of using recombinant rNP for the IgG-ELISA is that it enables a direct comparison of antibody cross-reactivity among arenavirus rNPs, since antigen preparations of all arenavirus rNPs tested are performed using the same method [51]. Rabbit anti-sera raised against LCMV-rNP and LASV-rNP show cross-reactivity to LASV-rNP and LCMV-rNP, respectively, indicating that rabbit antibodies against rNPs of Old World arenaviruses cross-react with rNPs of other Old World arenaviruses (Table 1) [51]. Similarly, rabbit anti-sera generated against JUNV-NP show cross-reactivity to the LASV-rNP and LCMV-rNP, although the reaction is weak. However, rabbit anti-sera against LASV-NP and LCMV-NP show a negative reaction to the JUNV-rNP (Table 1) [51], indicating that rabbit antibodies against JUNV (a pathogenic New World arenavirus) NP might cross-react with the Old World arenavirus NP, whereas antibodies against Old World arenavirus NPs may not be able to react with pathogenic New World arenavirus NPs.

The rNP-based IgG-ELISA has also been used for the characterization of a mouse monoclonal antibody (MAb). Nakauchi *et al.* [50] have investigated the cross-reactivity of MAbs against JUNV rNP to pathogenic New World arenavirus rNPs, as well as LASV rNP. MAb C11-12 reacts at the same level with the rNPs of all of the pathogenic New World arenaviruses, including JUNV, GTOV, MACV, SABV, and CHPV, indicating that this MAb recognizes an epitope conserved among pathogenic New World arenaviruses. Another MAb, C6-9, reacts specifically with the rNP of JUNV, but does not react with those of the other pathogenic New World arenaviruses [50]. This indicates that MAb C6-9 recognizes a JUNV-specific epitope. None of these MAbs reacts with the rNP of the human pathogenic Old World arenavirus LASV. Thus, the MAb C11-12 is considered to be a broadly reactive MAb against New World arenaviruses, whereas MAb C6-9 is JUNV-specific. These findings have been confirmed by detailed epitope analyses using peptide mapping [50]. Similarly, the cross-reactivity of MAbs against LASV rNP has been analyzed [51]. MAb 4A5 cross-reacts with the Mopeia virus (MOPV) but not with the LCMV rNP. MAb 6C11 cross-reacts with LCMV rNP, while MAb 2-11 does not cross-react with LCMV rNP [51].

Table 1. Anti-serum reactivity for rNPs of different arenaviruses in IgG ELISAs.

Rabbit anti-serum	Reactivity for rNP from		
	LASV	LCMV	JUNV
anti-LASV NP	++	+	−
anti-LCMV NP	+	++	−
anti-JUNV NP	+	+	++

It is important to evaluate whether rNP-based ELISA is useful for the diagnosis of human VHF cases. The specificity of the LASV-rNP-based IgG ELISA has been confirmed by using sera obtained from Lassa fever patients [51]. The Lassa fever patients' sera show a highly positive reaction in the LASV-rNP-based IgG-ELISA, but sera from patients with Argentine hemorrhagic fever (AHF), which is caused by JUNV, do not. The serum from an AHF patient showed a highly positive reaction in the JUNV-rNP-based IgG-ELISA [49]. In addition, it was shown that, using sera obtained from AHF cases, the results of the JUNV rNP-based IgG ELISA correlate well with an authentic JUNV antigen-based IgG ELISA [49]. An IgM-capture ELISA using purified LASV-rNP as an antigen has been developed in the same way as in previous reports [54,57] and detects an LASV-IgM

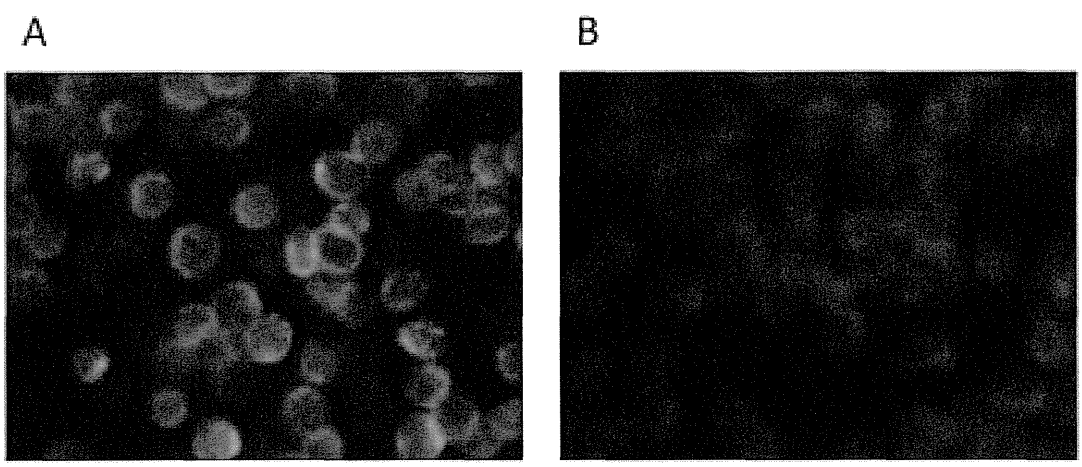
antibody [58]. In addition, immunoblot assays based on N-terminally truncated LASV rNP have been developed for detecting IgG and IgM antibodies against LASV. These methods may provide a rapid and simple Lassa fever test for use under field conditions [47].

3.2. Antibody Detection IFA

An IFA using virus-infected cells is a common antibody test for VHF viruses [59–63]. To avoid the use of highly pathogenic viruses for the antigen preparation, mammalian cells expressing recombinant rNP have been developed [51,57,64–68]. Lassa virus NP antigen for IFA can be prepared simply as described [51]. Briefly, the procedure involves (1) transfecting HeLa cells with a mammalian cell expression vector inserted with the cloned NP cDNA; (2) expanding the stable NP-expressing cells by antibiotic selection; (3) mixing the rNP-expressing cells with un-transfected HeLa cells (at a ratio of 1:1); (4) spotting the cell mixtures onto glass slides, then drying and fixing them in acetone.

