improvement (decreased β-D-glucan). In esophageal candidiasis, endoscopic improvement (eradication of plaque) and clinical improvement (in odynophagia, retrosternal pain, etc.) were observed. Some patients infected with Candida spp. with low susceptibility to FLCZ, including Candida albicans, Candida krusei, and Candida glabrata, showed clinical and/or mycological response to treatment with micafungin.

The efficacy of micafungin was exhibited in patients who had received prior antifungal treatment that was considered to be ineffective or toxic. This may indicate that many refractory or intolerant patients could be treated with micafungin.

Although the accurate optimal dose for each disease could not be shown in this study because the maximal dose in the protocol was increased from 25 mg/d to 75 mg/d and 150 mg/d stepwise during the course of the study according to the results from a maximal tolerated dose (MTD) study being

conducted at the same period of time in the US, based on these results the recommended starting dose of micafungin was considered as 50 to 150 mg/d for aspergillosis (but 150 mg/d will be recommended especially for IPA), 25 mg/d or more for candidemia, and 50 mg/d or more for esophageal candidiasis. These doses are also considered to be the optimal doses, respectively.

The diagnostic criteria are now presented by the European Organization for Research and Treatment of Cancer as an international consensus (32). However, it is difficult to define proven, probable, or possible fungal infection in this study. The definition of the diagnosis in our study allowed us to use positive results of serological tests, although their sensitivity and specificity are still insufficient. However, serological tests are frequently used in Japan, because positive culture results are still rare, and invasive examinations such as histopathological tests are difficult to perform in severely ill patients. Therefore, empiric antifungal treatment is usually started when a serological test is positive. Of the 6 patients with candidemia included in the efficacy evaluation, 1 patient was diagnosed on the basis of a positive serological result. Patients who received the study drug for less than 7 d were excluded from efficacy analysis. Considering the effects on mouse models of fungus infection and the blood levels obtained in human pharmacokinetic study, more than 7 d of continuous treatment might be necessary for micafungin to exert its action. This exclusion criterion was applied to 2 patients with IPA, and to 2 with candidemia (1 with negative blood culture). For reference, we conducted re-analysis including the patients formerly excluded only because of insufficient duration of treatment (less than 7 d) as 'ineffective' cases (2 patients with IPA and 1 candidemia), and we excluded 1 candidemia patient with a negative blood culture from the original analysis. As a result, the overall clinical response rates were 50% (6/12) for IPA, 55% (24/44) for all aspergillosis, 83% (5/6) for candidemia, and 71% (10/14) for all candidiasis, showing no major variation in the efficacy of the micafungin.

Overall, micafungin was well tolerated. Adverse events related to micafungin occurred in only 21 patients. Most of these adverse events were of mild or moderate severity, with the exception of 1 case of serious neutropenia. No adverse event showed a dose-related occurrence, suggesting that micafungin can be used without concern for serious dose-limiting adverse events.

Caspofungin, also an antifungal belonging to echinocandin family has recently been used in the market and its efficacy in IPA and esophageal candidiasis has been reported (12, 13). Although micafungin showed an efficacy in aspergillosis and candidiasis in this open study, this effect needs to be confirmed by a large-scale study, including a double-blind comparative study. A recent comparative study revealed that micafungin was superior to FLCZ in prevention of fungal infection in patients who had undergone a hematopoietic stem cell transplantation (33), and the drug also showed an efficacy to esophageal candidiasis in patients with HIV (34). With regard to safety, caspofungin is known to have a potential for drug interaction with a certain immunosuppressant. Dosage of caspofungin also needs to be reduced in subjects with poor liver function (12). However, micafungin is unlikely to have such potential for drug interaction, and the necessity of dose reduction in hepatic insufficiency. From these accumulating results, it is expected that micafungin is a useful drug in this field.

It is concluded that treatment with micafungin as monotherapy seems to be effective and safe in patients with deep-seated mycosis, suggesting that micafungin may play an important role in the treatment of fungal infections.

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Characterization of Monoclonal Antibodies to Marburg Virus Nucleoprotein (NP) That can be Used for NP-Capture Enzyme-Linked Immunosorbent Assay

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After the first documented outbreak of Marburg hemorrhagic fever identified in Europe in 1967, several sporadic cases and an outbreak of Marburg hemorrhagic fever have been reported in Africa. In order to establish a diagnostic system for Marburg hemorrhagic fever by the detection of Marburg virus nucleoprotein, monoclonal antibodies to the recombinant nucleoprotein were produced. Two clones of monoclonal antibodies, MAb2A7 and MAb2H6, were efficacious in the antigen-capture enzyme-linked immunosorbent assay (ELISA). At least 40 ng/ml of the recombinant nucleoprotein of Marburg virus was detected by the antigen-capture ELISA format. The epitope of the monoclonal antibody (MAb2A7) was located in the carboxy-terminus of nucleoprotein from amino acid position 634 to 647, while that of the MAb2H6 was located on the extreme region of the carboxy-terminus of the Marburg virus nucleoprotein (amino acid position 643–695). These monoclonal antibodies strongly interacted with the conformational epitopes on the carboxy-terminus of the nucleoprotein. Furthermore, these two monoclonal antibodies were reacted with the authentic Marburg virus antigens by indirect immunofluorescence assay. These data suggest that the Marburg virus nucleoprotein-capture ELISA system using the monoclonal antibodies is a promising technique for rapid diagnosis of Marburg hemorrhagic fever. ***J. Med. Virol. 76:111–118, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: Marburg virus; antigen-capture ELISA; monoclonal antibody; diagnosis

INTRODUCTION

Marburg virus (MBGV) infections cause one of the most severe forms of hemorrhagic fevers with a high mortality rate [Feldmann et al., 1996; Anonymous, 1999; Bausch et al., 2003]. MBGV belongs to the genus Marburg-like virus, family Filoviridae. Ebola viruses (EBOVs), which belong to genus Ebola-like virus, family Filoviridae, also cause Ebola hemorrhagic fever with a high mortality rate [Peters and LeDuc, 1999]. The first documented outbreak of Marburg hemorrhagic fever occurred in the former West Germany and the former Yugoslavia in 1967 [Martini et al., 1968]. The outbreak occurred among scientists and technicians who had handled monkeys, or their tissues, imported from Uganda [Martini et al., 1968]. Thirty-two patients were affected and seven died in the outbreak. After the first documented outbreak, three sporadic cases of Marburg hemorrhagic fever were reported in Zimbabwe (1975) and Kenya (1980 and 1987), resulting in the diagnosis of six patients with Marburg hemorrhagic fever [Smith et al., 1982; Conrad et al., 1987; Feldmann et al., 1996; Johnson et al., 1996]. Human to human transmission of Marburg hemorrhagic fever was documented in an outbreak in South Africa after patients with Marburg hemorrhagic fever had been transferred there from Zimbabwe [Gear et al., 1975]. Three of the six patients in

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these sporadic outbreaks died. From 1998 to 1999, there was a large outbreak of Marburg hemorrhagic fever in the Democratic Republic of Congo, former Zaire [Bausch et al., 2003]. More than 100 patients with Marburg hemorrhagic fever were reported in that outbreak and the mortality rate was more than 50%.

Marburg hemorrhagic fever should be confirmed by virus isolation, detection of virus antigen and/or virus genome, or by detection of specific immunoglobulin M (IgM) and immunoglobulin G (IgG) using samples from patients. Virus isolation, electron microscopic examination, and reverse-transcription polymerase chain reaction (RT-PCR) are used for the diagnosis of Marburg virus infection [Ksiazek et al., 1999; Sanchez et al., 1999]. Although antigen-capture ELISA systems have been used for the diagnosis of Ebola hemorrhagic fever [Ksiazek et al., 1999], no antigen-capture ELISA for Marburg virus has yet been used for confirmation of infection.

In the present study, monoclonal antibodies to the recombinant nucleoprotein (rNP) of MBGV were generated and an antigen-capture ELISA using these monoclonal antibodies was developed. These monoclonal antibodies efficacious were characterized.

MATERIALS AND METHODS

Cell Culture

Hybridomas and their parental cell line, P3/Ag568, were maintained in PRMI 1640 (Invitrogen life technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (Invitrogen), and antibiotics (streptomycin and penicillin G, Invitrogen). Hypoxanthine-aminopterin-thymidine supplement (Invitrogen) was added to the medium for selection of hybridomas, as recommended by the supplier. High five (Tn5) insect cells were maintained in TC100 (Invitrogen) supplemented with 10% FBS, 2% tryptose phosphate broth (Difco, Detroit, MI), and kanamycin (Invitrogen).

