antibody screening in Africa. Trop. Geogr. Med. **38**: 209–214.
23) World Health Organization. 1976. Ebola haemorrhagic fever in Sudan, 1976. Bull. World Health Organ. **56**: 247–270.
24) World Health Organization. 1978. Ebola haemorrhagic fever in Zaire, 1976. Report of an international commission. Bull. World Health Organ. **56**: 271–293.
25) World Health Organization. 1992. Viral haemorrhagic fever in imported monkeys. Wkly. Epidemiol. Rec. **67**: 142–143.

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Genetic Diversity of the M RNA Segment among Crimean-Congo Hemorrhagic Fever Virus Isolates in China

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The complete nucleotide sequences of the medium (M) segment of seven Chinese isolates of Crimean-Congo hemorrhagic fever (CCHF) virus were determined. The M-segment RNA of CCHF virus comprises 5356–5377 nucleotides depending on the isolate and encodes a protein comprising 1689–1697 amino acids in a viral complementary sense. Phylogenetic analysis of the M segment showed that the Chinese CCHF virus isolates were clustered into three groups, one of which was more closely related to a Nigerian isolate. Pairwise comparison of a precursor protein showed that amino-terminal regions comprising 250 amino acids were extraordinary heterogeneous, with a 22.4% identity in amino acids being observed between the most distantly related isolates. Since all the viruses were isolated from 1966 to 1988 within a restricted area in the Xinjiang Autonomous Region in western China, the results indicate that a multisource virus population is endemic in this region.

Key Words: CCHF virus; China; M-RNA; sequence.

INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) virus is a member of the genus *Nairovirus* in the family *Bunyaviridae* and causes severe hemorrhagic fever in humans resulting in a mortality rate of 10–50% (Gonzalez-Scarano and Nathanson, 1996; Schwarz *et al.*, 1997). CCHF virus is transmitted to humans by bites from Ixodid ticks, especially the genus of *Hyalomma*. The virus is also transmitted to humans either by direct contact with blood or tissues from infected animals, mainly sheep, or by patient's blood, vomit containing blood, or respiratory secretions (Swanepoel *et al.*, 1985; Schwarz *et al.*, 1996). The latter human-to-human infection sometimes causes nosocomial outbreaks of CCHF (Joubert *et al.*, 1985; Le Guenno, 1997). Outbreaks of CCHF have been documented in Africa, the Middle East, Eastern Europe, and western Asia (Suleiman *et al.*, 1980; Joubert *et al.*, 1985; Yen *et al.*, 1985; Altaf *et al.*, 1998; Khan *et al.*, 1997; Gonzalez *et al.*, 1990). In China, the disease is known to be endemic in the Xinjiang Autonomous Region (Yen *et al.*, 1985).

Phylogenetic analysis based on the partial sequence of the S RNA segment of CCHF virus isolates around the world demonstrated that the viruses can be subgrouped into three genetic groups; however, there is no correlation between genetic group and geographic origin of the viruses or year of virus isolation (Rodriguez *et al.*, 1997). The complex phylogenetic picture of CCHF viruses is probably due to the introduction of virus strains from different geographic areas through the importation of virus-infected livestock animals carrying ticks or the migration of virus-infected (or tick-infested) birds (Gonzalez-Scarano and Nathanson, 1996; Rodriguez *et al.*, 1997).

CCHF virus possesses a negative-sense RNA genome consisting of three RNA segments: the large (L), medium (M), and small (S) segments. The S segment encodes a nucleoprotein (NP), the M segment encodes a glycoprotein precursor, which is cleaved into mature glycoproteins, G1 and G2, and the L segment encodes RNA polymerase (Schmaljohn, 1996). In the case of the Hazara virus, another member of the CCHF serogroup of Nairoviruses, a third structural glycoprotein of approximately 45 kDa was identified apart from G1 and G2, but its coding strategy has not yet been analyzed (Foulke *et al.*, 1981). Only Dugbe Nairovirus has been analyzed for the nucleotide sequence and coding strategy of the M-segment RNA (Marriott *et al.*, 1992). The processing of the Dugbe virus M-segment precursor protein is rather complex compared to that of the other Bunyaviruses; the 5′ end of the open reading frame (ORF) encodes a nonstructural protein of 70 kDa, and G2 may be gener-

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TABLE 1

Origin of Chinese CCHF Virus Isolates

Isolate No.	Year of isolation	Geographical origin	Source	M segment RNA (nuc)/ORF (aa)*
66019	1966	Bachu	Patient	5368/1689
7001	1970	Bachu	Patient	5377/1697
75024	1975	Aksu	Patient	5356/1689
7803	1978	Bachu	Patient	5356/1689
79121	1979	Bachu	Long-eared Jerboa	5377/1697
8402	1984	Bachu	Tick	5367/1689
88166	1988	Bachu	Patient	5367/1689

* Nucleotide numbers of the M-RNA/amino acid numbers in the M-RNA-encoded ORF.

ated from its carboxyl-terminal region (Marriott *et al.*, 1992).

In the present study, we determined the complete nucleotide sequences of the M-segment RNA of seven Chinese CCHF virus isolates. Analysis of the genetic characteristics of the M segment of CCHF virus showed that Chinese CCHF virus isolates were clustered in three groups, one of which included the Nigerian tick isolate IbAr 10200. The results suggest that a multisource virus population is endemic in a restricted area in the Xinjiang Autonomous Region of China. Furthermore, we found that the amino-terminal regions of the large open reading *frame* (ORF) comprising approximately 250 amino acids (aa) were extraordinary heterogeneous. Comparison of the ORF between CCHF and Dugbe viruses suggests a common coding strategy of the M segment of Nairoviruses, i.e., a nonstructure protein located at the amino-terminal region of the ORF, followed by G2 and G1.

RESULTS

Sequence of M segment of Chinese isolates of CCHF virus

Bachu county in the Xinjiang Autonomous Region of China has been a CCHF endemic area at least since the 1960s, with a CCHF outbreak documented virtually every year. The viruses were therefore mainly isolated from patients or ticks in this region (Yen *et al.*, 1985; Gonzalez-Scarano and Nathanson, 1996). One exception is isolate 79121, which was isolated from a rodent, a long-eared Jerboa, living in a desert area. The seven CCHF virus strains used in the study were isolated in the western desert regions, Bachu and Aksu counties, in the Xinjiang Autonomous Region between 1966 and 1988. The origins of the viruses are summarized in Table 1. All seven isolates of CCHF virus were isolated by injecting the specimens intracerebrally into suckling mice. RT-PCR was performed to amplify the five parts of the M-segment RNA and the amplimers were directly sequenced. Each extremity of the RNA was amplified by either the 5'RACE or the 3'RACE method and sequenced.

The M-segment RNA of CCHF virus comprises 5356–5377 nucleotides depending on the isolate; i.e., 5356 nucleotides for isolates 75024 and 7803, 5367 nucleotides for 8402 and 88166, 5368 nucleotides for 66019, and 5377 nucleotides for isolates 7001 and 79121 (Table 1). Twenty-nine nucleotides of the 5′ and 39 nucleotides of the 3′ extremity of the viral RNA are conserved among isolates. The 5′ and 3′ ends are complementary to each other and may form a stable base-paired panhandle structure at a calculated free energy of −30.1 kcal/mol (Fig. 1). The 3′-extremity sequence of the M-segment RNA matches that of the S-segment RNA perfectly for 11 bases and partially for 21 bases, while the 5′-extremity sequence of the M segment matches that of the S segment perfectly for 12 bases and partially for 23 bases (Fig. 1).

The M RNA segment encodes a protein comprising either 1689 (for isolates 66019, 75024, 7803, 8402 and 88166) or 1697 (for isolates 7001 and 79121) amino acids (Table 1), with a molecular weight of approximately 187 kDa, in a viral complementary sense.

The results demonstrate that there is an extraordinary hypervariable region at the amino-terminal region within the ORF of CCHF virus. Pairwise comparison of the ORF showed that the amino-terminal regions comprising 250 amino acids were quite heterogeneous, with an identity

```
            U   G G                                  GUCU
5'-UCUCAAAGA AUA U GCGGCACG CAGU C  AAG GUUAA
   ::::::::: ::: : ::::::::: :::: :  ::: :::::
3'-AGAGUUUCU UAU A CGCCGUGC GUCA G  UUC CAAUU
            U   G A       A    U CA   A    GAAA

vRNA 3' ends:
S 3'-AGAGUUUCUUUGUGCACGGCGAAUGCGGG
     *********** ** *** **
M 3'-AGAGUUUCUUUAUGAACGCCGUGCAGUCA

vRNA 5' ends:
S 5'-UCUCAAAGAUAUCGUUGCCGCACAGCCCU
     ************ ** ** ****
M 5'-UCUCAAAGAUAUAGUGGCGGCACGCAGUC

CCHFV vs Dugbe (M segment)

vRNA 3' ends:
CCHFV 3'-AGAGUUUCUUUAUGAACGCCGUGCAGUC
       ********* **** ******** *
Dugbe 3'-AGAGUUUCUGUAUGGACGCCGUGUAUGU

vRNA 5' ends:
CCHFV 5'-UCUCAAAGAUAUAGUGGCGGCACGCAGU
       ********** **********
Dugbe 5'-UCUCAAAGAUAGCGUGGCGGCACAGCAA
```

FIG. 1. (1) Possible base-pairings of the 3′ and 5′ ends of the M segment of CCHF virus isolate 7001. (:) indicates a complementary residue. The calculated free energy of the structure is −30.1 kcal/mol. (2) Comparison of the ends of CCHF virus M and S segments, or the ends of the M segment of CCHF virus and Dugbe virus. (*) indicates an identical residue.

TABLE 2

Identity within the Variable Region (1–250 aa)/(1–812/839 nuc) and within the Conserved Region (251–1683 aa)/(813/840–5360/5377 nuc)

% Identity in amino acid	% Identity in nucleotides							
	10200	75024	7803	66019	8402	88166	7001	79121
				Variable region				
10200		83.2	83.2	68.1	65.6	65.9	52.5	52.5
75024	71.8		98.8	68.2	66.8	66.8	52.1	52.1
7803	71.8	97.2		68.3	66.4	66.4	51.0	51.0
66019	46.5	48.0	48.8		73.5	73.5	51.7	51.7
8402	44.4	45.5	45.5	57.7		99.5	51.4	51.4
88166	44.8	45.9	45.9	58.5	99.2		51.3	51.3
7001	28.4	30.1	27.2	22.4	26.8	27.2		100
79121	28.4	30.1	27.2	22.4	26.8	27.2	100	

% Identity in amino acid	% Identity in nucleotides								
	10200	75024	7803	8402	88166	66019	7001	79121	Dugbe
					Conserved region				
10200		89.3	89.2	82.7	82.8	82.8	74.3	74.2	53.5
75024	94.7		99.5	83.5	83.4	83.2	74.2	74.1	53.4
7803	94.2	99.4		83.3	83.2	83.1	74	73.9	53.3
8402	92.4	93.5	93.0		99.6	85.7	75.1	74.9	53.7
88166	92.2	93.3	92.8	99.5		85.6	74.9	74.8	53.7
66019	91.2	92.3	91.8	93.5	93.2		74.9	74.9	53.2
7001	82.6	83.0	82.5	83.5	83.4	81.9		99.8	52.9
79121	82.4	82.6	82.1	83.2	83.1	81.7	99.5		52.9
Dugbe	37.3	36.6	36.5	37.5	37.5	37.0	36.9	36.9	

as low as 22.4% observed between the most distantly related isolates (Table 2), while the remaining region of approximately 1400 amino acids was well conserved with a 81.7% identity observed between the most distantly related isolates.

Since the amino and carboxyl termini of the G1 and G2 of CCHF virus have not been determined, the precise location of these glycoproteins within the ORF is not known. However, based on the amino acid alignment of the ORF of Dugbe virus, the amino terminus of which has been determined (Marriott *et al.*, 1992), the amino terminus of the CCHF virus is F(L/S)D(S/N)(I/T)(A/V)(K/R)GMK located from amino acid residue 1054 (for isolates 7001 and 79121) or 1046 (for the other isolates). If this is the case, the G1 of the CCHF virus comprises 644 amino acids with a predicted molecular weight of 72.2 kDa (for isolates 7001 and 79121) or 72.6 kDa (for the other isolates) and with three to four potential N-linked glycosylation sites. These molecular weights are similar to that of 75 kDa obtained from the authentic CCHF virus G1 (Clerx *et al.*, 1981). As with Dugbe virus G1 (Marriott *et al.*, 1992), the predicted amino terminus of the G1 of the CCHF virus does not directly follow a hydrophobic region, but rather locates at approximately 50 amino acids downstream of the nearest hydrophobic region, a potential signal sequence. Thus, it is likely that the mature G1 of Nairoviruses including the CCHF virus arises by proteolytic processing after a signal cleavage event.