In the IFA specific for LASV-NP, antibody positive sera show characteristic granular staining patterns in the cytoplasm (Figure 2) [69], thus making it easy to distinguish positive from negative samples. The specificity of the assay has also been confirmed by using sera obtained from Lassa fever patients [51]. In addition, an IFA using JUNV rNP-expressing HeLa cells has been developed to detect antibodies against JUNV, and the assay has been evaluated by using AHF patients' sera [70]. The LASV-rNP-based antibody detection systems such as ELISA and IFA are suggested to be useful not only for the diagnosis of Lassa fever, but also for seroepidemiological studies of LASV infection. In our preliminary study, approximately 15% of the sera collected from 334 Ghanaians and less than 3% of 280 Zambians showed positive reactions in the LASV-rNP-based IgG ELISA [58]. These results are in agreement with the fact that Lassa fever is endemic to the West African region, including Ghana, but less in the East African region.

Figure 2. Staining patterns of the LASV-rNP-expressing HeLa cells obtained from the sera of a Lassa-NP-immunized monkey (**A**) and control serum (**B**) in an IFA.



4. Antigen-Capture ELISA

For the diagnosis of many viral infections, PCR assays have been shown to have an excellent analytical sensitivity, but the established techniques are limited by their requirement for expensive equipment and technical expertise. Moreover, the high degree of genetic variability of the RNA viruses, including arenavirus and bunyavirus, poses difficulties in selecting primers for RT-PCR assays that can detect all strains of the virus. Since the sensitivity of the Ag-capture ELISA is comparable to that of RT-PCR for several virus-mediated infectious diseases, including Lassa fever and filovirus hemorrhagic fever [51,71–73], the Ag-capture ELISA is a sophisticated approach that can be used for the diagnosis of viral infections. Ag-capture ELISAs detecting viral NP in viremic sera have been widely applied to detect various viruses, since they are the most abundant viral antigens and have highly conserved amino acid sequences [50,51,54,71,72,74,75]. Polyclonal anti-sera or a mixture of MAbs present in the ascetic fluids from animals immunized for HFVs have been used for capture-antibodies in the Ag-capture ELISA [36,76–79]. MAbs recognizing conserved epitopes of the rNP are also used as capture antibodies since they have a high specificity for the antigens, and an identification of the epitopes of these MAbs is of crucial importance for the assessment of the specificity and cross-reactivity of the assay system [50,51,53,54,71,75]. In order to develop a sensitive diagnostic test for Lassa fever and AHF, rNPs of LASV and JUNV (see above) have been prepared, and newly established MAbs against them have been characterized and used for Ag-capture ELISAs [50,51]. The Ag-capture ELISA using MAb 4A5 has been confirmed to be useful in the detection of authentic LASV antigen in sera serially collected from hamsters infected with LASV [51]. The sensitivity of the MAb 4A5-based Ag-capture ELISA was similar to that of conventional RT-PCR, suggesting that the Ag-capture ELISA can be efficiently used in the diagnosis of Lassa fever [51]. Therefore, the MAb 4A5- based Ag-capture ELISA is considered to be useful in the diagnosis of Lassa fever. Also, by using MAbs raised against the rNP of JUNV, Ag-capture ELISAs specific for JUNV and broadly reactive to human pathogenic New World arenaviruses have been developed [50]. The Ag-capture ELISA using MAb E4-2 and C11-12 detected the Ags of all of the pathogenic New World arenaviruses tested, including JUNV. On the other hand, the Ag-capture ELISA using MAb C6-9 detects only the JUNV Ag. Considering that the symptoms of JUNV infection in humans are indistinguishable from those due to other pathogenic New World arenaviruses, the Ag capture ELISA using MAb C6-9 may be a useful diagnostic tool, especially for AHF [50].

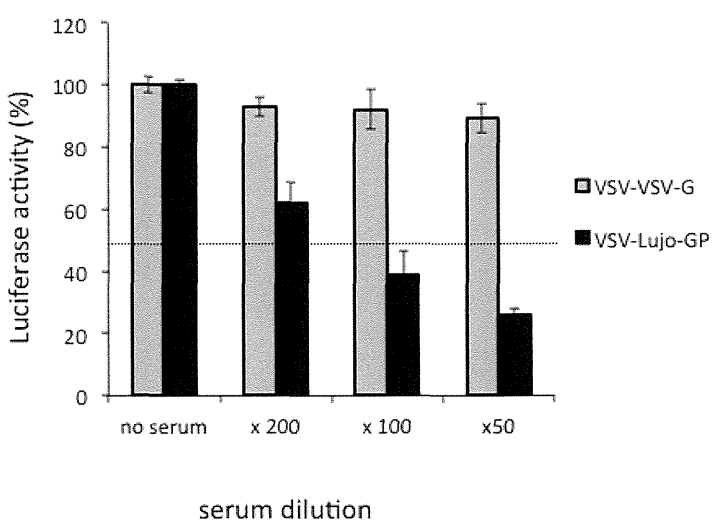
5. Neutralization Assays Based on VSV Pseudotypes

The virus neutralization assay is accepted as the "gold standard" serodiagnostic assay to quantify the antibody response to infection and vaccination of a wide variety of viruses associated with human diseases [80–86]. The presence of neutralizing antibodies is a reliable indicator of protective immunity against VHF [87–89]. The most direct method for detection of neutralizing antibodies against HFVs is by plaque reduction neutralization tests using infectious viruses. However, because of the high pathogenicity of HFVs to humans and the strict regulation of select agents, only a limited number of laboratories are able to perform such neutralization tests. For many HFVs, replication-incompetent pseudotyped virus particles bearing viral envelope protein (GP) have been shown to mimic the

respective HFV infections, thus, neutralization assays using the pseudotypes may be advantageous in some laboratory settings for the detection of antibodies to HFVs without the need for heightened biocontainment requirements.