Authentic MBGV Antigen

Radiation-inactivated and acetone-fixed authentic MBGV (the Musoke strain)-infected Vero E6 cells spotted on slides for immunofluorescence testing were kindly supplied to T.K. in 1987 by Dr. J.B. McCormick, former chief of the Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, GA.

rNP of MBGV

A full-length rNP of MBGV tagged with a 6× histidine at the amino-terminus was expressed using a baculovirus system [Saijo et al., 2001]. The DNA corresponding to each truncated NP fragment was amplified with the primers from a cDNA clone of NP of MBG provided by H.-D. Klenk, Philipps-University, Marburg, Germany. The full-length rNP of MBGV consisted of 695 amino acid residues. The truncated rNP fragments of the carboxy-terminal region of MBGV (MBG-NP/C-half, amino acid positions 341–695) was also expressed in an *Escherichia coli* (*E. coli*) system as a fusion protein with glutathione *S*-transferase (GST) as described previously [Saijo et al., 2001]. Smaller fragments of the rNP in the extreme carboxy-terminus (MBG-NP8, amino acid position 595–695) were also expressed as a form of GST-fusion protein at the amino-terminal portion in the *E. coli* system transformed with the respective pGEX-2T vector (Amersham Biosciences Corp., Piscataway, NJ) inserted with the corresponding DNA of the MBGV nucleoprotein. The data on the primers used for making the corresponding DNA regions are shown in Table I. The nucleotide sequence of the inserted DNAs was determined to exclude PCR errors.

The full-length rNP of MBGV was purified with the N^{2+}-resin purification system (Qiangen GmbH, Hilden, Germany). The GST-MBG-NP/C-half was also purified with glutathione Sephalose 4B (Amersham Biosciences Corp.). The protein concentration of the purified rNP of MBGV was measured by the Bradford method using

TABLE I. Primers Used in the Present Study

Sequence[a]	Name	Nucleotide position[b]	Direction
GGTGATTGATCAGAACCTATAAGATC	MBG-NP 8F	1771–1796	Forward
CAGGAATGATCAAGGATGAGGGAAGCC	MN8/2F	1819–1845	Forward
CTCTGGAATTCATGTTTGCAGAAGATCA	MN8/2R	1944–1971	Reverse
TTCACATGATCAGAGGATAATCAGCAG	MN8/3F	1864–1890	Forward
CAGGAATTCAGTATTCCTCAACGAGGGC	MN8/3R	1984–2011	Reverse
AAGAAGTGATCAACTTTCCTTTATC	MN8/4F	1915–1939	Forward
TTCATGAATTCACATGTCGGGCCAATC	MN8/4R	2035–2061	Reverse
GCTGAATTCTCTGGACTACAAGTTC	MBG-NP 8R	2079–2103	Reverse
CAGGGATCCTGGCCACAAAGAGTG	M8/2F-6	1885–1908	Forward
AATGGATCCCAAAGAGTGGTGAC	M8/2F-8	1891–1913	Forward
CCAGGATCCGTGGTGACAAAGAAG	N8/2F-10	1897–1920	Forward
GCAGAAGAATTCAAGGATAAAGGAAAG	M8/3R–6	1928–1954	Reverse
GATCATGAATTCAAAGGAAAGTTCTAC	M8/3R–8	1922–1948	Reverse
TAGGAGAATTCAAAGTTCTACCCTTC	M8/3R–10	1917–1942	Reverse

[a]Restriction sites are underlined.
[b]*Nucleotide position* is counted from the initiation ATG codon of the nucleotide gene of Marburg virus (GemBack Accession No. X68495).

Protein Assay™ (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

rNP of EBOV

The full-length rNP of Zaire Ebola virus was expressed by the baculovirus system and purified as described previously [Saijo et al., 2001].

Establishment of Monoclonal Antibodies

BALB/c mice were immunized three times with the purified GST-MBG-NP/C-half. Spleen cells were obtained 3 days after the last immunization and fused with P3/Ag568 cells using polyethylene glycol (Invitrogen). The culture supernatants of the hybridoma cells were screened by ELISA with purified GST-MBG-NP/C-half as an antigen. Monoclonal antibodies were purified from the culture supernatant with an MAb Trap GII antibody purification kit (Amersham Biosciences Corp.) according to the manufacturer's instructions. The isotypes of the monoclonal antibodies were determined with a Mouse Monoclonal Antibody Isotyping Kit (Invitrogen). The concentration of each purified monoclonal antibody was also determined by the Bradford method using Protein Assay (Bio-Rad Laboratories) according to the manufacturer's instructions.

Polyclonal and Monoclonal Antibodies

The polyclonal antibody was induced in rabbits by immunization with the purified rNP of MBGV expressed in the baculovirus system [Saijo et al., 2001]. Rabbit and mouse sera collected before immunization were used as controls.

A monoclonal antibody to rNP of EBOV (3-3D), which is efficacious in the EBOV nucleoprotein-capture ELISA, was used [Niikura et al., 2001].

Antigen-Capture ELISA

Purified monoclonal antibody was coated on microwell immunoplates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at >100 ng/well in 100 μl of PBS at 4°C overnight, followed by blocking with PBS containing 5% nonfat milk and 0.05% Tween-20 (PBST-M) for 1 hr at room temperature (RT). After the plates were washed with phosphate-buffered saline solution (PBS) containing 0.05% Tween-20 (PBST), 100 μl of samples containing serially diluted rNP of MBGV were added and the plates were incubated for 1 hr at 37°C. The plates were then washed with PBST, and 100 μl of rabbit polyclonal antibody raised against rNP of MBGV diluted 1:500 with PBST-M was added to each well. After 1 hr incubation at 37°C, the plates were washed with PBST and horseradish peroxidase (HRPO)-conjugated goat anti-rabbit IgG (Zymed Laboratories, Inc., South San Francisco, CA) was added. The plates were incubated for 1 hr at RT. After another extensive wash with PBST, 100 μl of ABTS substrate solution [4 mM 2,2-azino-di[3-ethylbenzthiazolinesulfate(6)] solution; 2.5 mM hydrogen superoxygenphosphate (pH 4.2)] (Roche Diagnostics, Mannheim, Germany) was added and the optical density (OD) was measured at a wavelength of 405 nm with a reference wavelength 490 nm after 30 min of incubation at 37°C. As a negative control, mock antigen-inoculated wells were tested. The adjusted OD values (OD_{405}) were calculated by subtracting the OD of the negative control well from the corresponding OD values.

Western Blotting

The monoclonal antibodies were tested for reactivity to the recombinant rNP fragments by Western blotting. Briefly, the expressed rNP fragment series were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the separated proteins were transblotted to the nitrocellulose membrane (Millipore, Bedford, MA). The monoclonal antibodies that reacted with the blots were detected with HRPO-conjugated gout anti-mouse IgG (Zymed) and peroxidase substrate (POD substrate, Wako Pure Chimeical, Tokyo, Japan). The monoclonal antibody to GST produced in our laboratory was also used. The SDS–PAGE gels were prepared in the same way as those for Western blotting and were stained by Coomassie staining solution for visualization.

Indirect Immunofluorescence Assay (IFA)

The authentic MBGV for IFA were reacted with each of the monoclonal antibodies or control mouse serum at 37°C for 1 hr in humidified conditions. Slides were then washed with PBS and the antigens were reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (Zymed). As a control, the antigens were simultaneously reacted with either the polyclonal antibody to the rNP of MBGV or a control rabbit serum at 37°C for 1 hr in a humidified condition. The slides were washed with PBS and the antigens were reacted with FITC-conjugated goat anti-rabbit IgG antibody (Zymed). After washing with PBS, the fluorescein-signal was observed under an immunofluorescent microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

Generation of Monoclonal Antibodies

Five hybridoma clones secreting IgG antibodies to GST-MBG-rNP/C-half were produced. The secreted monoclonal antibodies were purified and tested for reactivity to the rNP of MBGV in IgG-ELISA. Four monoclonal antibodies reacted with the rNP of MBGV in IgG-ELISA. Two of these four monoclonal antibodies secreted from the hybridoma clones, 2A7 and 2H6, could be used in the antigen-capture ELISA format. These two monoclonal antibodies were designated as MAb2A7 and MAb2H6, respectively. The isotype of MAb2A7 and MAb2H6 was IgG1.

Antigen-Capture ELISA Using the Monoclonal Antibodies

The antigen-capture ELISA with MAb2A7 or MAb2H6 detected at least 40 ng/ml of the purified rNP of Marburg virus, while the ELISA with mock-antibody (PBS) showed a negative reaction (Fig. 1). Furthermore, the ELISA did not react with negative control samples that did not contain rNP of MBGV.

Definition of Epitopes Recognized by the Monoclonal Antibodies

The MAb2A7 and MAb2H6 reacted with both GST-MBG-NP/C-half and GST-MBG-NP8 in Western blotting. Smaller fragments of the MBG-NP8 region were expressed and the reactivity of these monoclonal antibodies to these fragments was analyzed (Fig. 2).