CCHF virus isolates comprise three genetic groups

Neighbor-joining *phylo*genetic analysis of the amino acid sequences of the ORF in the M segment of seven Chinese isolates and a Nigerian isolate, IbAr 10200, was performed using the ClustalW program. Dugbe virus was used as an outgroup of the tree. As shown in Fig. 2, the phylogenetic tree topology shows three groups. Group 1 contains isolates 7001 and 79121, group 2 contains isolates 66019, 8402, and 88166, and group 3 contains isolates 75024, 7803, and the Nigerian IbAr 10200. This topology was also obtained by a neighbor-joining phylogenetic analysis of conserved partial region of the M-segment sequences (data not shown). The tree based on the M-segment sequence suggests that the genetic distance of CCHF virus isolates does not correlate with geographic origin, the year of isolation, and the hosts.

DISCUSSION

We report here the complete sequence of the M-segment RNA of seven CCHF virus isolates from China. The M-segment sequence of a Nigerian isolate of CCHF virus, IbAr 10200, was previously deposited in GenBank.

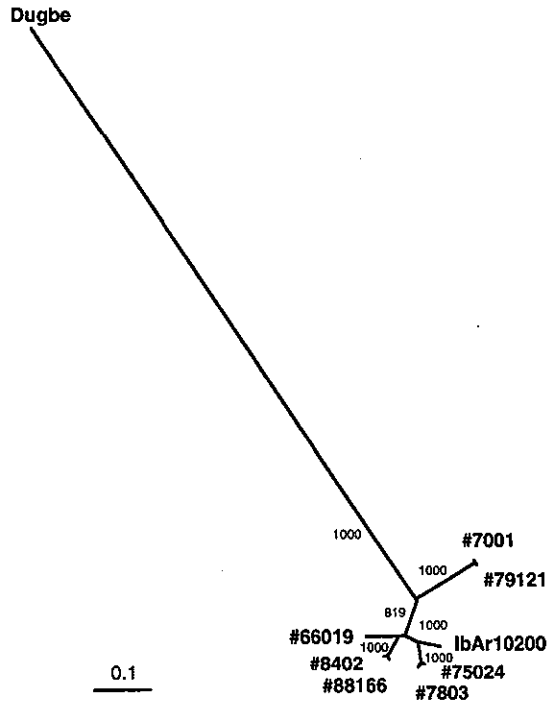

FIG. 2. Phylogenetic tree for the CCHF virus based on the entire amino acid sequence of the M-segment-encoded protein. Seven Chinese isolates and a Nigerian isolate, IbAr 10200, of CCHF virus were analyzed by a neighbor-joining method using ClustalW program (Higgins *et al.*, 1991). Dugbe virus was used as an outgroup of the tree. The tree was drawn using TreeView program (Page, 1996). The numbers at the nodes are the bootstrap confidence level for 1000 replicates. The scale indicates the number of substitutions per site.

However, as with other Nairoviruses, little is known about the coding strategy of the M-segment RNA, processing of the precursor protein, and identification of the conserved and variable regions within the protein encoded in the M segment. This has been partially analyzed for Dugbe virus belonging to the Nairobi sheep disease serogroup of the Nairoviruses. The M-segment RNA of Dugbe virus encodes a large precursor protein of G1 and G2 in a viral complementary sense. This precursor of Dugbe virus comprises 1551 amino acids, with a predicted molecular weight of 173.3 kDa, which is far larger than those of G1 (73 kDa) and G2 (35 kDa). The G2 of Dugbe virus was incompletely mapped to the carboxyl-terminal half of the 70-kDa nonstructural protein (Marriott *et al.*, 1992), which corresponds to the amino-terminal half of the large precursor. The G1 of Dugbe virus was mapped to the carboxyl terminus of the large precursor and its amino terminus did not follow a predicted signal sequence (Marriott *et al.*, 1992), but rather resides at approximately 50 amino acids downstream of the nearest hydrophobic region. According to the amino acid alignment of the precursor protein of Dugbe virus, the amino terminus of G1 of which was determined to be Phe at amino acid residue 897 of the precursor (Marriott *et al.*, 1992), the amino terminus of CCHF virus is Phe at amino acid residue 1054 (for isolates 7001 and 79121) or 1046 (for the other isolates). If this is the case, the CCHF virus G1 comprises 644 amino acids with a predicted molecular weight of 72.2–72.6 kDa and with three to four potential N-linked glycosylation sites, which is similar to that of 75 kDa estimated from the authentic CCHF virus G1. The predicted amino terminus of the CCHF virus G1 does not follow a hydrophobic region, but rather locates at approximately 50 amino acids downstream of the nearest hydrophobic region to function as a potential signal sequence. This figure is similar to that of the Dugbe virus G1, and a further amino-terminal trimming of the G1 by an unknown protease may occur following the cleavage of a signal peptide.

In the case of Rift Valley fever (RVF) virus, belonging to genus *Phlebovirus*, the coding strategy of the M RNA segment is somewhat similar to that of Nairoviruses. The M segment encodes a preglycoprotein or nonstructural protein (NS_M) at the amino terminus in a single ORF in a viral complementary sense, which is then followed by G2 and G1. The NS_M region of RVF virus has five in-frame initiation codons preceding G2, and these initiation codons play a functional role in the processing of G1 and G2 (Kakach *et al.*, 1988, 1989; Schmaljohn *et al.*, 1989; Suzich *et al.*, 1990). Since the amino and carboxyl termini of CCHF virus G2 have not yet been identified, we could not determine the carboxyl terminus of the NS_M or preglycoprotein. However, at least four in-frame initiation codons exist within the amino-terminal 250 aa region of CCHF virus, indicating that the in-frame initiation codons in the Narovirus NS_M region play a similar functional role in the generation of G2 and/or G1 as those in RVF virus. A significant difference in the NS_M-G2 region between Nairoviruses and RVF virus is the localization of potential signal sequences in this region. The NS_M region of RVF virus has a potential signal sequence just upstream of G2 in addition to a potential signal at the amino terminus of NS_M, and the signal sequence just upstream of G2 is thought to be used for processing of this protein (Collett *et al.*, 1985). On the other hand, a potential signal sequence locates only at amino terminus of this NS_M-G2 region in CCHF and Dugbe viruses. These features seem to suggest that this amino-terminal signal sequence is used for the processing of CCHF virus G2 from a large precursor that includes the NS_M region initiated at the first or second initiation codon. Further analysis of the expression of the CCHF virus M-segment cDNAs carrying mutations or deletions of these multiple initiation codons may allow the verification of assumptions.

The amino-terminal region of the precursor protein comprising approximately 250 aa was quite heterogeneous among CCHF virus isolates. Since six of the seven Chinese CCHF virus isolates used in this study originated in Bachu county in the Xinjiang Autonomous Region, while isolate 75024 originated in Aksu, close to Bachu county, it is reasonable that isolates 7001 and

79121, 75024 and 7803, 8402 and 88166, respectively, are closely related to each other. However, the amino acid identity of the amino-terminal 250 aa region between isolate 66019 and 7001/79121 was only 22.4%, in contrast to the high identity of 83% in amino acids of the remaining about 1400 aa region. This suggests that the amino-terminal region per se has little functional significance and only functions to control the expression and processing of the G2 and G1.

Phylogenetic analysis of the M-segment encoded protein showed that Chinese CCHF virus isolates were clustered in three groups, one group consisting of isolates 7001 and 79121, the other group consisting of isolates 66019, 8402, and 88166, and the remaining group consisting of isolates 75024 and 7803. A Nigerian isolate, IbAr 10200, was clustered in the last group. A previous study of phylogenetic analysis based on partial S-segment sequences showed no correlation between genetic group and geographic origin of the virus or year of virus isolation (Rodriguez *et al.*, 1997). However, phylogenetic analysis of the S segment also indicated that the Chinese CCHF virus isolates were clustered in the same branch and apparently distinguishable from the IbAr 10200 (Rodriguez *et al.*, 1997). The sequences of the M segment of three (66019, 8402, 88166) of the Chinese isolates were recently reported (Ma *et al.*, 2001), and their sequence data were virtually the same as our data with minor differences. These minor differences may be due to differences in sequencing strategy, since we have directly determined the sequence from RT-PCR products while Ma and associates determined the sequence after cloning into a plasmid vector. They suggested that the Chinese CCHF virus isolates formed an independent phylogenetic branch distinguishable from IbAr 10200. This discrepancy between their results and ours may be due to the smaller number of sequence data analyzed in their study. In the meantime, our data strongly suggest that several distinct genetic variants of CCHF virus are endemic in a restricted area in the Xinjiang Autonomous Region in China. To more clearly understand how these diverse populations of CCHF virus are maintained in the restricted area, analysis of more virus samples from ticks, animals, and patients is required.

MATERIALS AND METHODS

Viruses

CCHF viruses, 66019, 7001, 75024, 7803, 79121, 8402, and 88166, were all isolated in China between 1966 and 1988 (the first two numbers indicate the year of isolation). All the isolates except 75024 originated in Bachu county in the Xinjiang Autonomous Region of China. Isolate 75024 originated from Aksu county, which neighbors Bachu county in the Xinjiang Autonomous Region. The CCHF virus isolates were intracerebrally inoculated into suckling mice and the brains were collected at 4–5 days after inoculation. The inoculation of the viruses was performed in a containment laboratory under the regulations of the Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine, China.

RNA isolation and first-strand cDNA synthesis

Total RNAs were isolated from brain homogenates using RNAZol B (TEL-TEST, Friendswood, TX) according to the manufacturer's instructions. cDNA was synthesized using a You-Prime-First-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ) with a random hexamer ($p(dN)_6$) as a primer.

PCR and sequencing

Primers were synthesized based on the sequence data of the Nigerian isolate, IbAr 10200 (GenBank Accession No. U 39455). PCR was performed using random hexamer-primed cDNAs of the isolates as templates, using an Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany). The fragments including extremity of the sequence were amplified by either the 5'RACE or 3'RACE method using a commercial kit (5'RACE System, Version 2.0, Life Technologies, Rockville, MD). The amplimers were purified on agarose gel and directly sequenced by the dideoxy chain terminator method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The complete sequences of the M segment of seven Chinese isolates of CCHF virus were deposited in DDBJ under the Accession Nos. AB069669–AB069675.

Sequence analysis

The alignment of nucleotide and deduced amino acid sequences was performed using DNASIS-MAC software (Hitachi Software Engineering Co., Ltd., Kanagawa, Japan). Phylogenetic analysis using neighbor-joining method was performed using the ClustalW program (Higgins *et al.*, 1991). The tree was drawn using the TreeView program (Page, 1996).

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REFERENCES

Altaf, A., Luby, S., Ahmed, A. J., Zaidi, N., Khan, A. J., Mirza, S., McCormick, J., and Fisher-Hoch, S. (1998). Outbreak of Crimean-Congo haemorrhagic fever in Quetta, Pakistan: Contact tracing and risk assessment. *Trop. Med. Int. Health* 3, 878–882.

Clerx, J. P., Casals, J., and Bishop, D. H. (1981). Structural characteristics of nairoviruses (genus *Nairovirus*, *Bunyaviridae*). *J. Gen. Virol.* 55, 165–178.

Collett, M. S., Purchio, A. F., Keegan, K., Frazier, S., Hays, W., Anderson, D. K., Parker, M. D., Schmaljohn, C., Schmidt, J., and Dalrymple, J. M. (1985). Complete nucleotide sequence of the M RNA segment of Rift Valley fever virus. *Virology* **144,** 228–245.

Foulke, R. S., Rosato, R. R., and French, G. R. (1981). Structural polypeptides of Hazara virus. *J. Gen. Virol.* **53,** 169–172.

Gonzalez, J. P., LeGuenno, B., Guillaud, M., and Wilson, M. L. (1990). A fatal case of Crimean-Congo haemorrhagic fever in Mauritania: Virological and serological evidence suggesting epidemic transmission. *Trans. R. Soc. Trop. Med. Hyg.* **84,** 573–576.

Gonzalez-Scarano, F., and Nathanson, N. (1996). Bunyaviridae. *In* "Fields Virology" (B. Fields, D. Knipe, and P. Howley, Eds.), pp. 1473–1504. Lippincott-Raven, Philadelphia, PA.

Higgins, D. G. Bleasby, A. J., and Fuchs, R. (1991). CLUSTAL V: Improved software for multiple sequence alignment. *Comput. Appl. Biosci.* **8,** 189–191.

Joubert, J. R., King, J. B., Rossouw, D. J., and Cooper, R. (1985). A nosocomial outbreak of Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part III. Clinical pathology and pathogenesis. *S. Afr. Med. J.* **68,** 722–728.

Kakach, L. T., Suzich, J. A., and Collett, M. S. (1989). Rift Valley fever virus M segment: Phlebovirus expression strategy and protein glycosylation. *Virology* **170,** 505–510.

Kakach, L. T., Wasmoen, T. L., and Collett, M. S. (1988). Rift Valley fever virus M segment: Use of recombinant vaccinia viruses to study Phlebovirus gene expression. *J. Virol.* **62,** 826–833.