The VSV-based vector has already been used to generate replication-competent recombinant VSVs to study of the role of GPs of various viruses [90–92]. Recent advances in producing pseudotype virus particles have enabled the investigation of the virus cell entry, viral tropism, and effect of entry inhibitors, as well as measurement of the neutralization titers, by using human immunodeficiency virus-, feline immunodeficiency virus-, murine leukemia virus-, or VSV-based vectors [86,93–103]. Pseudotypes based on VSV have advantages compared with other pseudotypes based on retroviruses for the following reasons. First, the pseudotype virus titer obtained with the VSV system is generally higher than that of the pseudotyped retrovirus system [104]. Second, the infection of target cells with a VSV pseudotype can be readily detected as green fluorescent protein (GFP)-positive cells at 7–16 h post-infection because of the high level of GFP expression in the VSV system [104,105]. In contrast, the time required for infection in the pseudotyped retrovirus system is 48 h [106,107], which is similar to the time required for infectious viruses to replicate to a level that results in plaque-forming or cytopathic effects in infected cells. A high-throughput assay for determining neutralizing antibody titers using VSV pseudotypes expressing secreted alkaline phosphatase [108,109] or luciferase (Figure 3) has also been developed.

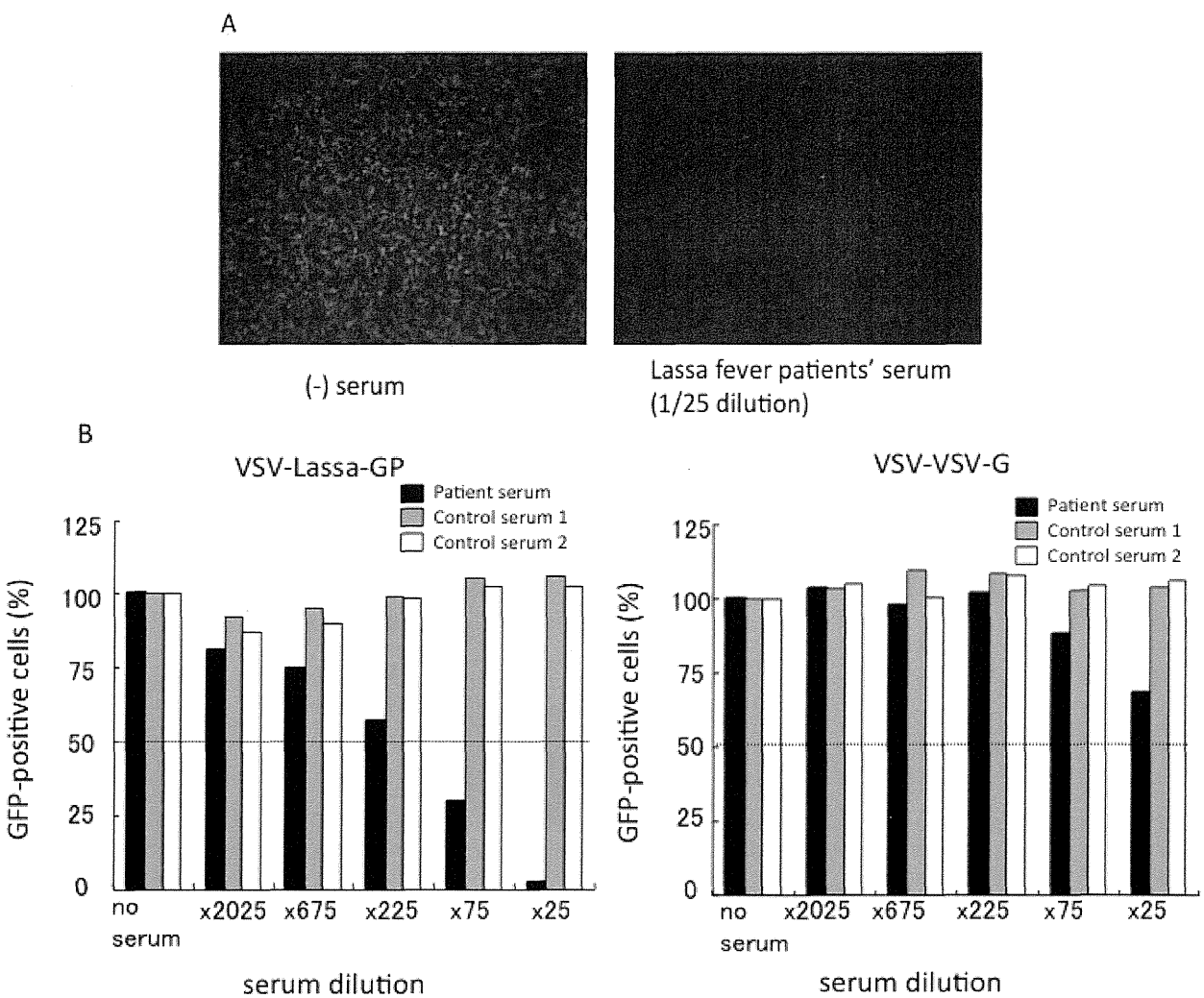
Figure 3. Neutralization assay for VSV-Lujo-GP. VSV-Lujo-GP or a control pseudotype (VSV-VSV-G) that expressed luciferase was incubated with serially diluted serum obtained from a rabbit immunized with Lujo-GPC, and was then inoculated in triplicate into Vero E6 cells. The luciferase activity (%) of each well compared to the negative control (no serum) is shown.



We have recently developed a VSV-based pseudotype bearing Lassa virus GP (VSV-LAS-GP) for the detection of neutralizing antibodies in the sera obtained from a Lassa fever patient. An example of the LASV neutralization assay using the VSV pseudotype is shown (Figure 4). In the presence of serum from Lassa fever patients, the number of GFP-positive cells (infectivity of VSV-LAS-GP) is significantly reduced compared with the number in the absence of the patient's serum (Figure 4A). The

control VSV pseudotype bearing VSV GP (VSV-VSV-G) is not neutralized by any sera. When the cut-off serum dilution is set at 50% inhibition of infectivity compared with the infectivity in the absence of the test serum, the neutralization titer of this patient's serum for VSV-LAS-GP is calculated to be 75 (Figure 4B). Likewise, a VSV-based pseudotype bearing the Junin virus GP has been developed for the detection of neutralizing antibodies from AHF patients' sera. The accuracy of the results of VSV-based neutralization assays has been confirmed by comparison with the results of the neutralization assay using live Junin virus [70].