The MAb2A7 reacted with all the fragments that contained the polypeptide of amino acid residues from amino acid position 626 to 653 (Fig. 2). Truncated polypeptides of this region were further designed and expressed as shown in Fig. 3. Reactivity of the monoclonal MAb2A7 to these fragments was evaluated (Fig. 3). The MAb2A7 reacted with the polypeptide, "WPQRVVTKKGRTFL (amino acid positions 632–645)" (Fig. 3). Although the epitope of the MAb2A7 was located in this region, MAb2A7 reacted with the MBG-NP8/1-5 (amino acid positions 595–695) more strongly than with the smaller fragments (Fig. 2), indicating that the reactivity of MAb2A7 with the nucleoprotein of MBGV was influenced by conformational and structural properties.

The MAb2H6 strongly reacted with the truncated polypeptides containing the extreme carboxy-terminal region and which were larger than the polypeptides mentioned above (Fig. 4). The smallest polypeptide to react with MAb2H6 was that consisting of amino acid residues (amino acid positions 643–695). MAb2H6 did not reacted with any of the polypeptides lacking the extreme carboxy-terminal region (amino acid positions 584–695) (Fig. 4). These results indicate that MAb2H6 reacted with the conformational epitope composed of the extreme carboxy-terminal region.

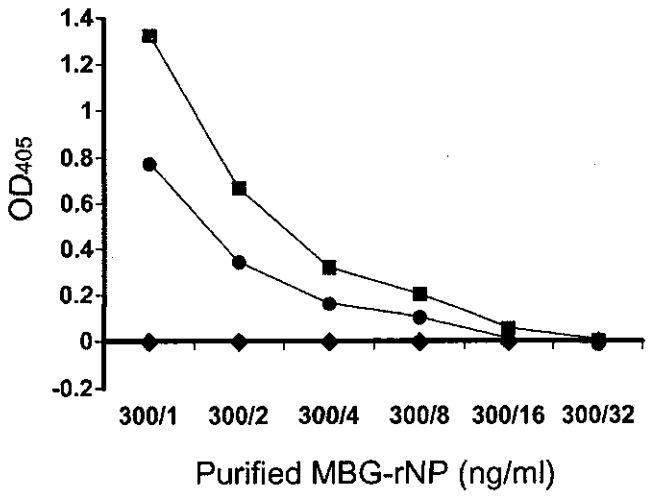

Fig. 1. Reactivity of each monoclonal antibody in the antigen-capture enzyme-linked immunosorbent assay (ELISA) format. Purified monoclonal antibodies (■, MAb2A7; ●, MAb2H6; ◆, mock) were coated onto the microplates as described in the text, and their ability to capture the rNP of Marburg virus (MBGV) was examined at various concentrations of MBGV rNP in the antigen-capture format.

Reactivity of the Monoclonal Antibodies to Authentic MBGV Antigen in IFA

MAb2A7 and MAb2H6 both reacted with the authentic MBGV antigens by the IFA test as well as the rabbit serum raised to rNP of MBGV. Negative control mouse and rabbit sera did not react with the authentic antigens (Fig. 5).

Reactivity of the Monoclonal Antibodies to rNP of EBOV

Neither of MAb2A7 and MAb2H6 reacted with the rNP of EBOV by both Western blotting and the antigen-detection ELISA, while MAb3-3D reacted with rNP of EBOV (Fig. 6). Both MAb2A7 and MAb2H6 reacted with rNP of MBGV by both the Western blotting and the antigen-detection ELISA, but MAb3-3D did not (Fig. 6).

DISCUSSION

Detection of MBGV antigen and amplification of viral genome of MBGV is necessary for rapid diagnosis of Marburg hemorrhagic fever. The application of RT-PCR and TaqMan PCR to MBGV genome amplification has been suggested [Sanchez et al., 1999; Drosten et al., 2002]. Although, serological diagnosis is also useful for the diagnosis of Marburg hemorrhagic fever, serum samples collected at both acute and convalescent phases are required, suggesting that serological diagnosis may not be suitable in certain areas and that it may not be applied in fatal cases without antibody responses. Therefore, a novel antigen-capture ELISA has been developed in the present study.

Two monoclonal antibodies (MAb2A7 and MAb2H6) developed in the study reacted with the authentic MBGV antigens by IFA (Fig. 5). At least 40 ng/ml of rNP of MBGV was detected in the MBGV nucleoprotein-capture ELISA using these monoclonal antibodies (Fig. 1). Unfortunately, the efficacy of the developed antigen-capture ELISA has not been validated using clinical samples, because clinical samples from patients with Marburg hemorrhagic fever at an acute phase were not available. It is generally accepted that antigen-capture ELISA is useful for the detection of viral antigens in blood and/or other organ tissue specimens collected not only from surviving patients but also from patients in whom the infection was fatal. The present study suggests that the antigen-capture ELISA using the unique monoclonal antibodies is a useful tool for diagnosis.

The nucleoprotein-detection ELISA systems for Zaire Ebola and Reston Ebola viruses were developed by

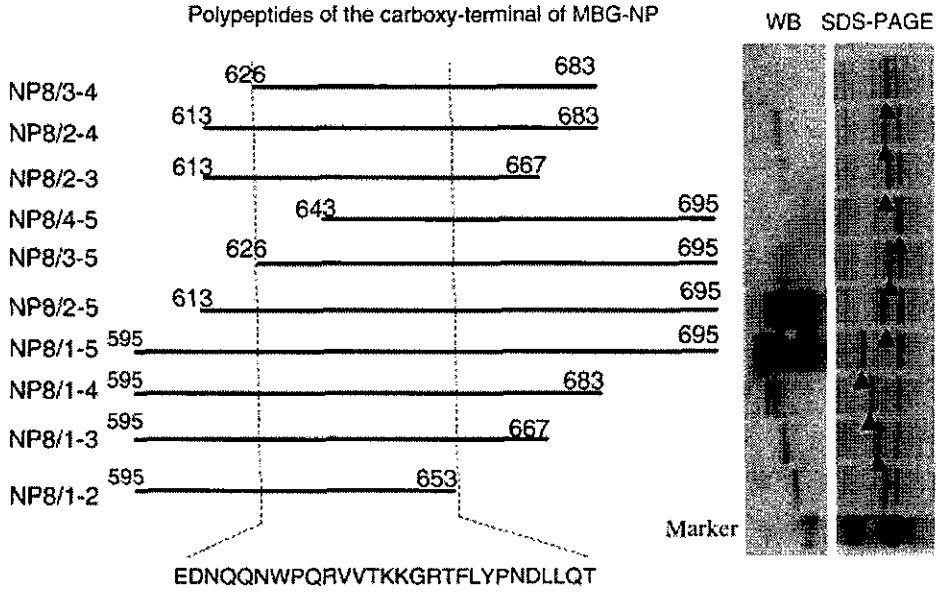

Fig. 2. Schematic representation of the truncated polypeptides (**left part**), expression levels of these polypeptides determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis (right part, SDS–PAGE), and reactivity of MAb2A7 to these polypeptides in Western blotting (WB) (**right part**, WB). The numbers shown for each schematic polypeptide indicate the amino acid positions of the MBGV nucleoprotein.

previous studies [Niikura et al., 2001; Ikegami et al., 2003]. Interestingly, the monoclonal antibodies, which could be used as antigen-capture antibodies in the antigen-capture ELISA systems, reacted with the carboxy-terminal regions of Ebola virus nucleoproteins. The monoclonal antibodies in the present study also reacted with a similar region of the nucleoprotein of MBGV. Although data are not shown here, none of monoclonal antibodies to nucleoproteins of Ebola and Marburg viruses, which reacted with regions other than the approximately 100 amino acid residues at the carboxy-terminal region, were found to be useful as antigen-capture antibodies in the antigen-capture ELISA. Thus, it is likely that the monoclonal antibodies to nucleoproteins of filoviruses useful in antigen-capture ELISA systems react with the carboxy-terminal region of the nucleoproteins.

The symptoms due to MBGV infections in humans and non-human primates are indistinguishable from those due to EBOV infections. Therefore, it was considered that reactivity of the monoclonal antibodies to the nucleoprotein of EBOV should be examined. It was confirmed that neither of monoclonal antibodies, 2A7 and 2H6, cross-reacted with nucleoprotein of EBOV

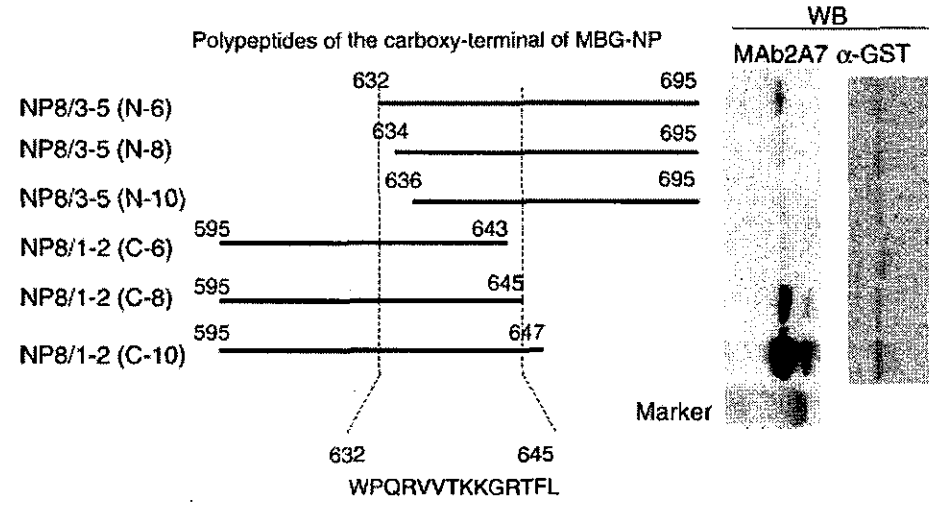

Fig. 3. Schematic representation of the truncated polypeptides (**left part**), and reactivity of MAb2A7 and anti-glutathione S-transferase (GST) monoclonal antibody to these polypeptides in WB. The numbers shown for each schematic polypeptide indicate the amino acid positions of the MBGV nucleoprotein.

