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Isolation of the Etiologic Agent of Korean Hemorrhagic Fever

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Lung tissues from 73 rodents (*Apodemus agrarius coreae*) gave specific immunofluorescent reactions when they reacted with sera from patients convalescing from Korean hemorrhagic fever. Similar staining was observed in the lungs of *A. agrarius* inoculated with acute-phase sera obtained from two patients with this disease. The unidentified agent was successfully propagated in adult *A. agrarius* through eight passages representing a cumulative dilution of $>10^{-17}$. Experimentally inoculated rodents developed specific fluorescent antigen in the lung, kidney, liver, parotid glands, and bladder. Organs, especially lungs, were positive beginning 10 days and continuing through 69 days after inoculation. The agent could not be cultivated in several types of cell cultures nor in laboratory animals. No fluorescence was observed when infected *A. agrarius* lung tissues were reacted with antisera to Marburg virus, Ebola virus, and several arenaviruses. Diagnostic increases in immunofluorescent antibodies occurred in 113 of 116 severe and 11 of 34 milder cases of clinically suspected Korean hemorrhagic fever. Antibodies were present during the first week of symptoms, reached a peak at the end of the second week, and persisted for up to 14 years. Convalescent-phase sera from four persons suffering a similar disease in the Soviet Union were also positive for antibodies.

Epidemic hemorrhagic fever was recognized for the first time in Korea in 1951 among soldiers of the United Nations [1]. Since that time it has been known as Korean hemorrhagic fever (KHF) and has remained endemic near the demilitarized zone between North and South Korea. In recent years it appears to have spread slowly in a southwesterly direction, and 100–800 hospitalized cases are diagnosed clinically each year (table 1).

The clinical manifestations of KHF were reviewed in detail more than 20 years ago [2]. Diseases that appear to be very similar to KHF have been described by Japanese investigators from Manchuria [3] and investigators from the Soviet Union [4] and Scandanavia [5, 6]. The disease has also been reported in several countries in Eastern Europe [7].

Many names have been used to describe what appears clinically to be the same disease: hemorrhagic fever with renal syndrome or hemorrhagic nephroso-nephritis in the Soviet Union, and nephropathia epidemica in northern Scandanavia. Although there is strong circumstantial evidence that the disease, whatever its name, is acquired through contact with rodents and/or their ectoparasites or excreta, elucidation of the specific nature of the syndrome and its natural history has been confounded by the inability to isolate, propagate, and characterize any etiologic agent. Thirty-odd years ago scientists in Japan and the Soviet Union reproduced the disease by inoculation of the sera and urine of patients into volunteers [8–10]. In at least one instance, infectious inocula were able to pass through bac-

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We dedicate this paper to the memory of Dr. Joseph Smadel, who provided the original stimulus and was an inspirational leader to so many of the small band of individuals who have worked on global problems associated with the syndrome of viral hemorrhagic fever.

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Table 1. Hospitalized cases of hemorrhagic fever in Korea, 1951–1976.

Year	No. of cases among			Total no. of cases
	Armed forces of the United States	Army of the Republic of Korea	Korean civilians	
1951	827	...	...	827
1952	833	...	...	833
1953	455	...	...	455
1954	307	...	19	326
1955	20	...	...	20
1956	28	26	...	54
1957	13	21	...	34
1958	15	20	...	35
1959	79	47	...	126
1960	10	185	...	195
1961	27	341	...	368
1962	29	311	...	340
1963	11	257	...	268
1964	22	205	18	245
1965	99	110	2	211
1966	36	82	11	129
1967	31	86	13	130
1968	28	102	13	143
1969	9	134	8	151
1970	13	221	85	319
1971	2	358	311	671
1972	0	203	186	389
1973	0	237	241	478
1974	0	251	170	421
1975	1	370	466	837
1976	4	304	521	829
Total	2,899	3,871	2,064	8,834
Fatal cases (%)	5	7	8	6.6

terial filters; therefore, the disease has been suspected of being caused by a virus. In one experiment in volunteers, a suspension of mesostigmatid mites obtained from field mice (*Apodemus agrarius*) caused hemorrhagic fever [11].

Many attempts have been made to isolate the etiologic agent of KHF and clinically similar diseases. A report from the Soviet Union of cultivation of an agent in cell cultures from patients with hemorrhagic nephroso-nephritis [12] has not been confirmed. In 1976 Lee and Lee succeeded in demonstrating an antigen in the lungs of the striped field mouse, *A. agrarius,* that gave immunofluorescent reactions with sera from patients convalescent from KHF [13]. We present here the first evidence that this antigen is produced by a replicating microbe. The essential materials employed were wild *A. agrarius,* convalescent-phase sera from severe, clinically "typical" cases of KHF, and fluorescein-conjugated human globulins prepared against human antiserum.