Figure 4. Neutralization assay for VSV-Lassa-GP. (**A**) VSV-LAS-GP was incubated with or without serum obtained from a Lassa patient, and then was inoculated into Vero cells. The GFP signal was observed under a fluorescence microscope. (**B**) VSV-LAS-GP or the control pseudotype (VSV-VSV-G) incubated with serially diluted patient serum or healthy control sera were inoculated into Vero E6 cells. The relative number of GFP-positive cells (%) compared with negative control cells (no serum) is shown.



The Lujo virus is a new member of the hemorrhagic fever-associated arenavirus family from Zambia and southern Africa, and the virus is classified as a BSL-4 pathogen [17]. The genome sequence analysis of the Lujo virus suggests that the virus is genetically distinct from previously characterized arenaviruses. In order to study the infectivity of this newly identified arenavirus, we have

recently developed a luciferase-expressing VSV pseudotype bearing Lujo virus GPC (VSV-Lujo-GP). As shown in Figure 3, infection with VSV-Lujo-GPC is specifically neutralized by rabbit anti-Lujo GPC serum. Thus, the VSV-Lujo-GP may be a useful tool not only for determining the neutralizing antibody titer within the serum, but also for exploring yet-to-be-defined cellular receptor(s) for Lujo virus infection or for screening inhibitors of the Lujo virus GP-mediated cell entry.

6. Conclusions

Hemorrhagic fever outbreaks caused by pathogenic arenaviruses result in high fatality rates. A rapid and accurate diagnosis is a critical first step in any outbreak. Serologic diagnostic methods for VHFs most often employ an ELISA, IFA, and/or virus neutralization assay. Diagnostic methods using recombinant viral proteins have been developed and their utilities for diagnosing of VHF have been reviewed. IgG- and IgM- ELISAs and IFAs using rNPs as antigens are useful for the detection of antibodies induced in the patients' sera. These methods are also useful for seroepidemiological surveys for HFVs. Ag-capture ELISAs using MAbs to the arenavirus rNPs are specific for the virus species or can be broadly reactive for New World arenaviruses, depending on the MAb used. Furthermore, the VSV-based pseudotype system provides a safe and rapid tool for measuring virus neutralizing antibody titers, as well as a model to analyze the entry of the respective arenavirus in susceptible cells without using live arenaviruses. Recent discoveries of novel arenavirus species [17,26,110] and their potential to evolve predominantly via host switching, rather than with their hosts [110,111], suggest that an unknown pathogenic arenavirus may emerge in the future, and that the diagnostic methods for VHF caused by arenaviruses should thus be further developed and improved.

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Conflict of Interest

The authors declare no conflict of interest.

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Analysis of the humoral immune responses among cynomolgus macaque naturally infected with Reston virus during the 1996 outbreak in the Philippines

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Abstract

Background: Ebolaviruses induce lethal viral hemorrhagic fevers (VHFs) in humans and non-human primates, with the exceptions of Reston virus (RESTV), which is not pathogenic for humans. In human VHF cases, extensive analyses of the humoral immune responses in survivors and non-survivors have shown that the IgG responses to nucleoprotein (NP) and other viral proteins are associated with asymptomatic and survival outcomes, and that the neutralizing antibody responses targeting ebolaviruses glycoprotein ($GP_{1,2}$) are the major indicator of protective immunity. On the other hand, the immune responses in non-human primates, especially naturally infected ones, have not yet been elucidated in detail, and the significance of the antibody responses against NP and $GP_{1,2}$ in RESTV-infected cynomolgus macaques is still unclear. In this study, we analyzed the humoral immune responses of cynomolgus macaque by using serum specimens obtained from the RESTV epizootic in 1996 in the Philippines to expand our knowledge on the immune responses in naturally RESTV-infected non-human primates.

Results: The antibody responses were analyzed using IgG-ELISA, an indirect immunofluorescent antibody assay (IFA), and a pseudotyped VSV-based neutralizing (NT) assay. Antigen-capture (Ag)-ELISA was also performed to detect viral antigens in the serum specimens. We found that the anti-$GP_{1,2}$ responses, but not the anti-NP responses, closely were correlated with the neutralization responses, as well as the clearance of viremia in the sera of the RESTV-infected cynomolgus macaques. Additionally, by analyzing the cytokine/chemokine concentrations of these serum specimens, we found high concentrations of proinflammatory cytokines/chemokines, such as IFNγ, IL8, IL-12, and MIP1α, in the convalescent phase sera.

Conclusions: These results imply that both the antibody response to $GP_{1,2}$ and the proinflammatory innate responses play significant roles in the recovery from RESTV infection in cynomolgus macaques.

Keywords: Ebola, Ebolavirus, Reston virus, Reston ebolavirus, Filovirus, Zoonosis, Humoral immune response, Cynomolgus macaque, Cytokine, Antibody

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Background

The family *Filoviridae* includes three genera, Ebolavirus, Marburgvirus, and Cuevavirus. The genus Ebolavirus currently has five members: Bundibugyo virus (BDBV), Ebola virus (EBOV), Reston virus (RESTV), Sudan virus (SUDV), and Taï Forest virus [1]. Filoviruses induce lethal viral hemorrhagic fevers (VHFs) in both humans and nonhuman primates, while RESTV infection in humans is probably subclinical, yet it also causes highly lethal VHF in macaques [2,3]. RESTV epizootics among cynomolgus macaques emerged in 1989, 1990, 1992, and 1996. In all of these epizootics, the cynomolgus macaques originated in a single primate breeding facility in the Philippines [4]. Although the natural reservoir of RESTV remains unknown, RESTV was isolated from pigs in the Philippines, in addition to porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type-2 in 2008 [5]. Considering the social impact of ebolaviruses, it is important to understand the endemic and epizootic status of RESTV in the Philippines.