Khan, A. S., Maupin, G. O., Rollin, P. E., Noor, A. M., Shurie, H. H., Shalabi, A. G., Wasef, S., Haddad, Y. M., Sadek, R., Ijaz, K., Peters, C. J., and Ksiazek, T. G. (1997). An outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates, 1994–1995. *Am. J. Trop. Med. Hyg.* **57,** 519–525.

Le Guenno, B. (1997). Haemorrhagic fevers and ecological perturbations. *Arch. Virol. Suppl.* **13,** 191–199.

Ma, B., Hang, C., and Papa, A. (2001). Sequencing and comparative analysis of the complete glycoprotein gene of three Crimean-Congo hemorrhagic fever virus Chinese isolates (in Chinese). *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* **15,** 105–111.

Marriott, A. C., el-Ghorr, A. A., and Nuttall, P. A. (1992). Dugbe Nairovirus M RNA: Nucleotide sequence and coding strategy. *Virology* **190,** 606–615.

Page, R. D. M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12,** 357–358.

Rodriguez, L. L., Maupin, G. O., Ksiazek, T. G., Rollin, P. E., Khan, A. S., Schwarz, T. F., Lofts, R. S., Smith, J. F., Noor, A. M., Peters, C. J., and Nichol, S. T. (1997). Molecular investigation of a multisource outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates. *Am. J. Trop. Med. Hyg.* **57,** 512–518.

Schmaljohn, C. (1996). Bunyaviridae: The viruses and their replication. *In* "Fields Virology" (B. Fields, D. Knipe, and P. Howley, Eds.), pp. 1447–1471. Lippincott-Raven, Philadelphia, PA.

Schmaljohn, C. S., Parker, M. D., Ennis, W. H., Dalrymple, J. M., Collett, M. S., Suzich, J. A., and Schmaljohn, A. L. (1989). Baculovirus expression of the M genome segment of Rift Valley fever virus and examination of antigenic and immunogenic properties of the expressed proteins. *Virology* **170,** 184–192.

Schwarz, T. F., Nitschko, H., Jager, G., Nsanze, H., Longson, M., Pugh, R. N., and Abraham, A. K. (1996). Crimean-Congo haemorrhagic fever in Oman. *Am. J. Trop. Med. Hyg.* **54,** 49–53.

Schwarz, T. F., Nsanze, H., and Ameen, A. M. (1997). Clinical features of Crimean-Congo haemorrhagic fever in the United Arab Emirates. *Infection* **25,** 364–367.

Suleiman, M. N., Muscat-Baron, J. M., Harries, J. R., Satti, A. G., Platt, G. S., Bowen, E. T., and Simpson, D. I. (1980). Congo/Crimean haemorrhagic fever in Dubai. An outbreak at the Rashid Hospital. *Lancet* **2,** 939–941.

Suzich, J. A., Kakach, L. T., and Collett, M. S. (1990). Expression strategy of a phlebovirus: Biogenesis of proteins from the Rift Valley fever virus M segment. *J. Virol.* **64,** 1549–1555.

Swanepoel, R., Shepherd, A. J., Leman, P. A., Shepherd, S. P., and Miller, G. B. (1985). A common-source outbreak of Crimean-Congo haemorrhagic fever on a dairy farm. *S. Afr. Med. J.* **68,** 635–637.

Yen, Y. C., Kong, L. X., Lee, L., Zhang, Y. Q., Li, F., Cai, B. J., and Gao, S. Y. (1985). Characteristics of Crimean-Congo hemorrhagic fever virus (Xinjiang strain) in China. *Am. J. Trop. Med. Hyg.* **34,** 1179–1182.

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Immunofluorescence Technique Using HeLa Cells Expressing Recombinant Nucleoprotein for Detection of Immunoglobulin G Antibodies to Crimean-Congo Hemorrhagic Fever Virus

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A HeLa cell line continuously expressing recombinant nucleoprotein (rNP) of the Crimean-Congo hemorrhagic fever virus (CCHFV) was established by transfection with an expression vector containing the cDNA of CCHFV NP (pKS336-CCHFV-NP). These cells were used as antigens for indirect immunofluorescence (IF) to detect immunoglobulin G antibodies to CCHFV. The sensitivity and specificity of this IF technique were examined by using serum samples and were compared to those of the IF technique using CCHFV-infected Vero E6 cells (authentic antigen). Staining of the CCHFV rNP expressed in HeLa cells showed a unique granular pattern similar to that of CCHFV-infected Vero E6 cells. Positive staining could easily be distinguished from a negative result. All 13 serum samples determined to be positive by using the authentic antigen were also determined to be positive by using CCHFV rNP-expressing HeLa cells (recombinant antigen). The 108 serum samples determined to be negative by using the authentic antigen were also determined to be negative by using the recombinant antigen. Thus, both the sensitivity and the specificity of this IF technique were 100% compared to the IF with authentic antigen. The novel IF technique using CCHFV rNP-expressing HeLa cells can be used not only for diagnosis of CCHF but also for epidemiological studies on CCHFV infections.

Crimean-Congo hemorrhagic fever virus (CCHFV) is a member of the genus *Nairovirus* in the family *Bunyaviridae* and is the causative agent of a severe hemorrhagic fever known as Crimean-Congo hemorrhagic fever (CCHF). The mortality rate of CCHF is as high as 50% in humans (8). CCHFV is prevalent from Africa through to the western part of China, including Eastern European and Middle Eastern countries (9). CCHFV is a tick-borne virus, and wild and domestic animals including sheep, cattle, goats, and ostriches are the reservoirs for zoonoses (8). The virus can be transmitted to humans either by bites of ixodid ticks (genus *Hyalomma*) or by direct contact with blood and tissues from viremic livestock (6, 21, 22; J. F. Saluzzo, J. P. Digoutte, M. Cornet, D. Baudon, J. Roux, and V. Robert, Letter, Lancet i:1179, 1984). The ticks are reservoirs and vectors for livestock, wild animals, and humans. CCHFV can also be spread from CCHF patients to other persons, resulting in nosocomial outbreaks (2, 7, 20, 25). Outbreaks of CCHF among shepherds, agricultural workers, and abattoir workers have often been reported (11, 21); therefore, these occupations present a high risk of infection in areas of CCHF endemicity. Because of the high mortality rate of CCHF and the person-to-person transmission, early diagnosis of suspected cases and use of appropriate barrier measures by health care workers are necessary for the prevention of nosocomial infections.

A CCHF epidemic in the United Arab Emirates was reported to have been caused by CCHFV introduced from Somalia and Nigeria through imported livestock and ticks (17). Furthermore, it is possible that a traveler with CCHF could, during the virus's incubation period, visit areas previously known to be free of CCHF. Therefore, development of diagnostic methods for CCHFV infections is necessary even in countries hitherto free from outbreaks of CCHF.

Because of its virulence to humans, CCHFV should be handled in a high-containment laboratory. This restriction makes it difficult to prepare diagnostic methods using live CCHFV. In the present study, we developed an indirect immunofluorescence (IF) technique using HeLa cells stably expressing recombinant nucleoprotein (CCHFV rNP) to detect specific immunoglobulin G (IgG). This new IF technique is useful both for the diagnosis of CCHF and for seroepidemiological studies on CCHFV infections.

MATERIALS AND METHODS

Virus and cell lines. CCHFV (Chinese strain 66019) isolated from a patient with CCHF in the western part of the Xinjiang Autonomous Region in 1966 (26) was used in this study. The S-segment RNA gene of strain 66019 has been deposited in GenBank under accession no. AJ010648. Vero E6 and HeLa 229 cell lines were purchased from the American Type Culture Collection and used. These cells were cultured in Dulbecco's minimum essential medium (DMEM; Life Technologies, Grand Island, N.Y.) containing 5% heat-inactivated fetal bovine serum and antibiotics such as penicillin and streptomycin (DMEM-5FBS).

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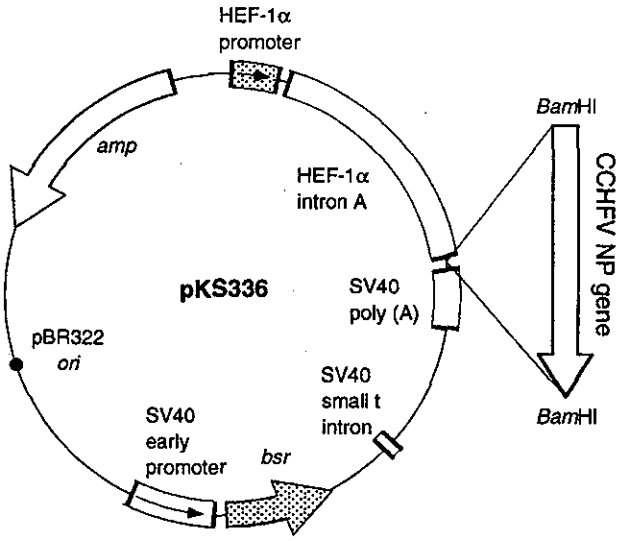

FIG. 1. Structure of pKS336-CCHFV-NP. The gene, a cDNA of the NP gene of CCHFV strain 8402, was inserted into the *Bam*HI restriction site within the multicloning site.

Sera. Twenty-five serum samples were collected from donors with or without a history of CCHF in the Xinjiang Autonomous Region (26), an area of CCHF endemicity in the People's Republic of China. Ninety-six serum samples collected from healthy Japanese adults were used as controls. Further, a monkey (*Macaca fascicularis*) was immunized with purified CCHFV rNP by using an adjuvant (Inject Alum; Pierce, Rockford, Ill.), and the serum was collected and used as a positive control. The monkey serum collected before the immunization was used as a negative control.

CCHFV NP expression vector. The pKS336 expression vector was constructed from the pSV2 *bsr* and pEF321 β-T plasmids (12, 13). A map of this vector is shown in Fig. 1. The nucleotide sequence of the vector is available in GenBank under accession no. AF403737. The blasticidin S deaminase (*bsr*) gene was under the control of the simian virus 40 early promoter. The DNA of interest was under the control of the human elongation factor-1α (HEF-1α) gene promoter.

The cDNA encoding the nucleoprotein (NP) was amplified by reverse transcription-PCR from the cDNA of the S segment of the Chinese CCHFV strain 8402 (accession no. AJ010649), with the designed primer set CCHF F/Bam (5′-GT GCT GGA TCC ATG GAG AAT AAA ATC-3′) and CCHF R/Bam (5′-C GGA TCC TCA GAT GAT GTT GGC AC-3′), in order to add the *Bam*HI restriction site (underlined) to both ends. PCR conditions were as follows: 5 cycles of denaturing at 94°C for 40 s, annealing at 40°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min; 15 cycles of denaturing at 94°C for 40 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min; and an additional extension at 72°C for 5 min. The PCR product was purified, digested with *Bam*HI, and subcloned into the *Bam*HI site of the pKS336 vector (Fig. 1). The CCHFV NP gene inserted into pKS336 (pKS336-CCHFV-NP) was confirmed to be in the correct orientation to the promoter, sequenced by using appropriate primers with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, Calif.), and confirmed to be identical to the original sequence.

Transfection. HeLa cells were transfected with pKS336-CCHFV-NP using the FuGENE 6 Transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The cells transfected with the plasmid were selected with 3 μg of blasticidin *S*-hydrochloride/ml in DMEM-5FBS. HeLa cell clones were analyzed for expression of CCHFV rNP by indirect IF using the monkey serum raised against CCHFV rNP. Cells expressing CCHFV rNP were subcloned and used as IF antigens.

Antigens. Vero E6 cells either infected with CCHFV (strain 66019) or mock infected were cultured for 4 days. CCHFV-and mock-infected cells were then harvested by trypsinization and washed with phosphate-buffered saline (PBS). The cells were spotted onto 14-well HT-coated slide glasses (AR Brown Co., Ltd., Tokyo, Japan) and fixed with acetone at room temperature for 5 min. CCHFV antigen-negative slides were prepared as a negative control for IF antigen. IF antigen slides were stored at −70°C until use. These IF antigens (CCHFV slides) were used as the authentic CCHFV antigens. CCHFV slides were prepared according to the regulations of the Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine.

Recombinant IF antigens were prepared as follows. HeLa cells expressing CCHFV rNP were mixed with parent HeLa cells at a ratio of 1:3 and washed with PBS. The cells were then spotted onto 14-well slide glasses, air dried, and fixed with acetone at room temperature for 5 min. CCHFV rNP slides were stored at −70°C until use. Antigen-negative slides were prepared similarly by using HeLa cells and were used as the control antigen for the IF test. These IF slides were thawed and dried immediately prior to use.