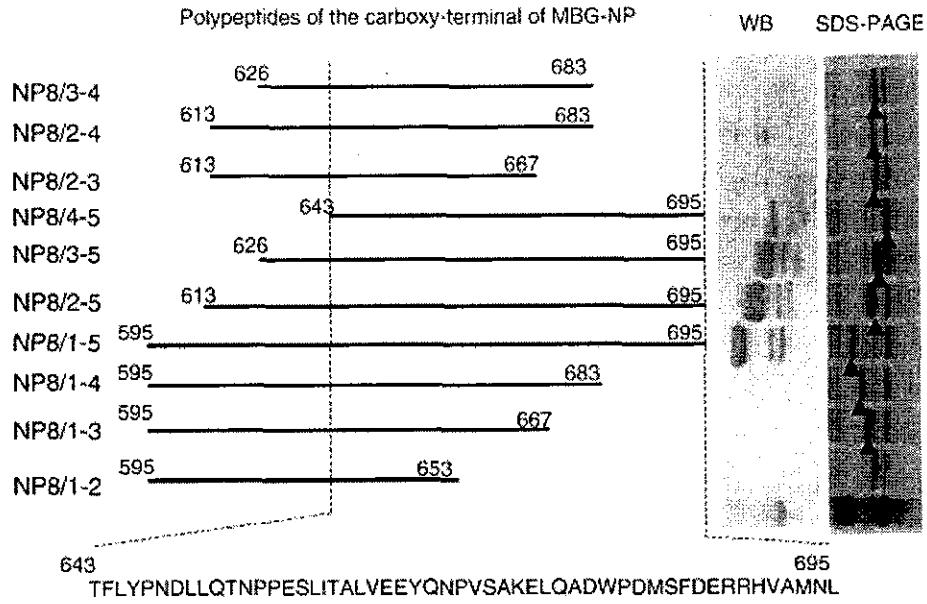

Fig. 4. Schematic representation of the truncated polypeptides (**left part**), expression levels of these polypeptides determined by SDS–PAGE analysis (right part, SDS–PAGE), and reactivity of MAb2H6 to these polypeptides in Western blotting (**right part**, WB). The numbers shown for each schematic *polypeptide* indicate the amino acid positions of the MBGV nucleoprotein.

(Fig. 6), indicating that the newly developed MBGV nucleoprotein-detection ELISA was specific for MBGV infections.

The amino acid sequence of the polypeptide recognized by MAb2A7 was conserved among Marburg virus isolates so far deposited in the GenBank, and there is no significant diversity in the amino acid sequences of the carboxy-terminal regions among Marburg virus isolates (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein, Accession nos. AAR85460, AAR85453,

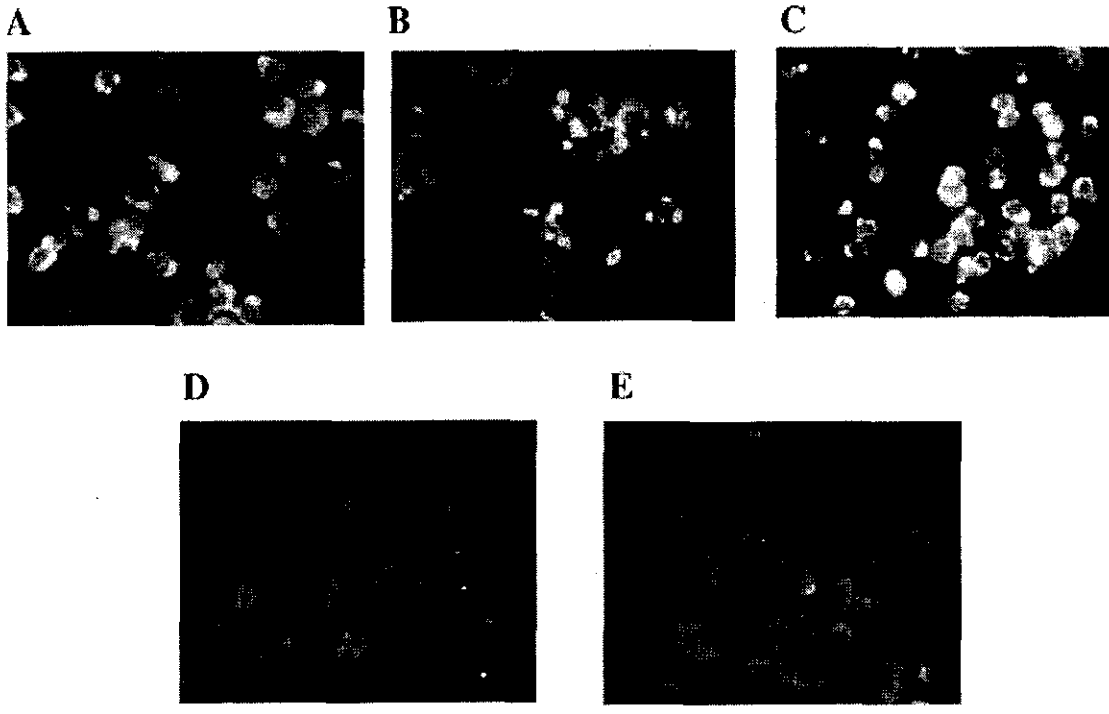

Fig. 5. Reactivity of MAb2A7 (**A**), MAb2H6 (**B**), anti-MBGV rNP rabbit serum (**C**), negative control mouse serum (**D**), and rabbit serum (**E**) to authentic MBGV antigens in Vero E6 cells in the immunofluorescence assay.

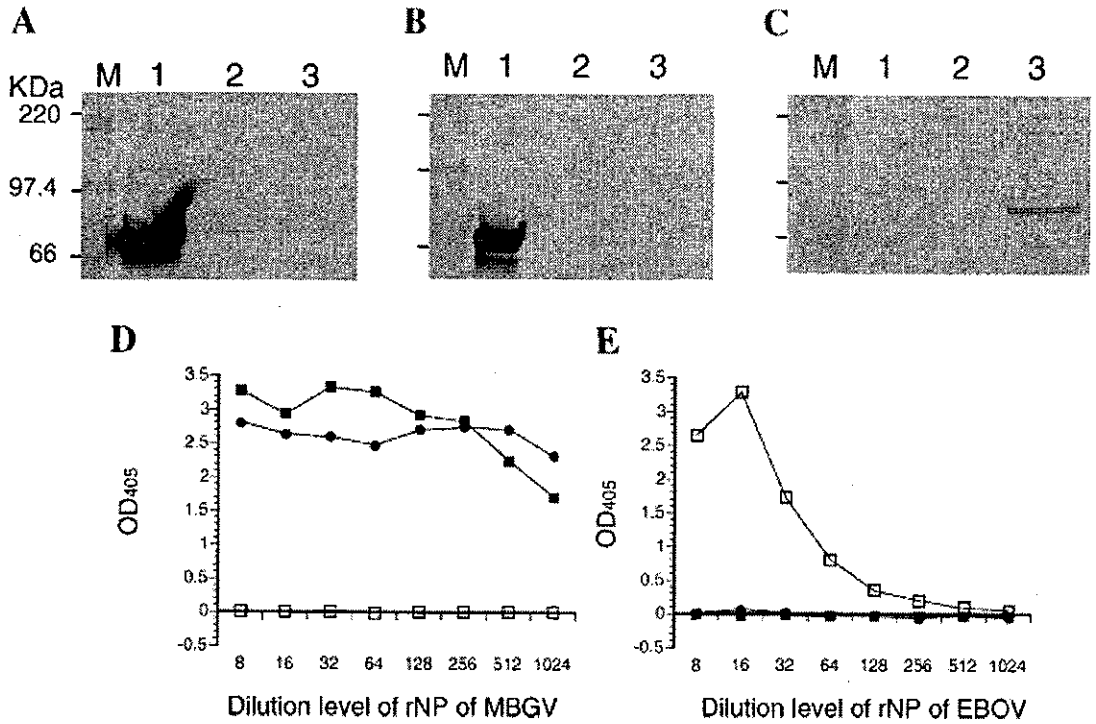

Fig. 6. Reactivity of the monoclonal antibodies to rNPs of MBGV and EBOV. Reactivity of MAb2A7 (**A**), MAb2H6 (**B**), and MAb3-3D (**C**) to MBGV rNP and EBOV rNP was examined in Western blotting. Lanes "M," "1," "2," and "3" indicate the lanes blotted with markers, GST-tagged MBG-NP/C-half, negative control antigen prepared from *Tn5* insect cells infected with the recombinant baculovirus without expression of foreign genes, and His-tagged rNP of EBOV, respectively [Saijo et al., 2001]. Reactivity of these monoclonal antibodies to the rNPs of MBGV (**D**) and EBOV (**E**) was also examined in the antigen-capture ELISA using MAb2A7 (■), MAb2H6 (●), or MAb3-3D (□).