In this study, we investigated the antibody responses of cynomolgus macaques that could be dead-end hosts for RESTV. Using serum specimens collected from cynomolgus macaques during a RESTV outbreak in the Philippines in 1996, we attempted to elucidate the significance of neutralizing antibodies to RESTV in viral clearance. We have previously established an enzyme-linked immunosorbent assay (ELISA) and an indirect immunofluorescent antibody assay (IFA) specific for RESTV nucleoprotein (NP) [6-8]. These assays are useful tools for investigating the signs of RESTV infection in cynomolgus macaques. In human cases, antibody responses against ebolaviruses have been analyzed extensively: IgG responses to NP and other structural proteins (e.g., VP40 and VP35) have been shown to correlate with asymptomatic and surviving cases, and neutralizing antibody responses targeting the ebolaviruses glycoprotein ($GP_{1,2}$) appear to be the major indicator of protective immunity [9].

On the other hand, proinflammatory cytokines/chemokines are known to play a major role in the pathogenesis of ebolaviruses infections in various species. Previous studies have shown an uncontrolled secretion of proinflammatory cytokines/chemokines to contribute to a fatal outcome in EBOV-infected humans [10] and cynomolgus macaques [11]. Strong proinflammatory cytokine/chemokine responses are also observed in convalescent or asymptomatic cases [12,13]. In RESTV-infected cynomolgus macaques, high viremia has been shown to induce the secretion of proinflammatory cytokines/chemokines [14]. However, there have so far only been a limited number of studies on the impact of proinflammatory cytokine/chemokine responses in the convalescent phase of RESTV infection.

In this study, we grouped the cynomolgus macaque samples based on the presence of RESTV NP-antigen in sera and analyzed the antibody reactions and cytokine/chemokine inductions to evaluate the presence of neutralizing antibody to RESTV. We found that the anti-$GP_{1,2}$ responses, but not the anti-NP responses, were closely correlated with the neutralization antibody responses, as well as the clearance of viremia, in the sera of RESTV-infected cynomolgus macaques. Additionally, a high concentration of proinflammatory cytokines/chemokines was detected in the convalescent phase specimens. These data suggest that both the anti-$GP_{1,2}$ responses and proinflammatory cytokines/chemokines play significant roles in the recovery from RESTV infection in cynomolgus macaques.

Results

RESTV NP-and $GP_{1,2}$-specific antibodies, neutralizing antibody responses, and the viral antigens in the cynomolgus macaque sera from the 1996 RESTV epizootic

Twenty-seven serum samples derived from cynomolgus macaques that were either found already dead or had been euthanized at the facility were available. The presence of RESTV NP antigens was evaluated by antigen-capture ELISA [15] or immunohistochemistry [3], while that of anti-RESTV NP IgG was evaluated using IgG ELISA and IFA methods [6-8]. RESTV NP antigens were detected in the liver in # 2182, 2612, 2615, 2669, 2739, 2921, 2644 and 2728, while RESTV NP was detected by antigen capture ELISA in the sera of #2182, 2612, 2408, 2615, 2669, 2739, 2921, 2721 and 2972. We therefore assumed that these cynomolgus macaques had suffered from the acute viremic phase of the disease. Seventeen of the 27 samples (#2408, 2615, 2669, 2739, 2921, 2728, 2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696, 2713 and 2194) were positive for anti-NP IgG in IgG ELISA, while these samples all reacted in IFA. On the other hand, two samples (#2644 and 2719) were only positive in IFA. We considered the samples as anti-NP IgG-positive when either ELISA or IFA showed positive reaction. As a result, a total of 19 samples had anti-NP IgG. Cynomolgus macaques with anti-NP IgG consisted of NP antigen-positive (#2408, 2615, 2669, 2739, 2921, 2644 and 2728) and NP antigen-negative groups (#2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713).

In order to examine whether the sera contained anti-$GP_{1,2}$ antibodies, we employed a $GP_{1,2}$-specific ELISA and IFA [16]. RESTV $GP_{1,2}\Delta TM$ prepared by a baculovirus expression system and RESTV $GP_{1,2}$-expressing HeLa cells were used as antigens for $GP_{1,2}$-specific ELISA and IFA, respectively. Anti-RESTV $GP_{1,2}$ IgG were detected in 10 (#2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713) out of the 27 serum samples according to ELISA (37%), whereas the

remaining 17 samples (63%) showed negative reactions. Nine serum samples positive for $GP_{1,2}$ antibodies in the IgG-ELISA also showed positive reactions in the IFA, while one serum sample (#2194) was only positive in the IFA. Serum samples showing positive reactions in either the $GP_{1,2}$-specific IgG-ELISA or IFA were considered to be anti-$GP_{1,2}$ positive (11/27, 41%, Table 1). Interestingly, the sera derived from cynomolgus macaques in the acute viremic phase did not contain any detectable anti-$GP_{1,2}$ IgG, although they often contained anti-NP IgG.

We next attempted to detect the neutralization (NT) antibody response in the sera of RESTV-infected cynomolgus macaques. The VSV pseudotype RESTV $GP_{1,2}$ (VSV-RESTV-$GP_{1,2}$/GFP) was used for the NT assay [17]. Twelve serum samples (12/27, 44%) neutralized the VSV-RESTV-$GP_{1,2}$/GFP infection, with NT titers ranging from 80 and 640 (#2721, 2972, 2180, 2181, 2189, 2190, 2191, 2195, 2404, 2693, 2696 and 2713) (Table 1). The anti-$GP_{1,2}$ IgG were not detectable in #2721 and #2972 by IgG ELISA, while those samples both had a neutralizing activity. These two specimens showed a positive response for viral antigen in the Ag-capture ELISA and were thus considered to be collected in an early seroconversion phase.