IF test. Human serum samples and monkey control serum samples were twofold serially diluted from 1:20 to 1:1,280 and placed on both CCHFV slides and CCHFV rNP slides. The slides were incubated under humidified conditions at 37°C for 1 h. After the slides were washed with PBS, the antigens on the slides were treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody (Zymed Laboratories, San Francisco, Calif.) at a dilution of 1:70. The FITC-conjugated anti-human IgG antibody was used not only for the human serum samples but also for the monkey control sera. After another wash with PBS, the slides were examined for staining patterns under a fluorescent microscope (Olympus, Tokyo, Japan) with an appropriate barrier and excitation filters for FITC visualization. CCHFV antibody-positive and -negative controls were included in each assay. The titers of tested samples were recorded as the reciprocals of the highest dilutions producing positive staining.

Statistical analysis. The differences in the antibody titers determined by the IF techniques using authentic and recombinant antigens were compared by the Wilcoxon signed-rank test. The relationship between the antibody titers determined by these two methods was evaluated by Spearman's rank correlation coefficient.

RESULTS

Immunofluorescence of CCHFV rNP-expressing HeLa cells. Positive-control monkey sera showed IF staining as a granular pattern, while negative-control sera showed no specific staining (data not shown). The antibody titers of the positive-control monkey serum determined by the IF techniques with recombinant and authentic antigens were both 160. Positive IF staining of CCHFV rNP antigens expressed in HeLa cells with a single CCHF patient serum sample is shown in Fig. 2a. CCHFV rNP was stained as granular aggregates (Fig. 2a), while control cells showed no staining (data not shown). The staining pattern of authentic virus antigens in Vero E6 cells with the same human serum sample was similar to that of CCHFV rNP in HeLa cells (Fig. 2b), while control cells failed to show any specific staining (data not shown).

Sensitivity and specificity of IF staining of CCHFV rNP in human serum samples. The sensitivity and specificity of the IF technique using CCHFV rNP slides were evaluated in comparison with those of the IF technique using authentic CCHFV antigens. Thirteen of 25 serum samples collected in an area of CCHF endemicity in the Xinjiang Autonomous Region showed a positive reaction to the IF technique using authentic CCHFV slides, while 12 serum samples showed a negative reaction. Ninety-six Japanese serum samples were also determined to be negative for CCHFV. All 13 serum samples determined to be CCHFV IgG antibody positive with authentic CCHFV slides also showed a positive reaction with CCHFV rNP slides. Furthermore, all 108 serum samples (comprising 12 Chinese and 96 Japanese samples) determined to be CCHFV IgG negative with the authentic CCHFV slides also showed a negative reaction with CCHFV rNP slides. These results indicate that both the sensitivity and the specificity of the IF technique using CCHFV rNP slides were 100%, compared to the IF technique using CCHFV slides.

The titers determined by the IF technique with CCHFV rNP

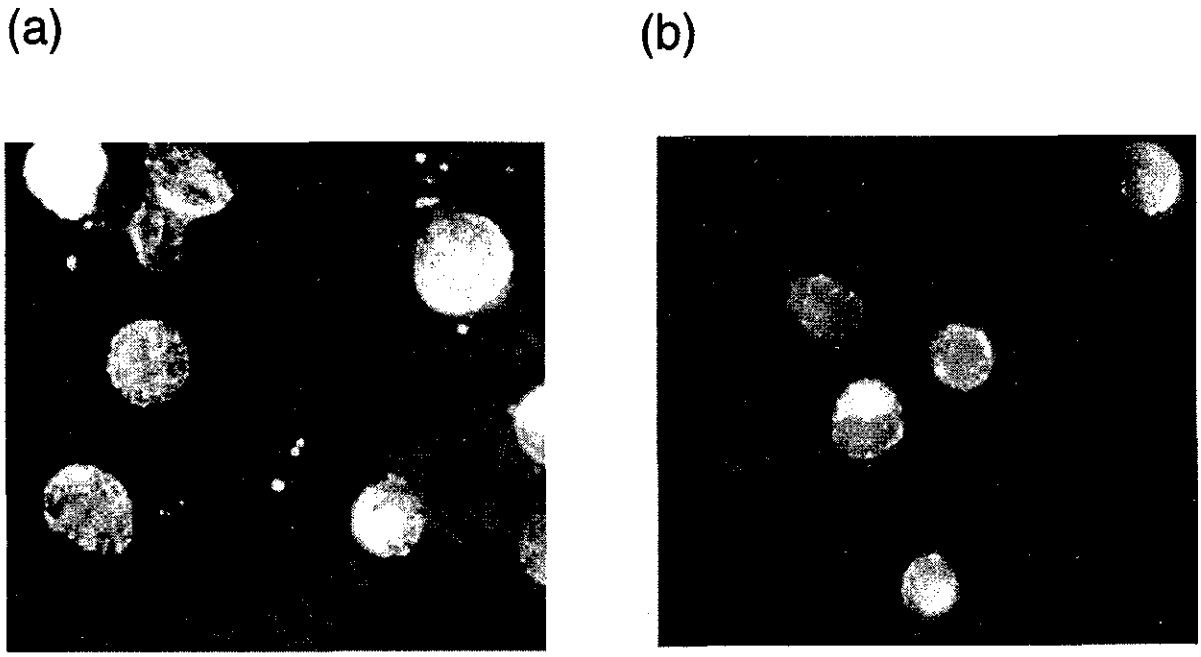

FIG. 2. Positive IF staining of CCHFV rNP-expressing HeLa cells (a) and CCHFV-infected Vero E6 cells (b) with a CCHF antibody-positive serum sample.

slides were positively correlated with those determined by the IF technique with authentic antigens ($P < 0.01$) (Fig. 3). The titers determined using CCHFV rNP slides were statistically significant at the same level as those determined using CCHFV slides ($P = 0.07$).

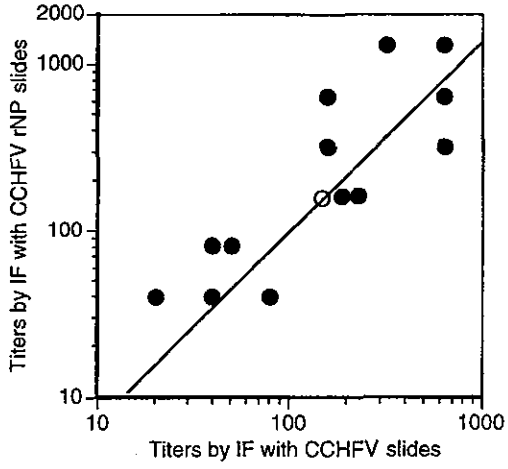

FIG. 3. Relationship between the titers of IgG antibody to CCHFV determined by IF using CCHFV slides and those determined by IF using CCHFV rNP slides. Serum from the monkey, which was immunized with the purified CCHFV rNP, was also tested by IF using authentic and recombinant antigens. The titers of the positive-control monkey serum sample (○) and the 13 CCHFV antibody-positive human serum samples (●) were plotted. Each data point represents one serum sample.

DISCUSSION

We established a HeLa cell line continuously expressing CCHFV rNP by using a novel vector, pKS336. The cells expressed CCHFV rNP in the cytoplasm in granular aggregate form, which was indistinguishable from that of CCHFV-infected Vero E6 cells (Fig. 2). The IF technique using these CCHFV rNP-expressing cells was highly sensitive and specific for the detection of IgG antibodies to CCHFV.

CCHFV-infected cells, e.g., Vero cells, or mouse brain cells have been mainly used as antigens for the detection of IgG to CCHFV (1, 5, 10, 16, 24). In one study, a CCHFV rNP was used for the detection of IgG antibodies to CCHFV (15). The investigators used CCHFV rNP derived from a European strain of CCHFV (AP92; GenBank accession no. U04958) as an antigen for an enzyme-linked immunosorbent assay (ELISA) and proved that CCHFV rNP was efficacious in detecting CCHFV antibodies in the ELISA. The amino acid homology of CCHFV rNP from strain 8401 with that from strain AP92 was 91.9%. It was revealed that the antibodies to Dugbe and Hazara viruses, related nairoviruses, did not cross-react with CCHFV rNP in the ELISA (15). Therefore, CCHFV rNP in HeLa cells seemed not to cross-react with the antibodies to these viruses in IF, although further study is needed. The sensitivity and/or specificity of the ELISA using CCHFV rNP was not evaluated in that report (15). In this paper, we confirmed the efficacy of CCHFV rNP as an antigen and also clarified the sensitivity and specificity of the IF with CCHFV rNP in comparison to the IF with authentic CCHFV antigen in detecting specific CCHFV antibodies.

It has been reported that IgG antibodies to CCHFV can be detected within 9 days in all patients with CCHF (3). IgG

antibodies to CCHFV were also demonstrated by the IF method within 8 days for two CCHF patients who were not treated with anti-CCHFV serum (19). The IgG and IgM antibodies to CCHFV were not detected in sera from patients with CCHF within the first 3 days from onset (3). Based on these results, we can diagnose a patient as having CCHF by detecting a significant rise in the titers of IgG antibody to CCHFV in serum samples obtained on the 9th day or later and within the first 3 days from onset. However, the IgG antibody response was weak or was not detected in fatal CCHF cases (3, 19). In this regard, antigen detection (14, 23), virus isolation, and reverse transcription-PCR (4, 17, 18) are also necessary for the rapid and accurate diagnosis of CCHF.

In summary, we established an efficacious IF technique using HeLa cells expressing CCHFV rNP for the diagnosis of CCHF and for seroepidemiological studies on CCHFV infections.

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REFERENCES

1. **Al Tikiriti, S. K., F. K. Hassan, I. M. Moslih, F. Jurji, M. I. A. Mahmud, and H. H. Tantawi.** 1981. Congo/Crimean haemorrhagic fever in Iraq: a seroepidemiological survey. J. Trop. Med. Hyg. 84:117–120.
2. **Burney, M. I., A. Ghafoor, M. Saleen, P. A. Webb, and J. Casals.** 1980. Nosocomial outbreak of viral hemorrhagic fever caused by Crimean hemorrhagic fever-Congo virus in Pakistan, January 1976. Am. J. Trop. Med. Hyg. 29:941–947.
3. ***Burt, F. J., P. A. Leman, J. C. Abbott, and R. Swanepoel.*** 1994. Serodiagnosis of Crimean-Congo haemorrhagic fever. *Epidemiol. Infect. 113:*551–562.
4. **Burt, F. J., P. A. Leman, J. F. Smith, and R. Swanepoel.** 1998. The use of a reverse transcription-polymerase chain reaction for the detection of viral nucleic acid in the diagnosis of Crimean-Congo haemorrhagic fever. J. Virol. Methods 70:129–137.
5. **Burt, F. J., R. Swanepoel, and L. E. O. Braack.** 1993. Enzyme-linked immunosorbent assays for the detection of antibody to Crimean-Congo haemorrhagic fever virus in the sera of livestock and wild vertebrates. Epidemiol. Infect. 111:547–557.
6. **Chapman, L. E., M. L. Wilson, D. B. Hall, B. LeGuenno, E. A. Dykstra, K. Ba, and S. P. Fisher-Hoch.** 1991. Risk factors for Crimean-Congo hemorrhagic fever in rural northern Senegal. J. Infect. Dis. 164:686–692.
7. **Fisher-Hoch, S. P., J. A. Khan, S. Rehman, S. Mirza, M. Khurshid, and J. B. McCormick.** 1995. Crimean Congo-haemorrhagic fever treated with oral ribavirin. Lancet 346:472–475.
8. **Gonzalez-Scarano, F., and N. Nathanson.** 1996. *Bunyaviridae*, p. 1473–1504. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven, *Philadelphia, Pa.*
9. **Hoogstraal, H.** 1979. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. J. Med. Entomol. 15:307–417.
10. **Johnson, B. K., D. Ocheng, A. Gichogo, M. Okiro, D. Libondo, P. M. Tukei, M. Ho, M. Mugambi, G. L. Timms, and M. French.** 1983. Antibodies against haemorrhagic fever viruses in Kenya populations. Trans. R. Soc. Trop. Med. Hyg. 77:731–733.
11. **Khan, A. S., G. O. Maupin, P. E. Rollin, A. M. Noor, H. H. Shurie, A. G. Shalabi, S. Wasef, Y. M. Haddad, R. Sadek, K. Ijaz, C. J. Peters, and T. G. Ksiazek.** 1997. An outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates, 1994–1995. Am. J. Trop. Med. Hyg. 57:519–525.
12. **Kim, D. W., T. Uetsuki, Y. Kaziro, N. Yamaguchi, and S. Sugano.** 1990. Use of the human elongation factor 1α promoter as a versatile and efficient expression system. Gene 91:217–223.
13. **Kimura, M., A. Takatsuki, and I. Yamaguchi.** 1994. Blasticidin S deaminase gene from *Aspergillus terreus* (BSD): a new drug resistance gene for transfection of mammalian cells. Biochim. Biophys. Acta 22:653–659.
14. **Logan, T. M., K. J. Linthicum, J. R. Moulton, and T. G. Ksiazek.** 1993. Antigen-capture enzyme-linked immunosorbent assay for detection and quantification of Crimean-Congo hemorrhagic fever virus in the tick, *Hyalomma truncatum*. J. Virol. Methods 42:33–44.
15. **Marriott, A. C., T. Polyzoni, A. Antoniadis, and P. A. Nuttall.** 1994. Detection of human antibodies to Crimean-Congo haemorrhagic fever virus using expressed viral nucleocapsid protein. J. Gen. Virol. 75:2157–2161.
16. **Morrill, J. C., A. K. Soliman, I. Z. Iman, B. A. M. Botros, M. I. Moussa, and D. M. Watts.** 1990. Serological evidence of Crimean-Congo haemorrhagic fever viral infection among camels imported into Egypt. J. Trop. Med. Hyg. 93:201–204.
17. **Rodriguez, L. L., G. O. Maupin, T. G. Ksiazek, P. E. Rollin, A. S. Khan, T. F. Schwarz, R. S. Lofts, J. F. Smith, A. M. Noor, C. J. Peters, and S. T. Nichol.** 1997. Molecular investigation of a multisource outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates. Am. J. Trop. Med. Hyg. 57:512–518.
18. **Schwarz, T. F., H. Nsanze, M. Longson, H. Nitschko, S. Gilch, H. Shurie, A. Ameen, A. R. M. Zahir, U. G. Acharya, and G. Jager.** 1996. Polymerase chain reaction for diagnosis and identification of distant variants of Crimean-Congo hemorrhagic fever virus in the United Arab Emirates. Am. J. Trop. Med. Hyg. 55:190–196.
19. **Shepherd, A. J., R. Swanepoel, S. P. Shepherd, P. A. Leman, N. K. Blackburn, and A. F. Hallett.** 1985. A nosocomial outbreak of Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part V. Virological and serological observations. S. Afr. Med. J. 68:733–776.
20. **Suleiman, M. N., J. M. Muscat-Baron, J. R. Harries, A. G. Satti, G. S. Platt, E. T. Bowen, and D. I. Simpson.** 1980. Congo/Crimean haemorrhagic fever in Dubai. An outbreak at the Rashid Hospital. Lancet ii:939–941.
21. **Swanepoel, R., A. J. Shepherd, P. A. Leman, and S. P. Shepherd.** 1985. A common-source outbreak of Crimean-Congo haemorrhagic fever on a dairy farm. S. Afr. Med. J. 68:635–637.
22. **Swanepoel, R., A. J. Shepherd, P. A. Leman, and S. P. Shepherd.** 1985. Investigations following initial recognition of Crimean-Congo haemorrhagic fever in South Africa and the diagnosis of 2 further cases. S. Afr. Med. J. 68:638–641.
23. **Swanepoel, R., J. K. Struthers, and G. M. McGillivray.** 1983. Reversed passive haemagglutination and inhibition with Rift Valley fever and Crimean-Congo hemorrhagic fever viruses. Am. J. Trop. Med. Hyg. 32:610–617.
24. **Swanepoel, R., J. K. Struthers, A. J. Shepherd, G. M. McGillivray, M. J. Nel, and P. G. Jupp.** 1985. Crimean-Congo hemorrhagic fever in South Africa. Am. J. Trop. Med. Hyg. 32:1407–1415.
25. **van Eeden, P. J., J. R. Joubert, B. W. van de Wal, J. L. King, A. de Kock, and J. H. Groenewald.** 1985. A nosocomial outbreak of viral hemorrhagic fever caused by Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part I. Clinical features. S. Afr. Med. J. 68:711–717.
26. **Yen, Y. C., L. X. Kong, L. Lee, Y. Q. Zhang, F. Li, B. J. Cai, and S. Y. Gao.** 1985. Characteristics of Crimean-Congo hemorrhagic fever virus (Xinjiang strain) in China. Am. J. Trop. Med. Hyg. 34:1179–1182.