NP_042025, AAQ55255, S44049, VHIWMV, P35263, 2110212A, P27588, CAA78114, CAA82536, AAA46563). Therefore, it is quite likely that the newly developed antigen-capture ELISA system for MBGV is useful for detecting most MBGV isolates, although further study is needed.

In conclusion, an MBGV nucleoprotein-detection ELISA system using unique monoclonal antibodies was developed, and the monoclonal antibodies useful for detecting nucleoprotein of the MBGV in the system were characterized. The combined use of the MBGV nucleoprotein-capture ELISA in the present study with the EBOV nucleoprotein-detection ELISA developed in a previous study [Niikura et al., 2001; Ikegami et al., 2003] may be useful for the diagnosis of viral hemorrhagic fevers due to filovirus infections.

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A Novel Role of IL-15 in Early Activation of Memory CD8$^+$ CTL after Reinfection[1]

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A rapid induction of effector functions in memory T cells provides rapid and intensified protection against reinfection. To determine potential roles of IL-15 in early expansion and activation of memory CD8$^+$ T cells in secondary immune response, we examined the cell division and cytotoxicity of memory CD8$^+$ T cells expressing $OVA_{257-264}/K^b$-specific TCR that were transferred into IL-15-transgenic (Tg) mice, IL-15 knockout (KO) mice, or control C57BL/6 mice followed by challenge with recombinant *Listeria monocytogenes* expressing OVA (rLM-OVA). In vivo CTL activities and expression of granzyme B of the transferred CD8$^+$ T cells were significantly higher in the IL-15 Tg mice but lower in the IL-15 KO mice than those in control mice at the early stage after challenge with rLM-OVA. In contrast, there was no difference in the cell division in IL-15 Tg mice and IL-15 KO mice compared with those in control mice. In vivo administration of rIL-15 conferred robust protection against reinfection via induction of granzyme B in the memory CD8$^+$ T cells. These results suggest that IL-15 plays an important role in early activation of memory CD8$^+$ T cells. *The Journal of Immunology,* 2005, 174: 3590–3597.

Antigen-specific memory CD8$^+$ T cell responses are of vital importance in protective immunity against re-exposure to intracellular pathogens (1–6). Memory CD8$^+$ T cells provided faster, more effective functions, including the secretion of cytokines and lysis of infected target cells, during secondary immune responses. The enhanced secondary immune response is thought to be due both to increased frequency of Ag-specific CD8$^+$ T cell precursors and to increased functional activities of memory T cells that respond to Ag in a manner qualitatively different from naive T cells (7–9). Studies using mitotic inhibitors have revealed that memory CD8$^+$ T cells can become cytotoxic without undergoing cell division after restimulation with Ag (10). Memory CD8$^+$ T cells were shown to become cytolytic more rapidly and to secret a greater amount of IFN-γ than naive T cells after in vitro Ag stimulation, although no significant difference was found between naive and memory CD8$^+$ T cells in their proliferative capacities (11). It has also been reported that memory CD8$^+$ T cells under in vitro conditions were more efficient and precious cytokine secretors, although they proliferated poorly (12). Thus, memory CD8$^+$ T cells can be reactivated to become cytotoxic without undergoing cell division.

IL-15 belongs to the four-helix bundle cytokine family and uses β- and γ-chains of IL-2R for signal transduction, and it thus has many properties that are the same as those of IL-2, despite the fact that it has no sequence homology with IL-2 (13–15). In contrast to IL-2, which is produced mainly by activated T cells, IL-15 is produced by a wide variety of tissues, including placenta, skeletal muscle, kidney, and macrophages upon stimulation with LPS (16, 17). IL-2 is known to be important in clonal expansion of Ag-specific CD8$^+$ T cells during primary immune response, whereas IL-15 is not mandatory for the expansion of CD8$^+$ T cells in the immune response. Recent studies have demonstrated that primary responses to lymphocytic choriomeningitis virus (LCMV)[3] and OVA were readily generated in IL-15 knockout (KO) mice or IL-15Rα KO mice to a level equal to that in control mice (18, 19). We have also reported that generation of listeriolysin O $(LLO)_{91-99}$-specific CD8$^+$ T cells in IL-15-transgenic (Tg) mice normally occurred after primary infection with *Listeria monocytogenes* (20). In contrast, IL-15 has potential roles in maintenance of memory phenotype CD8$^+$ T cells, which are capable of slowly dividing without Ag stimulation (18, 19). We have demonstrated that the number of LLO_{91-99}-positive memory CD8$^+$ T cells was significantly higher 6 wk after primary infection with *L. monocytogenes*, which resulted in increased levels of Bcl-2 expression and cell division without Ag stimulation (20). Thus, IL-15 plays an important role in long-term maintenance of Ag-specific memory CD8$^+$ T cells in an Ag-independent manner. In addition, there are several lines of evidence that IL-15 is capable of stimulating NK cells and CD8$^+$ CTL to exhibit increased cytotoxicities (21–24). IL-15 has been reported to directly up-regulate expression of cytotoxic molecules such as granzyme B and perforin, mimicking TCR cross-linking in the induction of cytotoxic molecules and cytotoxicity of memory CD8$^+$ T cells (25). These findings raise the possibility that IL-15 plays an important role in rapid elicitation of effector functions in

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; KO, knockout; LLO, listeriolysin O; Tg, transgenic; rLM-OVA, recombinant *L. monocytogenes* expressing OVA; PEC, peritoneal exudate cell; LN, lymph node; T_{CM}, central memory cell; T_{EM}, effector memory cell.

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memory CD8$^+$ T cells in re-exposure to microbial invasion, providing robust protection against reinfection.

In the present study, to elucidate potential roles of IL-15 in elicitation of effector functions of microbial Ag-specific memory CD8$^+$ T cells in secondary immune response, we examined the effector functions of Ag-specific memory CD8$^+$ T cells transferred into IL-15 Tg mice or IL-15 KO mice followed by infection with recombinant *L. monocytogenes* expressing OVA (rLM-OVA). We found that in vivo CTL activities and expression of granzyme B of the transferred CD8$^+$ T cells were significantly higher in the IL-15 Tg mice but lower in the IL-15 KO mice than those in control mice at the early stage after challenge with rLM-OVA. Furthermore, in vivo administration of exogenous IL-15 conferred protection against secondary infection via induction of cytotoxic molecules in the Ag-specific memory CD8$^+$ T cells. These results suggest that *IL-15 plays an important role in* early activation of Ag-specific memory CD8$^+$ T cells following secondary infection with microbes.

Materials and Methods

Mice

C57BL/6-background IL-15 Tg mice, which were constructed using originally described IL-15 cDNA, have been described previously (26). C57BL/6-background IL-15 KO mice were purchased from Taconic Farms. OT-I mice expressing the $OVA_{257-264}$/K^b-specific TCR and C57BL/6 Ly5.1-congenic mice were obtained from The Jackson Laboratory. To generate *Ly5.1$^+$ OT-I mice*, OT-I mice were crossed onto a B6-Ly5.1 background. All mice were used at 6–8 wk of age.

Microorganism

OVA expressing *L. monocytogenes* was kindly provided by Dr. Subash Sad (Institute for Biological Sciences, Ontario, Canada) (27). Bacterial virulence was maintained by serial passages in *C57BL/6* mice as described previously (20). Mice were infected i.p. with a sublethal dose of 5×10^5 CFU (~0.1 LD_{50}), or a lethal dose of 5×10^6 CFU (~1LD_{50}), or 5×10^7 CFU (~10LD_{50}) of rLM-OVA in 0.2 ml of PBS on day 0. The spleen and liver were removed from each mouse and separately placed in homogenizers containing 3 ml of HBSS. These samples were spread on trypto-soya agar plates, and colonies were counted after incubation for 24 h at 37°C.