All cynomolgus macaques at the facility were euthanatized regardless of clinical status and there was a possibility that some of the cynomolgus macaques had combined infection with simian hemorrhagic fever virus (SHFV) in the animal facility [18]. Therefore, immune responses against RESTV did not always reflect the

Table 1 Antibody responses and viremic status of Reston virus-infected cynomolgus macaques

Case ID	Anti-NP IgG		Anti-$GP_{1,2}$ IgG		NT	Ag-ELISA		Overall status	Dead or **euthanized
	ELISA	IFA	ELISA	IFA		liver	serum		
2182	-	<80	-	<80	-	+	+	Ag + NT -	euthanized
2612	-	<80	-	<80	-	+	+	Ag + NT -	euthanized
2408	+	10240	-	<80	-	*ND	+	Ag + NT -	#NR
2615	+	2560	-	<80	-	+	+	Ag + NT -	euthanized
2669	+	2560	-	<80	-	+	+	Ag + NT -	euthanized
2739	+	1280	-	<80	-	+	+	Ag + NT -	euthanized
2921	+	1280	-	<80	-	+	+	Ag + NT -	euthanized
2644	-	80	-	<80	-	+	ND	Ag + NT -	euthanized
2728	+	1280	-	<80	-	+	ND	Ag + NT -	euthanized
2721	-	<80	-	<80	80	+	+	Ag + NT +	euthanized
2972	-	<80	-	<80	160	+	+	Ag + NT +	dead
2180	+	1280	+	320	80	-	-	Ag - NT +	dead
2181	+	80	+	80	320	-	-	Ag - NT +	dead
2189	+	1280	+	320	320	-	-	Ag - NT +	NR
2190	+	160	+	640	640	-	-	Ag - NT +	NR
2191	+	640	+	320	320	-	-	Ag - NT +	NR
2195	+	2560	+	640	320	-	-	Ag - NT +	NR
2404	+	1280	+	160	160	-	-	Ag - NT +	dead
2693	+	160	+	<80	320	-	-	Ag - NT +	euthanized
2696	+	2560	+	160	320	-	-	Ag - NT +	euthanized
2713	+	5120	+	320	160	-	-	Ag - NT +	euthanized
2719	-	80	-	<80	-	ND		ND	NR
832	-	<80	-	<80	-	ND		ND	NR
888	-	<80	-	<80	-	ND		ND	NR
1134	-	<80	-	<80	-	ND		ND	NR
2636	-	<80	-	<80	-	ND		ND	euthanized
2194	+	5120	-	80	-	ND		ND	dead
No. of positive samples	17/27	19/27	10/27	10/27	12/27 (44%)	11/21 (52%)			
	19/27 (70%)		11/27 (41%)						

*ND: not determined, **euthanized: monkeys were euthanized regardless of clinical manifestation. #NR: not recorded, *Ag* +: antigen positive, *Ag* -: antigen negative, *NT*+: neutralization antibody positive, *NT*-: neutralization antibody negative. The specimens with case IDs written in italics were considered in acute phase. The specimens with case IDs written in bold were considered in convalescent phase.

clinical manifestation. For these reasons, we defined "convalescent" or "non-convalescent" based only upon serological findings.

It is noteworthy that, among the serum samples that were positive for viral antigen and negative for the NT antibody (Ag +, NT -), all nine serum samples were negative for anti-$GP_{1,2}$ antibodies, whereas only two samples were negative for anti-NP antibodies. On the other hand, all of the ten serum samples that were negative for viral antigen and positive for the NT antibody (Ag -, NT +) were positive for both anti-$GP_{1,2}$ and anti-NP antibodies. This finding indicates that the anti-$GP_{1,2}$ antibody may therefore increase in cynomolgus macaques in the convalescent phase, while anti-$GP_{1,2}$ antibody is rarely detectable in the acute viremic phase of infection.

Multiplex assay for cytokines and chemokines in the cynomolgus macaque sera

Ebola virus infection triggers the expression of several proinflammatory cytokines/chemokines [11,19,20]. To examine whether the convalescence from the RESTV infection correlates with the circulating proinflammatory cytokines/chemokines, eleven RESTV-infected cynomolgus macaque serum samples were subjected to a multiplex cytokine analysis. Since we do not know when the infection occurred for each cynomolgus macaque, it is still unclear whether the sera represented an early or late stage of infection. We used seven convalescent phase sera (Ag-, NT +: #2404, 2181, 2189, 2693, 2696, 2713, 2180), and four acute viremic phase sera (Ag +, NT -: #2182, 2612, 2739, 2921). Among the 27 serum samples, only these 11 serum samples were available for multiplex assay. Since the sera were heat-inactivated at 56°C for 30 min prior to being subjected to the multiplex analysis, some cytokines, such as GM-CSF and IL-2, which were previously shown to be elevated in some RESTV infected cynomolgus macaques [14], could not be measured in the assay.

We found that concentrations of several proinflammatory cytokines/chemokines (e.g., IFNγ, IL8, IL-12, IL-1ra, and MIP1α) were significantly higher in convalescent than in acute phase sera (Figure 1). This observation is similar to the previous studies showing elevated concentrations of proinflammatory cytokines/chemokines in the convalescent or asymptomatic human cases [12,13]. In contrast, the concentrations of the five cytokines/chemokines (e.g., IFNα, IP-10, MIP1β, IL-6, and TNFα) did not differ significantly between the two groups (Figure 2). Furthermore, the concentration of MCP-1, one of the proinflammatory chemokines, was lower in the convalescent than in the non-convalescent sera (Figure 2). These data indicated that IFN-γ, IL-8, IL-12, IL-1ra or MIP1α might therefore be involved with the host immune responses in the convalescent phase of RESTV infection.

Discussion

We previously developed a RESTV NP-specific IgG-ELISA and IFA that proved to be useful for the seroepidemiological studies of cynomolgus macaques during the RESTV epizootic in the Philippines in 1996 [6-8]. The assays based on recombinant NP are sensitive for the detection of RESTV-specific antibodies. On the other hand, anti-$GP_{1,2}$ antibodies are elicited in EBOV-infected human cases and are believed to have protective roles against lethal EBOV infection [21,22]. In the present study, in order to gain insight into the IgG responses during the recovery from infection with RESTV, anti-NP, anti-$GP_{1,2}$, and neutralizing antibodies and the level of viremia in the serum specimens were analyzed. The data presented herein showed that the anti-$GP_{1,2}$ response, rather than the anti-NP response, was correlated with both the lack in viremia and the neutralizing activities in the sera of RESTV-infected cynomolgus macaques. There may be at least two possibilities for the lack of anti-$GP_{1,2}$ IgG in the acute phase samples. It is known that soluble GP (sGP), which does not contain membrane anchor, is secreted during ebolaviruses infection, and it can absorb the anti-$GP_{1,2}$ antibodies [23]. The other possibility is that apoptosis of lymphocytes is induced during RESTV infection and the resulting host immune responses may thus be abrogated. Although the precise mechanism of action is still unknown, it is likely that no IgG responses to RESTV $GP_{1,2}$ are induced in the cynomolgus macaques during the acute phase of infection.