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Recombinant Nucleoprotein-Based Enzyme-Linked Immunosorbent Assay for Detection of Immunoglobulin G Antibodies to Crimean-Congo Hemorrhagic Fever Virus

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The full-length nucleoprotein of Crimean-Congo hemorrhagic fever virus (CCHFV; 482 amino acid residues) was expressed as a His-tagged recombinant protein (His-CCHFV rNP) in the baculovirus system. The His-CCHFV rNP was efficiently expressed in insect cells and purified by Ni^{2+} column chromatography. Using this substrate, an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) was developed. We evaluated the sensitivity and specificity of the IgG ELISA, using serum samples previously determined to be antibody positive or negative by immunofluorescence tests on CCHFV-infected Vero E6 cells. We found very good correlation between the two tests: 87% for the positive sera (13 of 15) and 99% for the negative sera (107 of 108). These results indicate that the new IgG ELISA using His-CCHFV rNP has high sensitivity and specificity for detecting CCHFV antibodies. The CCHF patients' sera with high titers reacted only with the NP fragment containing amino acid residues between 201 and 306 in Western blotting. It is known that amino acid homologies are high in this region among various isolates. Thus, it is expected that this ELISA can detect antibodies not only for Chinese strains of CCHFV but also for other strains circulating in the world. These results suggest that the IgG ELISA system developed with the recombinant CCHFV NP is a valuable tool for diagnosis and epidemiological investigations of CCHFV infections.

Crimean-Congo hemorrhagic fever virus (CCHFV) belongs to the family *Bunyaviridae* (genus *Nairovirus*) and causes severe hemorrhagic symptoms in humans (7). CCHF is distributed worldwide with the exception of the American continents. There have been many outbreaks of CCHF in countries from South Africa to the western part of China, including Eastern European and Middle Eastern countries (8). CCHFV is a tick-borne virus which is transmitted by ticks from the genus *Hyalomma* (7). Humans are usually infected with CCHFV either through the bites of infected ticks or by direct contact with virus-contaminated tissues or blood. CCHF outbreaks have been reported among agricultural workers, abattoir workers, and shepherds who handle livestock animals such as sheep, goats, and ostriches (10, 21). Furthermore, nosocomial, or in-house, CCHF infections have also been reported among caregivers (2, 6, 20, 23).

It was reported that the epidemic of CCHF in the United Arab Emirates was caused by imported livestock and ticks from Somalia and Nigeria (17). Although there has been no definite evidence that CCHFV is imported from an outbreak area to CCHFV-free countries through CCHFV-infected humans, it is possible that the virus could be introduced to outbreak-free areas through CCHFV-infected ticks, humans, and animals.

In the present study, we developed an enzyme-linked immunosorbent assay (ELISA) to detect CCHFV-directed immunoglobulin G (IgG) by using the recombinant nucleoprotein (rNP). We demonstrated that this new ELISA system has high sensitivity and specificity in detecting CCHFV antibody in human sera in comparison to the indirect immunofluorescence (IIF) method using authentic viral antigen. The results suggest the usefulness of this IgG ELISA for serological diagnosis and epidemiological studies of CCHFV infections.

MATERIALS AND METHODS

Cells and viruses. The Vero E6 cell line was purchased from the American Type Culture Collection and cultured in Eagle's minimum essential medium containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin). Tn5 insect cells were also used for the expression of CCHFV rNP in a baculovirus system. Tn5 insect cells were cultured in TC-100 (Life Technologies, Rockville, Md.) supplemented with 10% fetal bovine serum, 2% tryptose phosphate broth (Becton Dickinson Microbiology Systems, Sparks, Md.), and kanamycin. CCHFV (Chinese strain 66019) isolated from a patient with CCHF in the western part of the Xinjiang Autonomous Region, People's Republic of China, in 1966 was used in the study (24).

Sera. Twenty-five serum samples were collected from human subjects in the area where CCHF is endemic, the western part of the Xinjiang Autonomous Region. Two serum samples collected from patients with CCHF in the convalescent phase were provided to us by T. G. Ksiazek, Special Pathogens Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC), Atlanta, Ga. Ninety-six serum samples collected from Japanese volunteers who had no past history of travel to the area where CCHF is endemic were used as controls. An anti-CCHFV rNP polyclonal rabbit serum was raised in a rabbit previously immunized with purified CCHFV rNP in the form of a

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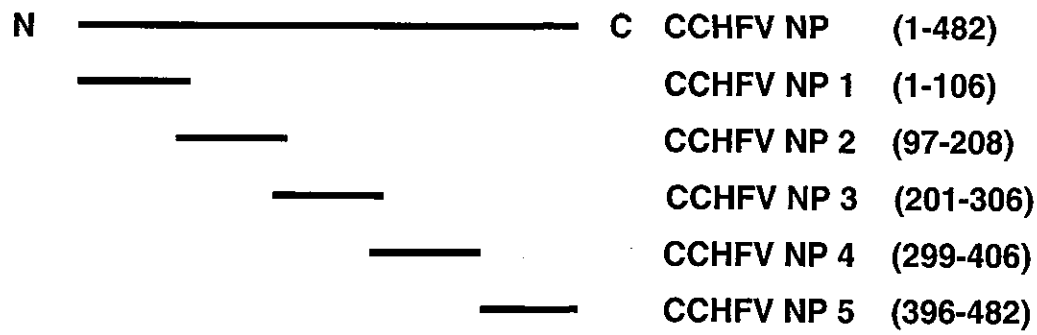

FIG. 1. The entire NP and truncated NP fragments of CCHFV. The truncated NP fragments were expressed as fusion proteins with GST on the N-terminal side. The designations and amino acid positions of the truncated NP fragments are indicated.

mixture with adjuvant (Inject Alum; Pierce, Rockford, Ill.). Further, a monkey (*Macaca fascicularis*) was immunized with purified CCHFV rNP using the adjuvant Inject Alum, and serum was collected and used as a positive control. The monkey serum collected before immunization was used as a negative control. The method of expression and purification of CCHFV rNP is described below. A monoclonal antibody directed against glutathione *S*-transferase (GST) protein was generated by fusing the spleen cells collected from BALB/c mice immunized with purified GST-tagged protein with mouse myeloma cells (the P3/Ag568 cell line) and was used in these experiments.

Recombinant transfer vector. In order to construct our transfer vector, an entire cDNA clone of NP from CCHFV Chinese strain 8402 was used. The DNA of CCHFV NP was amplified by PCR from the source using primers CCHF-NP/F(HI) (5'-GTGCTGGATCCATGGAGAATAAAATCG-3') and CCHF-NP/R(HI) (5'-CGGATCCTCAGATGATGTTGGCACTG-3') (the *Bam*HI restriction sites are underlined in both sequences). The PCR conditions were 5 cycles of denaturation at 94°C for 40 s, annealing at 40°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min; 15 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min; and additional extension at 72°C for 5 min. The amplified DNA (1.6 kbp) was digested with *Bam*HI and subcloned into the *Bam*HI site of pQE30 vector DNA (QIAGEN GmbH, Hilden, Germany) to construct pQE30-CCHFV NP. The inserted CCHFV NP DNA was sequenced using appropriate primers with ABI PRISMTM 310 genetic analyzer (PE Applied Biosystems, Foster City, Calif.) and was confirmed to be in proper orientation to the promoter and identical to the original sequence. The DNA fragment of CCHFV NP with a histidine (His) tag was isolated from the plasmid pQE30-CCHFV NP by digestion with *Eco*RI and *Sal*I. Then, it was repaired for blunting with Klenow enzyme and ligated into the blunt-ended cloning site of pAcYM1 (15). The resultant recombinant transfer vector with the correct orientation to the promoter was designated pAcYM1-His-CCHFV NP.

Generation of recombinant baculovirus. Tn5 insect cells were transfected with mixtures of purified *Autographa californica* nuclear polyhedrosis virus (*Ac*MNPV) DNA and recombinant pAcYM1-His-CCHFV NP following the procedures described by Kitts et al. (11) with the modification of Matsuura et al. (15). Recombinant baculovirus was isolated. The baculovirus, which expresses a His-tagged recombinant NP of CCHFV (His-CCHFV rNP), was designated *Ac*-His-CCHFV NP. A baculovirus (*Ac*-ΔP) which lacks polyhedrin expression was used as a control virus.

Expression of His-CCHFV NP. *Ac*-His-CCHFV NP-infected Tn5 cells were incubated at 26°C for 72 h. Then, the cells were washed twice with cold phosphate-buffered saline (PBS) solution and lysed in cold PBS solution containing 1% Nonidet P-40 (NP-40). The cell lysate was centrifuged at 13,000 × *g* at 4°C for 10 min. The supernatant fraction was collected as a source of His-CCHFV NP for purification. The His-CCHFV rNP was purified using an Ni^{2+} resin purification system (QIAGEN GmbH) according to the manufacturer's instructions. Expression of His-CCHFV rNP was analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (12% polyacrylamide) stained with Coomassie blue.

Expression of truncated NPs. In order to determine the antigenic regions within the NP, we expressed overlapping fragments of NP (Fig. 1). The DNA corresponding to each of the truncated NP fragments was amplified with the designed primer sets. The amplified DNA was subcloned into the *Bam*HI and *Eco*RI cloning sites of plasmid pGEX-2T (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Each insert was sequenced and confirmed to be in a correct frame and identical to the original sequence. The GST-tagged NP fragments were expressed in an *Escherichia coli* (BL21) system.