Abs and reagents

FITC-conjugated anti-CD44 (IM7), anti-CD69 (H1.2F3), anti-CD25 (7D4), anti-Ly6C (AL-21), and anti-IFN-γ (XMG1.2); PE-conjugated anti-Vα2(B20.1), anti-CD62L (MEL-14), CD122 (5H4), and CD132 (4G3); CyChrome-conjugated anti-*CD8α (53-6.7) and streptavidin*; allophycocyanin-conjugated streptavidin; and biotin-conjugated anti-Ly5.1 (A20) were purchased from BD Biosciences. PE-labeled anti-human granzyme B mAb and isotype control Ab were obtained from Caltag Laboratories. CFSE was purchased from Molecular Probes. $OVA_{257-264}$ H-2K^b tetramers were purchased from MBL.

Analysis of intracellular granzyme B synthesis

Spleen cells from infected mice were stained for surface markers for 30 min at 4°C and then subjected to intracellular cytokine staining using the Fast Immune Cytokine System according to the manufacturer's instructions (BD Biosciences). For intracellular staining for granzyme B expression, *the cells were stained with* PE-labeled anti-human granzyme B or an isotype control for 30 min at room temperature, and the fluorescence of the cells was analyzed using a flow cytometer.

Generation of memory OT-I cells

Memory OT-I cells were generated as follows. Purified CD8$^-$ T cells (2×10^6) of splenocytes from naive OT-I mice *were injected i.v. into naive* C57BL/6 hosts. Twenty-four hours later, these mice were infected with 5×10^5 CFU rLM-OVA. After 40 or more days, these mice were sacrificed, and the number of transgenic memory cells was determined by staining with anti-CD8 mAb, anti-Ly5.1 mAb, and $OVA_{257-264}$ H-2K^b tetramer. In some experiments, mice harboring memory OT-I cells were injected i.p. with various doses of rIL-15 or PBS for control.

Analysis of T cell proliferation following rLM-OVA infection in vivo

CD8$^+$ T cells from naive OT-I mice or rLM-OVA-immune C57BL/6 mice harboring memory OT-I cells were purified by depleting nylon wool-enriched splenocytes with B220, CD4, and MHC II MicroBeads by MACS (*Miltenyi Biotec*) *to* >90% purity. For the analysis of naive or memory OT-I cell proliferation following rLM-OVA infection in vivo, purified CD8$^+$ T cells containing 2×10^6 naive OT-I cells or 5×10^5 memory OT-I cells were labeled with CFSE as described previously (20). Proliferation of transferred cells was visualized by FACS analysis of their CFSE profile. Transferred OT-I cells were identified by staining with a mAb to Ly5.1, CD8, and the Vα2 or $OVA_{257-264}K^b$ tetramer.

In vivo cytotoxicity assay

Analysis of in vivo cytolytic activity was carried by a protocol similar to those previously reported (28, 29). B6-Ly5.1$^+$Ly5.2$^+$ splenocytes were divided into two populations and labeled with a high concentration (5 μM) and a low concentration (0.5 μM) of CFSE. Next, *CFSE^{high} cells were* pulsed with 5 μg/ml $OVA_{257-264}$ peptide for 1 h at 37°C and CFSE^{low} cells remained nonpulsed. After washing, these groups were mixed in equal proportions and then injected i.v. into mice infected with rLM-OVA 2 days previously. Spleens from recipients were taken 5 h later for flow cytometric analysis to measure in vivo killing activities. Percent specific lysis was calculated according to the formula [1 − (ratio primed/ratio unprimed) × 100], where the ratio unprimed = percent CFSE^{low}/percent CFSE^{high} cells remaining in noninfected recipients, and ratio primed = percent CFSE^{low}/percent CFSE^{high} cells remaining in infected recipients.

Results

Expansion and activation of naive CD8$^+$ T cells in naive IL-15 KO or IL-15 Tg hosts in primary response to L. monocytogenes

We first examined the role of IL-15 in expansion and activation of naive CD8$^+$ T cells during primary immune responses using a system of adoptive transfer of OT-I Tg CD8$^+$ T cells (Ly5.1$^+$) that express a TCR specific for H-2K^b-restricted $OVA_{257-264}$ epitopes into naive Ly5.2$^+$ IL-15 Tg, IL-15 KO, or control C57BL/6 hosts that were subsequently infected with rLM-OVA. The absolute numbers of OT-I cells in the spleens of IL-15 KO and IL-15 Tg mice, as assessed by staining with an H-2K^b tetramer coupled with an OVA-derived *SIINFEKL peptide or* with anti-Vα2 mAb, were almost the same as those in control mice on day 5 or 7 after primary infection with rLM-OVA (Fig. 1*A*). The absolute numbers of OT-I cells in the peritoneal exudate cells (PEC) and lymph node (LN) from IL-15 Tg and IL-15 KO mice were also the same as those in control mice after primary infection (data not shown). We next examined that expansion of OT-I cells in IL-15 Tg and IL-15 KO mice during the primary response. CFSE-labeled naive OT-I cells were adoptively transferred i.v. into naive IL-15 KO mice, IL-15 Tg mice or C57BL/6 mice that were subsequently infected with rLM-OVA. As shown in Fig. 1*B*, OT-I cells began to proliferate equally in all groups on day 4, and a massive expansion of OT-I cells occurred on day 5 after rLM-OVA infection. Although all of the cells were CFSE negative at this stage, indicating that most of the cells had divided five to eight times. Thus, we confirmed by using an adoptive transfer system that IL-15 is not essential for expansion of Ag-specific effector CD8$^+$ T cells after primary infection.

To determine the role of IL-15 in activation of effector CD8$^+$ T cells during primary immune response, we examined the expression levels of activation markers and granzyme B of OT-I cells after primary infection with rLM-OVA. The OT-I cells, of CD44^{low}, CD122 (IL-2 Rβ)^{low}, and CD132(cγ)^{low} phenotypes, did not contain intracellular granzyme B in IL-15 KO and IL-15 Tg mice on day 3 and were equivalently increased on OT-I cells on day 5 after rLM-OVA infection (Fig. 1*C* and data not shown). Only the CFSE-negative OT-I cells from each mouse contained

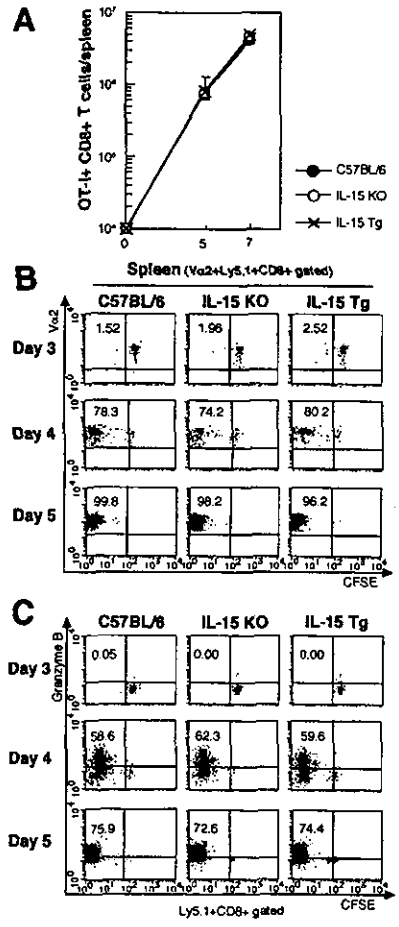

FIGURE 1. IL-15 is not required for expansion during primary $CD8^+$ T cell response. *A*, Purified naive OT-I cells ($Ly5.1^+$) from splenocytes were adoptively transferred i.v. into naive IL-15 KO mice, IL-15 Tg mice, or control C57BL/6 mice ($Ly5.2^+$) that were infected with rLM-OVA 24 h later. On days 5 and 7 after rLM-OVA infection, splenocytes from these mice were stained with $OVA_{257-264}$ MHC class I tetramer or anti-$V\alpha2$ mAb, anti-Ly5.1 mAb, and anti-CD8mAb. Absolute numbers of OVA-specific $CD8^+$ T cells in the spleen were calculated on days 5 and 7 after rLM-OVA infection. Data are presented as mean ± SD for five mice. *B*, CFSE-labeled naive OT-I cells ($Ly5.1^+$) were adoptively transferred into naive IL-15 KO, IL-15 Tg, and control C57BL/6 mice 1 day before rLM-OVA infection. Proliferation of specific T cells ($V\alpha2^+Ly5.1^+CD8^+$) was assessed in the spleen by flow cytometry at indicated time points. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on $V\alpha2^+Ly5.1^+CD8^+$ cells. *C*, Splenocytes were isolated and analyzed for the expression of CFSE and intracellular granzyme B direct ex vivo on the indicated days after infection. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on $Ly5.1^+CD8^+$ cells.

high levels of granzyme B, indicating that naive OT-I cells required clonal expansion to differentiate into fully functional effector CTL that secrete cytotoxic molecules. We further analyzed the ability of transferred naive OT-I cells to produce IFN-γ upon restimulation with peptide on day 5 after rLM-OVA infection. The proportions of intracellular IFN-γ production by OT-I cells in the spleens from IL-15 Tg and IL-15 KO mice were almost the same as those in control mice (data not shown). These results indicated that Ag-specific naive $CD8^+$ T cells might expand and acquire effector function in an IL-15-independent manner during primary *Listeria* infection.