Since the cynomolgus macaques at the facility where the RESTV epizootic occurred were euthanized, sequential serum specimens from each cynomolgus macaque were not available. It is also difficult to determine when each cynomolgus macaque became infected with RESTV. We found that three specimens that have anti-GP antibodies were obtained from dead monkeys (#2180, 2181 and 2194), however, it is difficult to conclude whether RESTV infection caused their death because of the possibility of succumbing to infection by SHFV or some other agents. It is predicted that, among the serum samples examined here, nine were acute phase samples because they were positive in Ag-ELISA or immunohistochemistry [3,15] (Table 1). On the other hand, all but one (serum #2194) of the anti-$GP_{1,2}$-positive serum samples were Ag negative. Therefore, these cynomolgus macaques were considered to be in the convalescent phase of RESTV infection. In this regard, the presence of the anti-$GP_{1,2}$ antibody is thought to be a useful indicator for convalescence in cynomolgus macaques infected with RESTV.

Aberrant proinflammatory cytokines/chemokines are a significant factor implicated in the disease progression of EBOV- and SUDV-infected human cases and experimentally

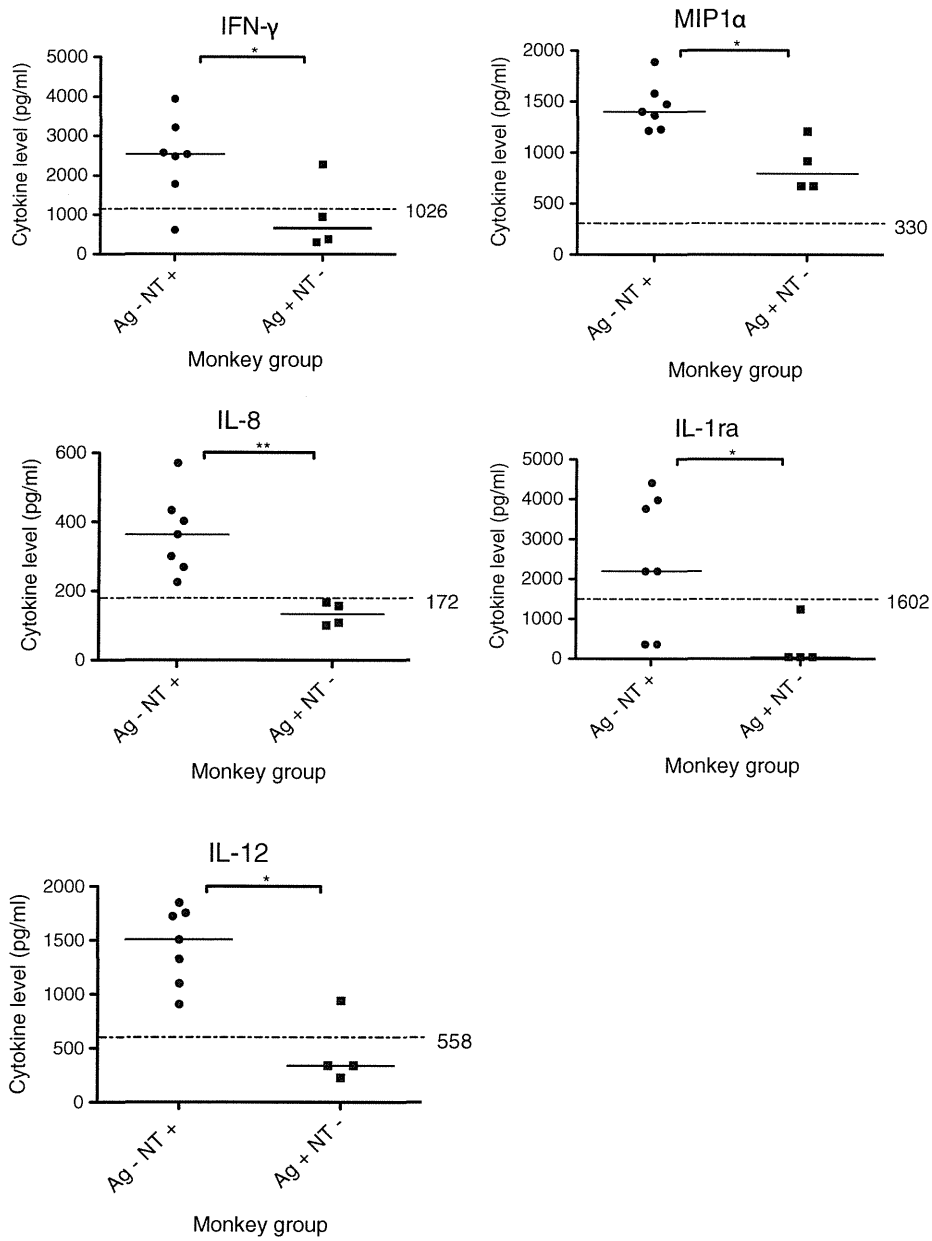

Figure 1 Profiles of the serum cytokine and chemokine concentrations in Reston virus-infected cynomolgus macaques. The serum concentrations of IFN-γ, IL-8, IL-12, MIP1α, and IL-1ra. The concentrations of these proinflammatory cytokines/chemokines (IFN-γ, IL-8, IL-12, MIP1α) and the anti-inflammatory cytokine (IL-1ra) were significantly higher in convalescent (Ag - NT +) than in non-convalescent sera (Ag + NT -). Each dot represents one sample, and dashes (-) represent the median values. * indicates $p < 0.05$, ** indicates $p < 0.005$ (Mann Whitney test). Broken lines and the numbers written aside indicate the average concentrations of cytokine and chemokine in negative control cynomolgus macaques (n = 13).