Western blotting. His-CCHFV rNP and the recombinant NP fragments were tested for reactivity to the serum samples by Western blotting. *E. coli* (BL21) cell lines expressing each of the NP fragments were resuspended in an appropriate amount of PBS solution and sonicated at full power for 1 min. The sonicated cell suspensions and His-CCHFV rNP were then adjusted to 1× SDS sample buffer (0.0625 M Tris [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue) and boiled for 10 min. Equal quantities of samples were loaded onto an SDS–12% PAGE gel (4% stacking gel). The protein bands were then electroblotted onto nitrocellulose membrane. The blots were incubated in PBS containing 0.05% Tween-20 (TPBS) and 5% skim milk (M-TPBS) at room temperature for 1 h to block nonspecific sites. The blots were incubated with CCHF patients' sera (1:100 dilution), with the rabbit serum to His-CCHFV rNP (1:500 dilution), or with the monoclonal antibody to GST protein for 1 h at room temperature. After being washed in TPBS, the membrane was incubated with secondary antibodies (horseradish peroxidase [HRPO]-conjugated goat anti-human IgG antibody, HRPO-conjugated goat anti-rabbit IgG, and HRPO-conjugated goat anti-mouse IgG [1:1,000 dilution]; ZYMED Laboratories, San Francisco, Calif.). Antigen levels were visualized by reaction with POD immunostain set (Wako BioProducts, Inc., Tokyo, Japan).

ELISA. ELISA was performed as previously described except for the antigen preparation (12, 18). Briefly, ELISA plates were coated with the predetermined optimal quantity of purified His-CCHFV rNP (approximately 100 ng/well) at 4°C overnight. Then, each well of the plates was inoculated with 200 μl of M-TPBS and incubated for 1 h for blocking. The plates were washed three times with TPBS and then inoculated with the test samples (100 μl/well), which were diluted fourfold from 1:100 to 1:6,400 with M-TPBS. After a 1-h incubation period, the plates were washed three times with TPBS, and then the plates were inoculated with goat anti-human IgG antibody labeled with HRPO (1:1,000 dilution; ZYMED Laboratories). After a 1-h incubation period, the plates were washed and 100 μl of ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany) was added to each well. The plates were incubated for 30 min at 37°C, and the optical density (OD) was measured at 405 nm with reference at 490 nm. The adjusted OD was calculated by subtracting the OD of the noncoated wells from that of the corresponding wells. The mean and standard deviation (SD) were calculated from those of 96 control sera. The cutoff value for the assay was defined as the mean plus 3 SD.

Positive- and negative-control monkey sera were tested in each ELISA for verification.

IIF. The antibody titer to CCHFV was determined by the IIF method, using CCHFV (strain 66019)-infected Vero E6 cells. Briefly, Vero E6 cells infected with CCHFV were washed with PBS and air dried on 14-well HT-coated slide glasses (AR Brown Co., Ltd., Tokyo, Japan). The cells were then fixed in cold acetone for 10 min and used as antigens. Serum samples were serially twofold diluted with PBS from 1:20 to 1:640. The diluted samples were put on the antigens and incubated for 1 h at 37°C under humidified conditions. The slides were washed with PBS and inoculated with fluorescein isothionate-labeled goat anti-human IgG antibody (1:70 dilution; ZYMED Laboratories). After the slides were washed with PBS, fluorescein isothionate signal was observed under an immunofluorescent microscope (Olympus, Tokyo, Japan).

Positive- and negative-control monkey sera were also tested by the IIF method in each IIF test for verification.

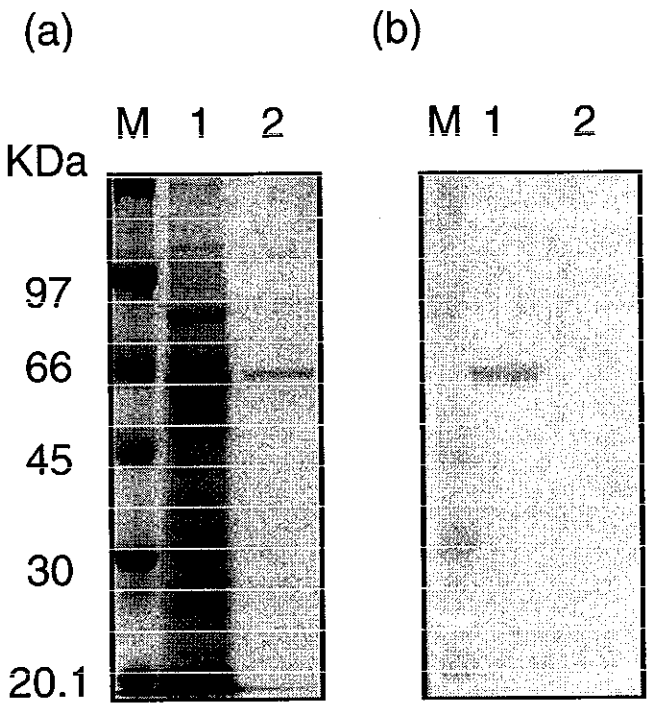

FIG. 2. SDS-PAGE (a) and Western blot (b) analyses of the expression of His-CCHFV NP in insect cells upon infection with *Ac*-His-CCHFV rNP and purified His-CCHFV rNP. (a) Crude material from the supernatant fraction of 1% NP-40–PBS lysate (lane 1) and purified His-CCHFV rNP (lane 2) are shown. M, molecular mass markers. (b) His-CCHFV rNP in 1% NP-40–PBS lysate was stained by Western blotting with CCHFV antibody-positive human serum (lane 1), while nothing in 1% NP-40–PBS lysate of *Ac*-ΔP-infected control insect cells was stained with the same serum (lane 2).

Statistical analysis. The antibody titers determined by the IIF test and the OD values determined by IgG ELISA with His-CCHFV rNP were compared by Spearman's correlation coefficient by rank.

Nucleotide sequence accession number. The complete nucleotide sequence of the Crimean-Congo hemorrhagic fever virus (8402 strain) NP gene used in this paper is registered in GenBank under accession no. AJ010649.

RESULTS

Expression and purification of His-CCHFV rNP. The expression of His-CCHFV rNP in *Ac*-His-CCHFV NP-infected insect cells was demonstrated by SDS-PAGE analysis. The His-CCHFV rNP was purified almost to homogeneity, as shown in Fig. 2a. The His-CCHFV rNP was specifically detected by Western blotting with CCHF patient's serum, indicating that the protein band visualized in Fig. 2a (lane 2) is the expressed recombinant His-CCHFV rNP with the antigenicity of the rNP of CCHFV (Fig. 2b).

Sensitivity and specificity of IgG ELISA with His-CCHFV rNP. The positive-control monkey sera showed positive reactions in an IIF test with a titer of 160, while the negative-control monkey serum showed a negative reaction. The OD value of the positive-control monkey serum by ELISA with His-CCHFV rNP was 0.885 at 1:400 dilution. Using 123 sera, this IgG ELISA with His-CCHFV rNP was tested for sensitivity and specificity compared with those of an IIF test with authentic CCHFV antigen. These serum samples had been determined to be CCHFV antibody positive or negative by IIF testing with CCHFV-infected Vero E6 cells. Fifteen serum samples consisting of 13 sera collected in the Xinjiang Autonomous Region and the 2 sera from CDC were determined to be positive by IIF testing, and 108 serum samples were determined to be negative by IIF testing.

TABLE 1. Relationship between results of IgG ELISA with His-CCHFV rNP and the IIF method using CCHFV-infected Vero E6 cells

ELISA result	No. of IIF results that were:	
	Positive	Negative
Positive	13	1[a]
Negative	2[b]	107

[a] This serum sample, which showed a negative reaction by the IIF method but a positive reaction by IgG ELISA, was collected from a subject in Xinjiang Autonomous Region, People's Republic of China.
[b] Antibody titers of these two sera to CCHFV were 40 and 80.

The mean and SD obtained using 96 Japanese sera at 1:400 dilution were 0.078 and 0.045, respectively. Therefore, the cut-off value of ODs at 1:400 dilution was determined to be 0.213. Thirteen of the 15 IIF-positive sera and 1 of the 108 IIF-negative sera were determined to be positive by IgG ELISA (Table 1). The 1 serum sample which showed a negative reaction by the IIF test but a positive reaction by IgG ELISA was among the 12 IIF-negative Chinese sera. All the Japanese control sera were determined to be antibody negative by this criterion. The sensitivity and specificity were thus calculated to be 87 and 99%, respectively.

Relationships between OD values determined by IgG ELISA and CCHFV antibody titers determined by the IIF method.

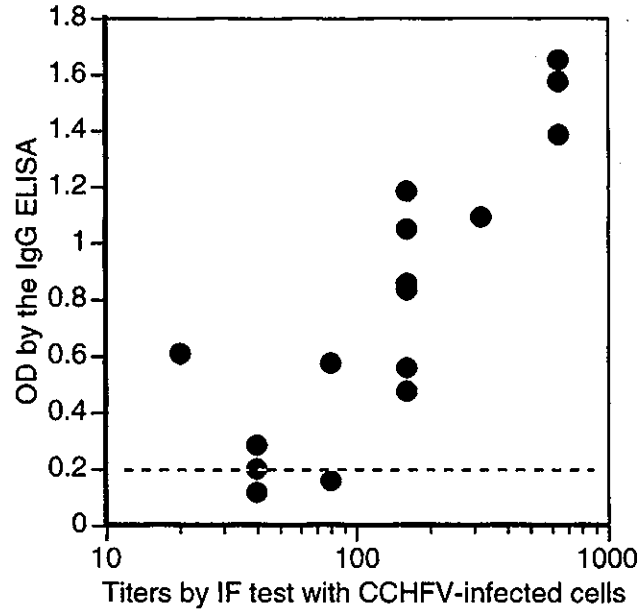

FIG. 3. Relationship between OD values at 1:400 by IgG ELISA with His-CCHFV rNP and antibody titers determined by the IIF method. Fifteen CCHFV antibody-positive sera and one positive-control monkey serum raised against His-CCHFV rNP serum were plotted.

TABLE 2. Reactions of anti-CCHFV serum samples to full-length rNP of CCHFV (His-CCHFV rNP) and truncated NP fragments

Sample	Species	Antibody titer to CCHFV by IIF	Reaction of NP fragment[b]					
			His-CCHFV rNP	GST-CCHFV NP[c]				
				1	2	3	4	5
MAb[a] to GST	Mouse		−	+	+	+	+	+
Anti-His-CCHFV rNP	Rabbit	>640	+	−	−	+	−	−
Serum 1	Human	>640	+	−	−	+	−	−
Serum 2	Human	320	+	−	−	+	−	−
Serum 3	Human	>640	+	−	−	+	−	−
Serum 4	Human	>640	+	−	−	+	−	−

[a] MAb, monoclonal antibody.
[b] Negative (−) and positive (+) reactions of tested sera to full-length or truncated NP fragments in Western blotting.
[c] The numbers 1 to 5 indicate the name of the truncated fragment (Fig. 1).

OD values determined by IgG ELISA were compared with antibody titers determined by the IIF method, using the 15 IIF-positive human sera and 1 positive-control monkey serum (Fig. 3). There was a significant positive correlation between the ODs at 1:400 dilution and the CCHFV antibody titers ($P < 0.01$).

Antigenicities of truncated CCHFV NPs. We attempted to localize the antigenic regions recognized by CCHFV antibodies. We selected four human serum samples, which had high antibody titers to CCHFV, and the rabbit polyclonal serum to His-CCHFV rNP. The full-length CCHFV rNP and the five overlapping truncated NP fragments were examined for antigenicity using these serum samples in Western blotting (Fig. 1). As shown in Table 2, all of the tested sera reacted only to GST-CCHFV NP3. No sera reacted to the other truncated NP fragments. These results indicate that GST-CCHFV NP3, the central region of CCHFV NP, has high antigenicity and suggest that the epitopes recognized by CCHFV antibodies within CCHFV NP are present only in this fragment.

DISCUSSION

Various methods, including ELISA, IIF testing, complement fixation, and reversed passive hemagglutination and inhibition methods, have been used for detecting CCHFV antibodies (1, 3, 5, 9, 16, 22). Nevertheless, all these methods require at one stage that live virus be manipulated, which necessitates the use of biosafety level 4 containment. The CCHFV rNP from CCHFV strain AP92 (GenBank accession no. U04958) expressed in the baculovirus system had been reported to be useful for the detection of specific antibody to the CCHFV NP, but the sensitivity and specificity of the IgG ELISA were not evaluated (14). In the present study, we expressed CCHFV rNP from CCHFV Chinese strain 8402. The amino acid homologies in CCHFV NP and CCHFV NP3 from CCHFV strain 8402 were 91.9 and 92.5%, respectively, with those from CCHFV strain AP92. We confirmed that the CCHFV rNP from a Chinese strain was also useful as an ELISA antigen for detecting specific antibodies in CCHF patients' sera. Furthermore, the sensitivity and specificity of the IgG ELISA using His-CCHFV rNP were determined in comparison to IIF testing with authentic viral antigen in the present study. Marriott and colleagues revealed that the antibodies to Dugbe and Hazara viruses, related nairoviruses, did not cross-react with CCHFV rNP in ELISA (14). Therefore, His-CCHFV rNP is considered not to be cross-reactive with the antibodies to these viruses in ELISA. The advantage of our IgG ELISA system is that the antigen can be prepared in a facility without a biosafety level 4 laboratory. Furthermore, as shown in Fig. 2, His-CCHFV rNP was soluble in 1% NP-40–PBS solution, and the His tag on the N terminus allowed purification of this protein. Based on those characteristics, this ELISA is a very attractive system compared to other methods commonly used worldwide.