Expansion and activation of memory $CD8^+$ T cells transferred into naive IL-15 KO or IL-15 Tg hosts in secondary response to L. monocytogenes

We next examined the role of IL-15 in expansion and activation of memory $CD8^+$ T cells during secondary immune responses using a system of adoptive transfer of memory OT-I cells into naive IL-15 Tg, IL-15 KO, and C57BL/6 hosts. Because the magnitude of the secondary expansion of memory $CD8^+$ T cells is related to the precursor frequency of Ag-specific memory $CD8^+$ T cells and because numbers of endogenous $OVA_{257-264}$-specific memory $CD8^+$ T cells in the spleen were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice than those in control mice >40 days after primary infection (our unpublished data), we used an adoptive transfer system in which the numbers of Ag-specific cells could be defined precisely before and during immune responses. We generated memory $CD8^+$ T cells in vivo by adoptive transfer of $Ly5.1^+$ naive OT-I cells into C57BL/6 mice ($Ly5.2^+$) followed by infection with rLM-OVA and purified the memory OT-I cells at 40 or more days after primary rLM-OVA infection. Memory OT-I cells were of $CD44^{high}$, $CD122^{high}$, $CD132^+$, $CD25^-$, and $CD69^-$ phenotypes (data not shown), representing resting memory $CD8^+$ T cells. Two subsets of memory $CD8^+$ T cells based on their anatomical location, expression of cell surface markers, and effector functions have been described (30, 31). Approximately 60% of the memory OT-I cells purified from spleens of recipients expressed CD62L, representing central memory cells (T_{CM}), and 40% were of $CD62L^-$ phenotype, representing effector memory cells (T_{EM}).

To determine the role of IL-15 in expansion of memory OT-I cells during the secondary immune response, memory OT-I cells were labeled with CFSE and adoptively transferred i.v. into naive IL-15 KO, IL-15 Tg, and C57BL/6 hosts. To compare expansion of memory $CD8^+$ T cell subsets after secondary infection, it was critical to demonstrate that total number of memory $CD8^+$ T cells was the same after adoptive transfer to recipient mice. At 24 h after adoptive transfer, total number of memory OT-I cells recovered from spleen in IL-15 Tg and IL-15 KO mice were almost the same as those in control mice (data not shown). Then these mice were challenged with a lethal dose of rLM-OVA and the proliferation of memory OT-I cells was analyzed after infection (Fig. 2*A*). As shown in Fig. 2*B*, none of the memory OT-I cells had divided by day 3 infection when transferred into naive IL-15 Tg, IL-15 KO, or C57BL/6 mice, showing kinetics similar to the kinetics of naive OT-1 cells after primary infection (Figs. 1*B* and 2*B*). A massive expansion of OT-I cells occurred in all groups on day 5 after *Listeria* infection. Thus, during the secondary response, there was no obvious difference in the CFSE profiles of memory OT-I cells in IL-15 Tg and IL-15 KO mice compared with those of control mice. These results suggest that IL-15 is not essential for proliferation of memory $CD8^+$ T cells during secondary immune response.

To determine the role of IL-15 in activation of memory $CD8^+$ T cells during secondary immune response, we analyzed the expression of granzyme B in memory OT-I cells in naive IL-15 Tg and IL-15 KO mice after rLM-OVA infection. The expression levels of intracellular granzyme B in memory OT-I cells were significantly lower in IL-15 KO mice but higher in IL-15 Tg mice than those in control mice on day 2 after rLM-OVA infection ($p < 0.05$, Fig. 2, *C* and *D*). On day 5 after reinfection, intracellular expression levels of granzyme B in the dividing memory OT-I cells in IL-15 Tg and IL-15 KO mice became comparable to those in control mice. We further analyzed the ability of transferred memory OT-I cells to produce IFN-γ upon restimulation with peptide on days 2 and 5 after rLM-OVA infection. The proportions of

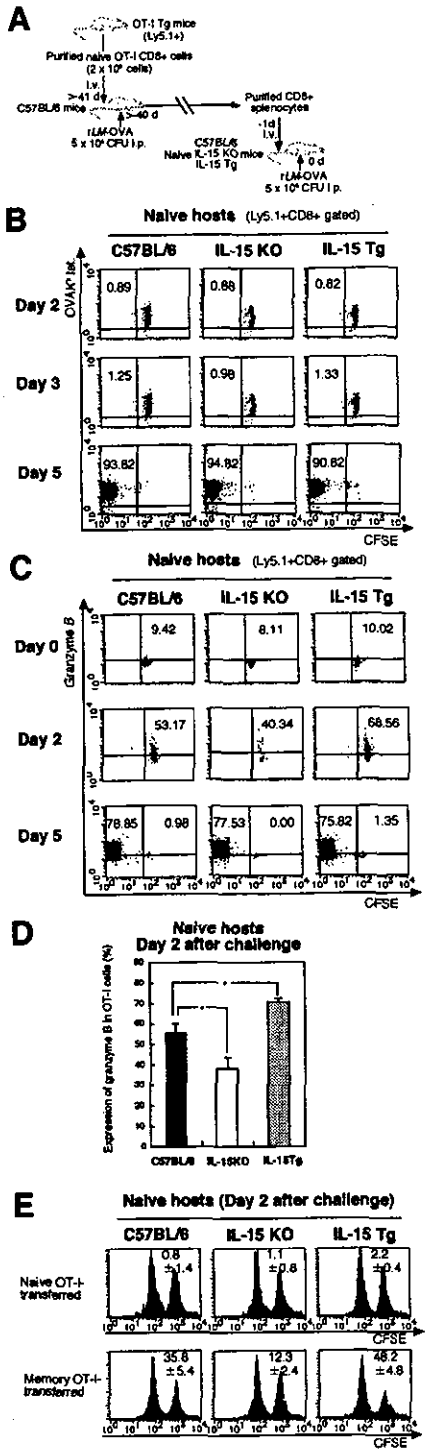

FIGURE 2. IL-15 plays an important role in the induction of cytotoxic molecules of Ag-specific memory $CD8^+$ T cells during secondary immune response in naive hosts. *A*, Purified naive OT-I cells ($Ly5.1^+$) were adoptively transferred into naive C57BL/6 hosts that were infected with rLM-OVA 24 h later. At 40 or more days after primary infection, $CD8^+$ T cells from these mice were purified by negative selection using MACS and labeled with CFSE. CFSE-labeled $CD8^+$ T cells containing 5×10^5 memory OT-I cells were adoptively transferred i.v. into naive IL-15 KO, IL-15 Tg, and control mice that were infected with rLM-OVA 24 h later. *B*, CFSE fluorescence of splenic OT-I cells was analyzed by flow cytometry by staining with anti-CD8 mAb, anti-Ly5.1 mAb, and OVA K^b tetramer at indicated time points. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on $Ly5.1^+CD8^+$ cells. *C*, Splenocytes from IL-15 KO, IL-15 Tg, or control mice harboring CFSE-labeled memory OT-I cells were isolated at indicated days before and after intracellular IFN-γ production by OT-I cells in the spleens from IL-15 Tg and IL-15 KO mice were almost the same as those in control mice (data not shown). These results demonstrate that IL-15 are involved in the induction of granzyme B in nondividing memory $CD8^+$ T cells at the early stage of secondary immune response.

To directly detect cytotoxic activity of OT-I cells in vivo, we measured the ability of naive and memory OT-I cells to eliminate fluorescent-labeled spleen cells pulsed with $OVA_{257-264}$ peptides at the early stage after *Listeria* infection. Naive or memory OT-I cells were adoptively transferred into naive IL-15 Tg mice, IL-15 KO mice, and control C57BL/6 mice that were infected with rLM-OVA 24 h later. We confirmed that naive IL-15 Tg mice, IL-15 KO mice, and control C57BL/6 mice had not developed any in vivo CTL activities on day 2 after rLM-OVA (data not shown). Consistently, none of the $OVA_{257-264}$ peptide-pulsed donor cells in any group of mice into which naive OT-I cells had been transferred were cleared from the spleens on day 2 after infection (Fig. 2*E*). In contrast, on day 2 after infection ~35% of $OVA_{257-264}$ peptide-pulsed donor cells were cleared from the spleens of C57BL/6 mice into which memory OT-1 cells had been transferred. In correlation with the expression of intracellular granzyme B of memory OT-I cells, in vivo CTL activity levels of memory OT-I cells were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice than those in control mice (Fig. 2*E*). These results suggest that IL-15 provides early activation of Ag-specific memory $CD8^+$ CTL via induction of cytotoxic molecules.