infected cynomolgus macaques [10,11,24]. In addition, a balanced proinflammatory response is believed to be a critical factor for determining the disease outcome [25,26]. We focused on the circulating inflammatory cytokines/chemokines in RESTV-infected cynomolgus macaques and examined their relationship with convalescence. We thus found the concentrations of several proinflammatory cytokines/chemokines, such as IFNγ, IL8, IL-12, and MIP1α, to be significantly higher in convalescent sera than in non-convalescent sera. Gupta et al. (2012) recently demonstrated that convalescent serum samples obtained from BDBV-infected human cases include high concentrations of IL-1α, IL1β, IL6, TNFα, and MCP-1 [13]. Although the exact profiles of proinflammatory cytokines/chemokines shown in our study are different from those reported by Gupta et al., these differences are considered most likely to be due to

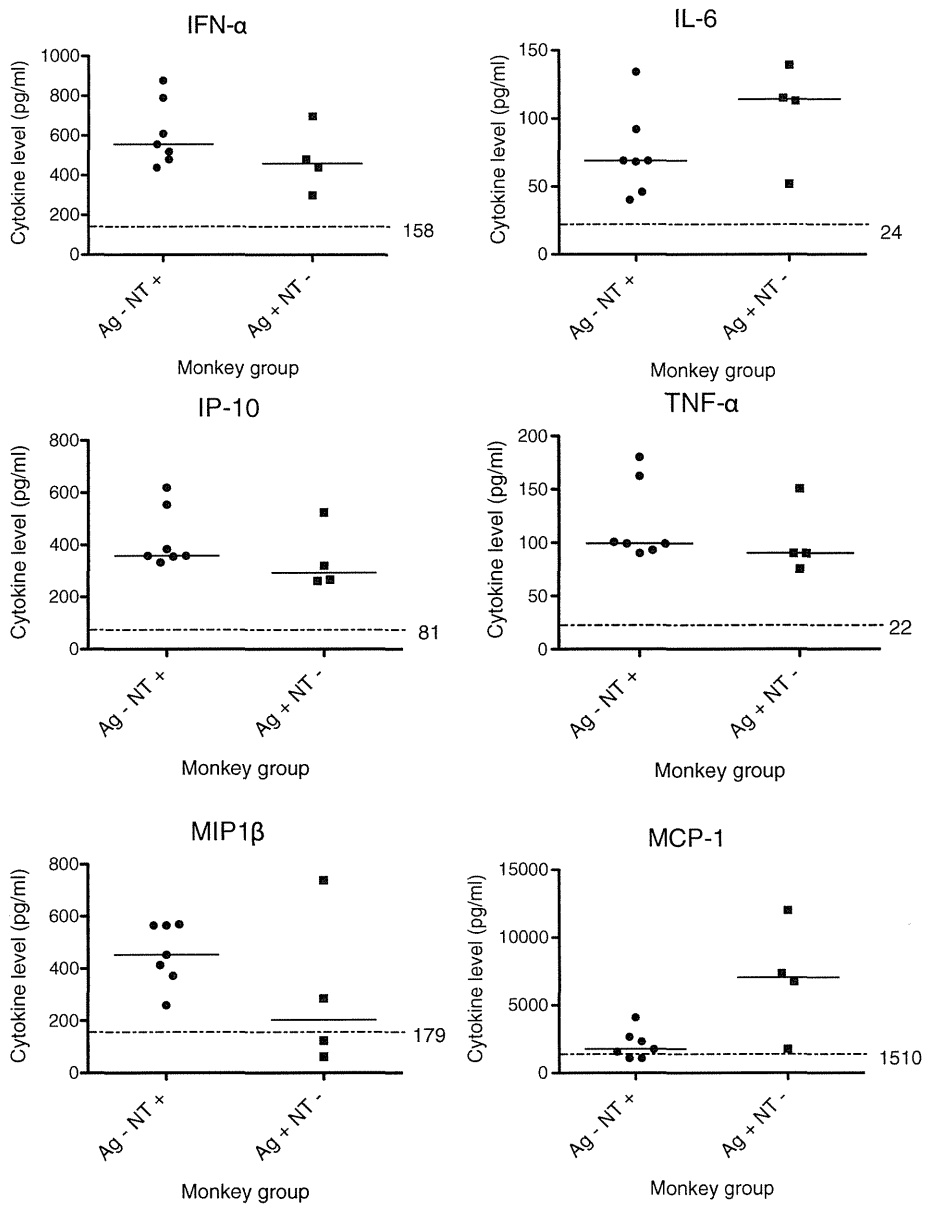

Figure 2 Profiles of the serum cytokine and chemokine concentrations in Reston virus-infected cynomolgus macaques. The IFN-α, IP-10, TNF-α, MIP1β, IL-6 and MCP-1 concentrations. The serum concentrations of these cytokines/chemokines (IFN-α, IP-10, TNF-α, MIP1β) did not differ significantly between the convalescent cases (Ag - NT +) and the non-convalescent cases (Ag + NT -). The concentration of the anti-inflammatory cytokine, MCP-1, was lower in convalescent than in non-convalescent cynomolgus macaques (Mann Whitney test). Each dot represents one sample, and dashes (-) represent the median values. Broken lines and the numbers written aside indicate the average concentrations of cytokine and chemokine in negative control cynomolgus macaques (n = 13).

differences among ebolaviruses (RESTV vs. BDBV), host species (cynomolgus macaques vs. humans), and differences related to the disease phase when the samples were obtained. It is possible that the upregulation of the proinflammatory innate immune responses contributed to the recovery from RESTV infection in cynomolgus macaques.

In rhesus monkeys experimentally infected with a lethal dose of EBOV, anti-inflammatory cytokines, such as IL-13 and IL-1ra, are highly elevated in the acute phase [11]. In human Ebola VHF patients, increased concentrations of IL-10 and IL-1ra have been shown in fatal cases, thus suggesting that the mixed anti-inflammatory response syndrome (MARS) contributes to the pathogenesis of the hemorrhagic fever caused by ebolaviruses. Since all of the cynomolgus macaques involved in the epizootic were euthanized at the affected facility, the actual fate of the cynomolgus macaques was not clear, and some might have survived the infection. Our data obtained using the sera