The main antigenic region of the NP was located on GST-CCHFV NP3, the central fragment of the NP between amino acids 201 and 306 (Table 2). The amino acid residues in this region were compared among CCHFV isolates, which included Chinese strains (GenBank accession no. AY029157, M86625, AF354296, AF358784, AF362080, AJ010648, and AJ010649) and non-Chinese strains (GenBank accession no. U88410, U88411, U88412, U88413, U88414, U88415, U88416, and U04958). The homologies were between 92 and 100%, indicating that this region is extremely highly conserved. The CCHFV NP3 had 97 to 100% homology to the other Chinese strains and 92 to 96% homology to the non-Chinese strains IbAr 10200, DAK8194, and UGANDA3010. Thus, it is likely that our IgG ELISA prepared with the rNP of CCHFV Chinese strains can detect antibodies not only to CCHFV Chinese strains but also to the other stains circulating in the world.

We believe that IgG ELISA alone is not enough for the accurate diagnosis of CCHF. Detection of IgM specific to CCHFV and CCHFV antigens is also important for early diagnosis of CCHF. Detection of IgM by the IIF method or by IgM capture ELISA using CCHFV rNP is in the process of validation in our laboratories. Virus isolation, CCHFV antigen detection ELISA, and reverse transcription-PCR are also useful methods for the diagnosis of CCHF (4, 13, 17, 19).

In summary, we developed an IgG ELISA system using CCHFV rNP and demonstrated that it has high sensitivity and specificity. Therefore, it is expected that this IgG ELISA will be useful for diagnosis and for seroepidemiological studies of CCHFV infections.

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REFERENCES

1. **Al Tikriti, S. K., F. K. Hassan, I. M. Moslih, F. Jurji, M. I. A. Mahmud, and H. H. Tantawi.** 1981. Congo/Crimean haemorrhagic fever in Iraq: a seroepidemiological survey. J. Trop. Med. Hyg. 84:117–120.
2. **Burney, M. I., A. Ghafoor, M. Saleen, P. A. Webb, and J. Casals.** 1980. Nosocomial outbreak of viral hemorrhagic fever caused by Crimean hemorrhagic fever-Congo virus in Pakistan, January 1976. Am. J. Trop. Med. Hyg. 29:941–947.
3. **Burt, F. J., P. A. Leman, J. C. Abbott, and R. Swanepoel.** 1994. Serodiagnosis of Crimean-Congo haemorrhagic fever. Epidemiol. Infect. 113:551–562.
4. **Burt, F. J., P. A. Leman, J. F. Smith, and R. Swanepoel.** 1998. The use of a

reverse transcription-polymerase chain reaction for the detection of viral nucleic acid in the diagnosis of Crimean-Congo haemorrhagic fever. J. Virol. Methods **70**:129–137.

5. **Burt, F. J., R. Swanepoel, and L. E. O. Braack.** 1993. Enzyme-linked immunosorbent assays for the detection of antibody to Crimean-Congo haemorrhagic fever virus in the sera of livestock and wild vertebrates. Epidemiol. Infect. **111**:547–557.
6. **Fisher-Hoch, S. P., J. A. Khan, S. Rehman, S. Mirza, M. Khurshid, and J. B. McCormick.** 1995. Crimean Congo-haemorrhagic fever treated with oral ribavirin. Lancet **346**:472–475.
7. **Gonzalez-Scarano, F., and N. Nathanson.** 1996. Bunyaviridae, p. 1473–1504. *In* B. Fields, D. M. Knipe, and P. Howley (ed.). Fields virology. 3rd ed. Lippincott-Raven, Philadelphia, Pa.
8. **Hoogstraal, H.** 1979. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. J. Med. Entomol. **15**:307–417.
9. **Johnson, B. K., D. Ocheng, A. Gichogo, M. Okiro, D. Libondo, P. M. Tukei, M. Ho, M. Mugambi, G. L. Timms, and M. French.** 1983. Antibodies against haemorrhagic fever viruses in Kenya populations. Trans. R. Soc. Trop. Med. Hyg. **77**:731–733.
10. **Khan, A. S., G. O. Maupin, P. E. Rollin, A. M. Noor, H. H. Shurie, A. G. Shalabi, S. Wasef, Y. M. Haddad, R. Sadek, K. Ijaz, C. J. Peters, and T. G. Ksiazek.** 1997. An outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates, 1994–1995. Am. J. Trop. Med. Hyg. **57**:519–525.
11. **Kitts, P. A., M. D. Ayres, and R. D. Possee.** 1990. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. Nucleic Acids Res. **18**:5667–5672.
12. **Ksiazek, T. G., C. P. West, P. E. Rollin, P. B. Jahling, and C. J. Peters.** 1999. ELISA for the detection of antibodies to Ebola viruses. J. Infect. Dis. **179**(Suppl. 1):S192-S198.
13. **Logan, T. M., K. J. Linthicum, J. R. Moulton, and T. G. Ksiazek.** 1993. Antigen-capture enzyme-linked immunosorbent assay for detection and quantification of Crimean-Congo hemorrhagic fever virus in the tick Hyalomma truncatum. J. Virol. Methods **42**:33–44.
14. **Marriott, A. C., T. Polyzoni, A. Antoniadis, and P. A. Nuttall.** 1994. Detection of human antibodies to Crimean-Congo haemorrhagic fever virus using expressed viral nucleocapsid protein. J. Gen. Virol. **75**:2157–2161.
15. **Matsuura, Y., R. D. Possee, H. A. Overton, and D. Bishop.** 1987. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. **68**:1233–1250.
16. **Morrill, J. C., A. K. Soliman, I. Z. Iman, B. A. M. Botros, M. I. Moussa, and D. M. Watts.** 1990. Serological evidence of Crimean-Congo haemorrhagic fever viral infection among camels imported into Egypt. J. Trop. Med. Hyg. **93**:201–204.
17. **Rodriguez, L. L., G. O. Maupin, T. G. Ksiazek, P. E. Rollin, A. S. Khan, T. F. Schwarz, R. S. Lofts, J. F. Smith, A. M. Noor, C. J. Peters, and S. T. Nichol.** 1997. Molecular investigation of a multisource outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates. Am. J. Trop. Med. Hyg. **57**:512–518.
18. **Saijo, M., M. Niikura, S. Morikawa, T. G. Ksiazek, R. F. Meyer, C. J. Peters, and I. Kurane.** 2001. Enzyme-linked immunosorbent assays for detection of antibodies to Ebola and Marburg viruses using recombinant nucleoproteins. J. Clin. Microbiol. **39**:1–7.
19. **Schwarz, T. F., H. Nsanze, M. Longson, H. Nitschko, S. Gilch., H. Shurie, A. Ameen, A. R. M. Zahir, U. G. Acharya, and G. Jager.** 1996. Polymerase chain reaction for diagnosis and identification of distant variants of Crimean-Congo hemorrhagic fever virus in the United Arab Emirates. Am. J. Trop. Med. Hyg. **55**:190–196.
20. **Suleiman, M. N., J. M. Muscat-Baron, J. R. Harries, A. G. Satti, G. S. Platt, E. T. Bowen, and D. I. Simpson.** 1980. Congo/Crimean haemorrhagic fever in Dubai. An outbreak at the Rashid Hospital. Lancet **ii**:939–941.
21. **Swanepoel, R., A. J. Shepherd, P. A. Leman, and S. P. Shepherd.** 1985. A common-source outbreak of Crimean-Congo haemorrhagic fever on a dairy farm. S. Afr. Med. J. **68**:635–637.
22. **Swanepoel, R., J. K. Struthers, and G. M. McGillivary.** 1983. Reversed passive hemagglutination and inhibition with Rift Valley fever and Crimean-Congo hemorrhagic fever viruses. Am. J. Trop. Med. Hyg. **32**:610–617.
23. **van Eeden, P. J., J. R. Joubert, B. W. van de Wal, J. L. King, A. de Kock, and J. H. Groenewald.** 1985. A nosocomial outbreak of viral hemorrhagic fever caused by Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part I. Clinical features. S. Afr. Med. J. **68**:711–717.
24. **Yen, Y. C., L. X. Kong, L. Lee, Y. Q. Zhang, F. Li, B. J. Cai, and S. Y. Gao.** 1985. Characteristics of Crimean-Congo hemorrhagic fever virus (Xinjiang strain) in China. Am. J. Trop. Med. Hyg. **34**:1179–1182.

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Histopathology of Natural Ebola Virus Subtype Reston Infection in Cynomolgus Macaques during the Philippine Outbreak in 1996

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Abstract: *We investigated the livers, spleens, kidneys and lungs collected from 24 cynomolgus macaques (Macaca fascicularis) naturally infected with Ebola virus subtype Reston (EBO-R) during the Philippine outbreak in 1996, in order to reveal the histopathologic findings. These macaques showed necrotic hepatocytes with inclusions, slight to massive fibrin deposition in splenic cords, depletion of lymphoid cells in the white pulp of the spleen, and fibrin thrombi in some organs. Immunohistochemical analysis using anti-leukocyte antigen L1 antibody revealed an increase in blood-derived macrophages/ monocytes in the livers, kidneys and lungs of EBO-R infected macaques. EBO-R NP antigens were detected in the macrophages/monocytes, endothelial cells and fibroblasts in the liver, spleen, kidney and lung. These results indicate that EBO-R infection is characterized by systemic coagulopathy and an increase in blood-derived macrophages/ monocytes in accordance with the EBO-R propagation in macrophages/monocytes.*

Key words: *cynomolgus macaque, Ebola virus, Histopathology, subtype Reston*

Introduction

Ebola virus belongs to the family *Filoviridae* and is divided into 4 subtypes: Zaire (EBO-Z), Sudan (EBO-S), Ivory Coast (EBO-IC), and Reston (EBO-R) [12]. EBO-R outbreaks have occurred in 1989 (Virginia, USA), 1990 (Texas, USA), 1992 (Siena, Italy), and 1996 (Texas, USA) among macaques imported from the Phil-

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ippines [7–9, 16, 19, 20, 23, 26, 32]. A high mortality rate among EBO-R-infected cynomolgus macaques was documented in each epidemic; however, there was no illness or fatalities among the serologically confirmed, infected humans [8, 9, 23, 32]. Histopathological findings of cynomolgus macaques naturally infected with EBO-R during the 1989 American outbreak have been reported [11, 15, 19]. EBO-R virions were demonstrated in circulating macrophages/monocytes, interstitial fibroblasts, interstitial macrophages, adipose cells, vascular endothelial cells, hepatocytes and adrenal cortical cells, but rarely in the renal tubules and type-II alveolar epithelial cells in the monkeys infected in the 1989 outbreak [15]. Simian hemorrhagic fever virus (SHFV), belonging to the family *Arteriviridae* was also isolated from some of the macaques during the EBO-R outbreaks [11, 16, 19]. However, the involvement of SHFV in the pathogenesis of EBO-R has not yet been clarified.

Experimental infection of non-human primates [4, 5, 13, 14, 18, 21, 28] and guinea pigs [10, 29] with EBO-Z demonstrated that EBO-Z propagates initially in mononuclear phagocytic system (MPS) cells, such as monocytes and macrophages, and subsequently in hepatocytes, adrenal cortical cells, endothelial cells, and fibroblasts in EBO-Z infected monkeys [28] and guinea pigs [10].

The histopathological findings of EBO-R infected cynomolgus macaques in the Philippine outbreaks have not yet been reported. In this study, we investigated the characteristic light microscopic findings of the livers, spleens, kidneys and lungs from 24 dead or sacrificed cynomolgus macaques during the EBO-R outbreak of 1996 in the Philippines, and demonstrated an increase of blood-derived macrophages/monocytes in EBO-R infected macaques.

Materials and Methods

Animals

The EBO-R epidemic occurred in a monkey breeding and export facility in the Philippines from March to September in 1996 [23, 26]. During the outbreak at this facility, some monkeys died of Ebola virus infection, and some monkeys held in pens with antigen-positive animals were sacrificed to control the spread of the virus.