Expansion and activation of memory $CD8^+$ T cells transferred into immunized IL-15 KO or IL-15 Tg hosts in secondary response to L. monocytogenes

As shown above, we examined the expansion and activation of memory $CD8^+$ T cells in the specific situation of secondary immune response by using an adoptive transfer system of memory OT-I cells into naive hosts. To examine the role of IL-15 in expansion and activation of memory OT-I cells in more physiological conditions of secondary immune response, we transferred memory OT-I cells into IL-15 Tg, IL-15 KO, or control C57BL/6 hosts that had been immunized with rLM-OVA and subsequently rechallenged with rLM-OVA (Fig. 3*A*). As shown in Fig. 3*B*, memory OT-I cells began to proliferate in all groups of immunized mice on day 2 after rLM-OVA reinfection. This expansion was much faster than that of memory OT-I cells transferred into naive hosts after secondary infection. A massive expansion of OT-I cells

reinfection and stained for expression of CFSE and intracellular granzyme B of memory OT-I cells. Dot plots are gated on donor cells ($Ly5.1^+CD8^+$), and the number indicated is the percentage of donor cells stained positive for granzyme B. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles. *D*, Intracellular expression of granzyme B in OT-I cells transferred into naive IL-15 KO, IL-15 Tg, or control mice on day 2 after infection. Data were obtained from three separate experiments, and each value shown is the mean + SD for five mice (*, $p < 0.05$). *E*, Memory or naive OT-I cells (5×10^5) were adoptively transferred into naive IL-15 KO mice, IL-15 Tg mice, or control C57BL/6 mice that were infected with rLM-OVA 24 h later. As target cells, spleen cells from naive mice ($Ly5.1^+Ly5.2+$) were pulsed with OVA peptides or left unpulsed and then injected i.v. into each infected mouse harboring memory or naive OT-I cells on day 2 after rLM-OVA infection. Histograms are gated on $Ly5.1^+Ly5.2^+$ cells in the spleen from infected mice. The values in the *right corner* of each panel represent the percentage of specific killing compared with nonpulsed cells. Data were obtained from three separate experiments, and each value shown is the mean ± SD for three experiments.

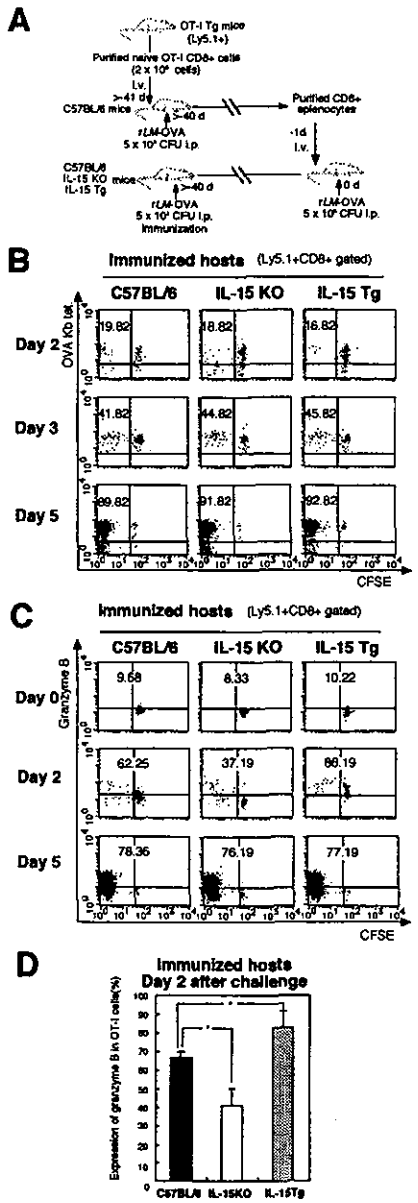

FIGURE 3. IL-15 plays an important role in the induction of cytotoxic molecules of Ag-specific memory $CD8^+$ T cells during secondary immune response in immunized hosts. *A*, CFSE-labeled memory OT-I cells ($Ly5.1^+$) generated in C57BL/6 mice were adoptively transferred i.v. into IL-15 KO, IL-15 Tg, and control mice that had been immunized with rLM-OVA 40 or more days previously. At 24 h after adoptive transfer, these mice were challenged with a lethal dose of rLM-OVA. *B*, CFSE fluorescence of splenic OT-I cells was analyzed by flow cytometry by staining with anti-CD8 mAb, anti-Ly5.1 mAb, and OVA K^b tetramer at indicated time points. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on $Ly5.1^+CD8^+$ cells. *C*, Splenocytes from IL-15 KO, IL-15 Tg, and control mice harboring memory OT-I cells were isolated at indicated days before and after reinfection and stained for expression of CFSE and intracellular granzyme B of memory OT-I cells. Dot plots are gated on donor cells ($Ly5.1^+CD8^+$), and the number indicated is the percentage of donor cells stained positive for granzyme B. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles. *D*, Intracellular expression of granzyme B in OT-I cells transferred into immunized IL-15 KO, IL-15 Tg, or control mice on day 2 after secondary infection. Data were obtained from three separate experiments, and each value shown is the mean +SD for five mice (*, $p < 0.05$).

occurred in all groups on day 5 after *Listeria* infection. Thus, during secondary *Listeria* infection, there was no obvious difference in the CFSE profiles of memory OT-I cells in IL-15 Tg and IL-15 KO mice compared with those of control mice. These results suggest that not only in the specific situation in naive hosts but also in a physiological situation in immunized hosts, IL-15 is not essential for proliferation of memory $CD8^+$ T cells during secondary infection.

We next analyzed the expression of granzyme B in memory OT-I cells in immunized IL-15 Tg and IL-15 KO hosts after rLM-OVA re-infection. The expression levels of intracellular granzyme B in memory OT-I cells not dividing were significantly lower in IL-15 KO mice but higher in IL-15 Tg mice than those in control mice on day 2 after rLM-OVA reinfection ($p < 0.05$, Fig. 3, *C* and *D*). Intracellular expression levels of granzyme B in the dividing memory OT-I cells in immunized IL-15 Tg and IL-15 KO mice became comparable to those in control mice on day 5 after reinfection. Taken together, these results demonstrate that IL-15 plays an important role in rapid elicitation of effector functions in nondividing memory $CD8^+$ T cells at the early stage after secondary infection.

Effects of exogenous IL-15 on activation of memory $CD8^+$ T cells and protection at the early stage after secondary infection

On day 40 after rLM-OVA infection, memory OT-I cells could be generated and maintained in IL-15 KO mice into which naive OT-1 cells had been transferred, albeit at lower degrees than those in control mice (our unpublished data). To test directly whether IL-15 is involved in the induction of granzyme B in memory $CD8^+$ CTL after reinfection, we examined the effects of in vivo administration of rIL-15 on expression of intracellular granzyme B in memory $CD8^+$ T cells after secondary infection in an IL-15-deficient environment. IL-15 KO mice were subjected to transfer of naive OT-I cells followed by rLM-OVA infection, and the IL-15 KO mice were rechallenged 40 days later with a lethal dose of rLM-OVA. rIL-15 was injected i.p. at 0 and 24 h after rechallenge with rLM-OVA (Fig. 4*A*). On day 2 after secondary infection, splenocytes were prepared and intracellular granzyme B staining was performed. As shown in Fig. 4*B*, strong induction of granzyme B was found in memory OT-I CTL from rIL-15-treated IL-15 KO mice compared with that in memory OT-I CTL from PBS-treated IL-15 KO mice after secondary infection. In contrast, there were no significant differences in the proportions of memory OT-I CTL between rIL-15-treated IL-15 KO mice and PBS-treated IL-15 KO mice on day 2 after reinfection (data not shown). Intracellular expression levels of granzyme B in endogenous $CD8^+$ T cells were also significantly increased in rIL-15-treated IL-15 KO mice compared with those in PBS-treated IL-15 KO mice on day 2 after secondary infection. We next measured the in vivo cytolytic activities of memory OT-I cells in rIL-15-treated IL-15 KO mice on day 2 after infection. In vivo CTL activity levels were significantly higher in rIL-15-treated IL-15 KO mice than in PBS-treated IL-15 KO mice after secondary infection (Fig. 4*C*). Moreover, to evaluate antilisterial immunity of memory CTL after treatment with rIL-15, the bacterial growth was determined in the spleen and liver from both rIL-15-treated IL-15 KO mice and PBS-treated IL-15 KO mice after secondary infection. As shown in Fig. 4*D*, both PBS-treated naive IL-15 KO mice and rIL-15-treated naive IL-15 KO mice had high bacterial loads in the spleens and livers on day 2 after infection. In contrast, immunized IL-15 KO mice exhibited antilisterial immunity, as shown by a reduction in bacterial counts in the spleens and livers after rechallenge. Administration of rIL-15 resulted in 10- to 30-fold reduction in bacterial loads in the spleens and livers of immunized IL-15 KO mice compared with