Tissues from 29 cynomolgus macaques (*Macaca fascicularis*) were histopathologically examined. Twenty-four of the 29 monkeys were infected with EBO-R, while the remaining 5 were uninfected. The EBO-R infection was previously confirmed by detection of the antigen in liver homogenates by antigen-capture enzyme-linked immunosorbent assay (ELISA) at the Research Institute for Tropical Medicine, Philippines [23]. Of the 24 EBO-R-infected macaques, 12 died of the virus infection, and 12 were sacrificed to prevent the viral spread. The available clinical signs of these macaques are shown in Table 1.

Light Microscopy

Tissue samples from the livers, spleens, lungs, and kidneys of the macaques were fixed in 10 % formalin. The tissues were embedded in paraffin, cut into 3-μm sections and stained with either hematoxylin and eosin (HE) or phosphotungstic acid hematoxylin (PTAH).

Immunohistochemistry

The paraffin sections were examined by immunohistochemistry using a commercial kit (VECTASTAIN elite ABC kit: Vector Laboratories, Inc. Burlingame, USA). They were pre-treated in 10 mM citrate buffer at 95°C for 15 min in a microwave (H2500 Microwave Processor, Energy Beam Sciences, Inc. Massachusetts, USA) prior to immunostaining. We used a rabbit polyclonal antibody to EBO-Z recombinant nucleoprotein (NP) [30] to detect EBO-R NP, since the rabbit serum showed considerable cross-reactivity with the EBO-R antigen in the indirect immunofluorescent assay, immunoglobulin G-ELISA and Western blotting (data not shown). We also used a mouse monoclonal antibody to a leukocyte antigen L1 protein (Novocastra Lab, Inc, Newcastle, UK). Biotinylated horse antibody to mouse IgG was used as secondary antibody for the primary antibodies. Then, peroxidase-conjugated streptavidin was reacted. Diaminobenzidine and hydrogen peroxide were used as a substrate for visualization. We counted the number of L1 antigen-positive cells in the liver, medulla of kidney, and lung in 5 fields at 50X magnification, using computer software (the public domain NIH Image program, National Institutes of Health, Bethesda, USA). The number of positive cells was compared with those of 5 non-infected monkeys by Student's t-test.

Table 1. Case history, viral antigen localization, and light microscopic findings in EBO-R infected macaques

	macaque No.	Recorded health status*	Viral antigen localization†	Inclusions in hepatocytes (liver)‡	Fibrin deposition in red pulp (spleen)§	Lymphoid cell depletion (spleen)	Hemorrhage in renal tubles (kidney)	Hemorrhage in alveoli (lung)	Thrombi formation¶
I. Sacrificed macaques (EBO-R infected)	2671	H	L(0), S(1), K(0), P(1)	–	+	–	–	–	L(1), S(1), K(0), P(0)
	2182	H, W	L(1), S(1), K(0), P(1)	–	+	–	–	–	L(1), S(1), K(0), P(0)
	2921	NR	L(1), S(1), K(0), P(N)	+	+	–	–	ND	L(1), S(1), K(0), P(N)
	2728	NR	L(1), S(1), K(1), P(1)	+	+	–	–	–	L(1), S(1), K(0), P(0)
	2739	NR	L(1), S(1), K(1), P(1)	2+	+	–	–	–	L(1), S(1), K(0), P(0)
	2644	NR	L(2), S(2), K(2), P(1)	2+	2+	–	+	–	L(1), S(1), K(0), P(0)
	2612	H	L(2), S(2), K(2), P(N)	2+	+	–	–	ND	L(1), S(1), K(1), P(N)
	956	S	L(2), S(2), K(2), P(1)	2+	2+	+	+	–	L(1), S(1), K(1), P(1)
	2400	NR	L(2), S(2), K(2), P(1)	2+	2+	+	–	–	L(1), S(1), K(0), P(1)
	2939	D	L(2), S(2), K(2), P(1)	2+	2+	+	+	–	L(1), S(1), K(1), P(1)
	2669	H	L(2), S(2), K(2), P(2)	2+	2+	+	–	+	L(1), S(1), K(0), P(1)
	2615	M	L(2), S(2), K(2), P(2)	2+	2+	+	–	+	L(1), S(1), K(0), P(0)
II. Died macaques (EBO-R infected)	2386	W	L(2), S(2), K(N), P(2)	2+	2+	+	ND	–	L(1), S(1), K(N), P(1)
	2377	NR	L(2), S(2), K(2), P(2)	2+	2+	+	+	–	L(1), S(1), K(1), P(1)
	2384	NR	L(2), S(2), K(N), P(2)	2+	2+	+	–	–	L(1), S(1), K(1), P(1)
	2938	S	L(2), S(2), K(2), P(2)	2+	2+	+	–	+	L(1), S(1), K(1), P(1)
	2338	NR	L(2), S(2), K(2), P(2)	2+	2+	+	+	–	L(1), S(1), K(1), P(1)
	2809	S	L(2), S(2), K(N), P(2)	2+	2+	+	–	–	L(1), S(1), K(1), P(1)
	2622	NR	L(2), S(2), K(2), P(2)	2+	2+	+	–	–	L(0), S(1), K(0), P(0)
	2319	NR	L(2), S(2), K(2), P(2)	2+	2+	+	–	–	L(1), S(1), K(1), P(0)
	2334	NR	L(2), S(2), K(2), P(N)	2+	2+	+	+	ND	L(1), S(1), K(1), P(N)
	2872	NR	L(2), S(2), K(2), P(2)	2+	2+	+	+	–	L(0), S(1), K(0), P(0)
	2882	NR	L(2), S(2), K(2), P(2)	2+	2+	+	+	–	L(1), S(1), K(1), P(1)
	2878	NR	L(2), S(2), K(2), P(2)	2+	2+	+	+	–	L(1), S(1), K(1), P(0)
III. Negative macaques (Uninfected)	2759	H	L(0), S(0), K(0), P(0)	–	–	–	–	–	L(0), S(0), K(0), P(0)
	2782	H	L(0), S(0), K(0), P(0)	–	–	–	–	–	L(0), S(0), K(0), P(0)
	2784	H	L(0), S(0), K(0), P(0)	–	–	–	–	–	L(0), S(0), K(0), P(0)
	2757	H	L(0), S(0), K(0), P(0)	–	–	–	–	–	L(0), S(0), K(0), P(0)
	2758	H	L(0), S(0), K(0), P(0)	–	–	–	–	–	L(0), S(0), K(0), P(0)

*, H: healthy, S: sick, M: moribund, D: diarrhea, W: wounded, NR: not recorded. †, L: liver, S: spleen, K: kidney, P: lung (N: not done, 0: not detected, 1: MPS cells, 2: MPS cells and endothelial cells). ‡, –: not detected, +: positive in a few hepatocytes, 2+: positive in many hepatocytes. §, –: not detected, +: minimal deposition, 2+:massive deposition. ¶, L: liver, S: spleen, K: kidney, P: lung (N: not done, 0: not detected, 1: thrombi were detected), ND: not done.

Results

Histopathologic findings in macaques that died of EBO-R infection

All the macaques that had died of Ebola virus infection during the outbreak showed similar pathological findings (Table 1). Moderate to severe infiltration of mononuclear cells was present in the portal areas in the livers. Hepatocytes contained irregular shaped intracytoplasmic acidophilic inclusion bodies, which were widely scattered throughout the lobules (Fig. 1a). Necrosis of hepatocytes was occasionally observed. A prominently increased number of mononuclear cells and fewer neutrophils were present in the hepatic sinuses and veins. Some of the mononuclear cells were enlarged and contained acidophilic inclusions in the cytoplasm. PTAH staining demonstrated fibrin thrombi in the sinuses.

In the white pulp of the spleen, the number of lymphoid cells was severely decreased, and hemorrhage was occasionally present in the follicle (Fig. 1b). The number of MPS cells in the red pulp cords was dramatically decreased. The splenic cords were prominently engorged with acidophilic materials (Fig. 1c). The acidophilic

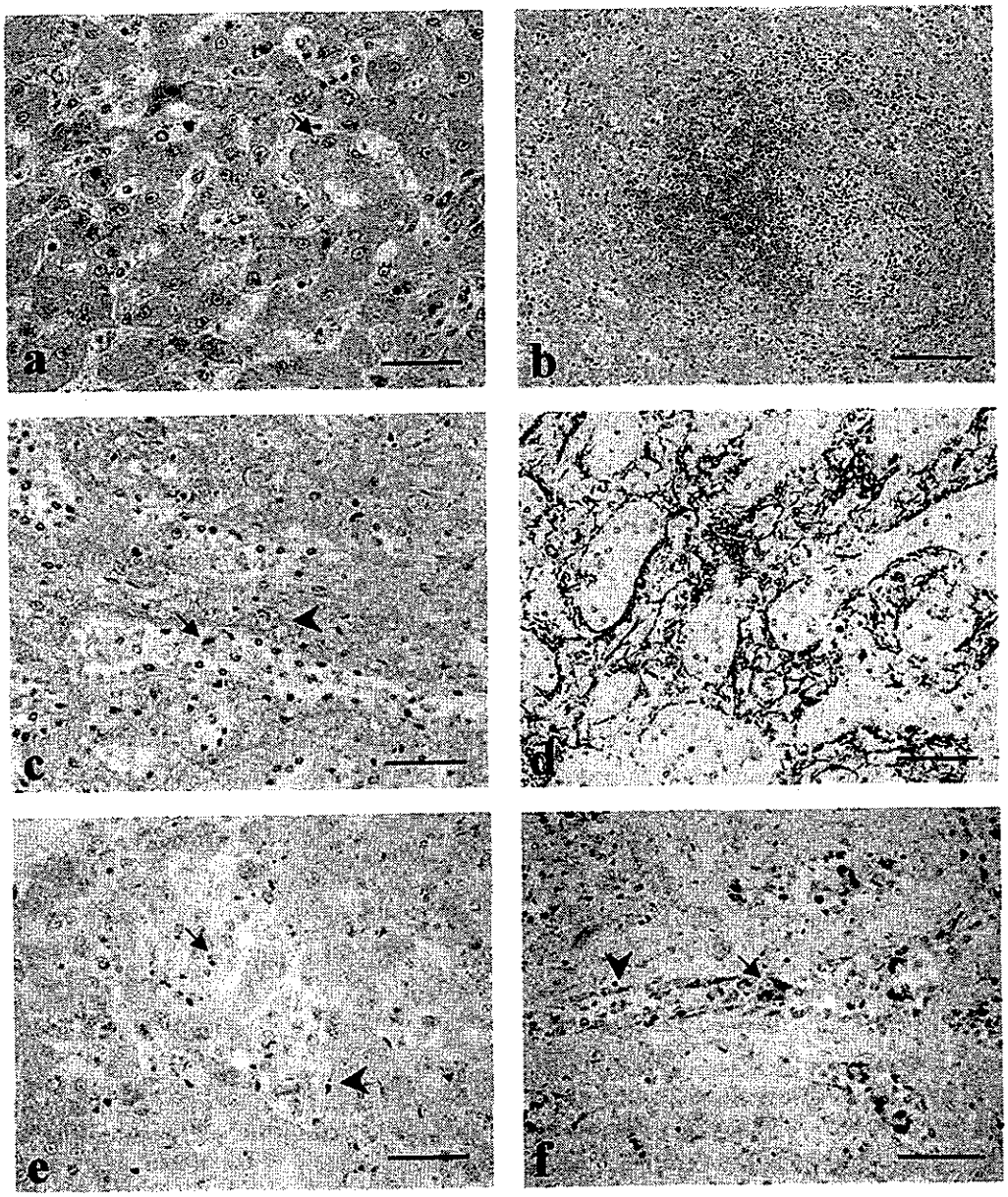

Fig. 1. Light microscopic lesions observed in macaques that died of EBO-R infection. (a) Liver, No. 2386 monkey. Some hepatocytes have irregular acidophilic inclusion bodies in the cytoplasm (arrow). HE. Bar = 50 μm. (b) White pulp in spleen, No. 2386. The number of lymphoid cells is prominently reduced in the follicle and hemorrhage is also observed. HE. Bar = 200 μm. (c) Red pulp in spleen, No. 2386. The number of constituent cells of the red pulp cord is prominently decreased. The red pulp cord is engorged, and acidophilic material (fibrin) is deposited (arrow head). Enlarged macrophages are seen in the splenic sinus (arrow). HE. Bar = 50 μm. (d) Red pulp in spleen, No. 2386. Fibrin deposition is demonstrated in the red pulp cord. PTAH. Bar = 50 μm. (e) Liver, No. 2377. EBO-R NP antigens are detected in the cytoplasm of macrophages / monocytes (arrow) in the sinus and endothelial cells (arrow head). Immunohistochemistry, Mayer's hematoxylin counterstain. Bar = 50 μm. (f) Red pulp in spleen, No. 2377. Macrophages (arrow) in the splenic sinus and endothelial cells (arrow head) have EBO-R NP antigens. Immunohistochemistry, Mayer's hematoxylin counterstain. Bar = 50 μm.