Materials and Methods

Project areas. Surveys were carried out in civilian villages where many cases of KHF have been reported each year. Six areas, namely, Yunchun, Puchun, Songnaeri, Jihengri, Chunsuri, and Kasangri of Kyungido, Korea, were surveyed. These areas are typical farm villages, located in mountainous districts and surrounded by rice paddies, streams, and mountains. All of these areas are located not far from the 38th parallel, north latitude.

Collection, identification, and processing of rodents. Rodents were captured from fields, uncultivated scrub vegetation, and near farm dwellings with use of wire-mesh live traps. Traps were set during late afternoon and examined at midnight and at dawn. Animals were transported live to the laboratory in Seoul, Korea, and identified according to the taxonomic scheme of Won [14]. Samples of blood were obtained by cardiac puncture of animals anesthetized with chloroform, and the animals were autopsied. Cervical lymph nodes, spleen, lung, liver, and kidneys were removed and weighed. Portions of each organ were triturated in borate-buffered saline, pH 8.0, containing 1% bovine plasma albumin (BSBA) for attempts at viral isolation, and the remainder was stored at −70 C.

Rodents used in laboratory studies. A. agrarius subspecies *coreae,* obtained in Chullanamado, Korea, and *A. agrarius* subspecies *jejudoica,* trapped on Jeju Island, south of the Korean peninsula, were used in laboratory studies. Neither area has ever registered cases of KHF. The animals weighed 20–30 g.

Source of the KHF agent employed. All experimental and diagnostic work was done with lung tissues originating from four naturally infected *A. agrarius coreae.* Designation, source, and date of obtainment of the strains are as follows: (*1*) strain 75-91, Puchun, October 6, 1975; (*2*) strain 76-66, Yunchun, May 6, 1976; (*3*)

strain 76-118, Songnaeri, June 23, 1976; and (*4*) strain 76-236, Songnaeri, October 7, 1976.

For fluorescent antibody (FA) tests frozen sections of lung tissue were cut to a thickness of 4 μm, air-dried on glass slides, fixed for 7 min in acetone, and stored at −70 C until needed. For titration of the infectious agent, 10% lung suspensions were prepared in BSBA and then clarified at 2,000 *g* for 20 min; the supernatant was used to inoculate cell cultures and animals. Titration end points in *A. agrarius* were calculated 20 days later by the method of Reed and Muench [15].

FA techniques. Since immunofluorescence employing fresh sections from naturally (initially) and experimentally (subsequently) infected *A. agrarius* and antisera from persons convalescent from KHF was the only system to emerge from this work that was capable of measuring infection, an early problem was that of reagent standardization. Block titrations were carried out with several human sera and sections from a single pair of infected rodent lungs with use of the indirect FA procedure. The titer of antibody in the serum was found to be 1:1,024, and the end-point dilution of conjugated antiglobulin in terms of acceptable brightness was most sharply determined when the serum dilution was held to 1:64 (later at 1:50 for convenience). Thus 1 antiglobulin unit was defined as the highest dilution giving distinct fluorescence at a serum dilution of 1:64 (or 1:50). Eight antiglobulin units were employed in all indirect FA tests. One unit of human serum antibody was, in turn, defined as the highest dilution of serum giving a distinct reaction in the presence of 8 antiglobulin units. When the indirect FA method was used to search for antigen, 16 units of serum antibody to the KHF agent were used in the initial reaction. Fluorescein isothiocyanate (FITC)-conjugated polyvalent immunoglobulins of goat origin prepared against human and guinea pig immunoglobulins were obtained from Hyland Laboratories, Costa Mesa, Calif.

Serum from a patient convalescent from KHF with a titer of 1:2,048 by the indirect FA test was fractionated by the method of Kabat and Meyer [16], adjusted to contain 10 mg of globulin/ml, and conjugated with FITC by the method of Marshall et al. [17]. Unbound dye was removed according to the method of Porath and Flodin [18], and the conjugate was absorbed by the technique of Coons et al. [19]. This direct conjugate had a titer of 1:32 against the infected *A. agrarius* lung tissue and was used at a dilution of 1:4 (8 antiglobulin units) to search for KHF-specific antigen in animal tissues and cell cultures. Conjugate was incubated with cells at 36 C for 30 min. Slides were washed three times for 5 min with continuous agitation in phosphate-buffered saline, pH 7.6, air-dried, and mounted with cover slips in glycerine-saline. They were examined in a transmission electron microscope (American Optical Co., Buffalo, N.Y.) with use of a mercury arc light source, a BG-12 exciter, and a 510-mm barrier filter. Incubation, washing, and mounting procedures for the two-step indirect FA test were basically similar.

Controls for the various FA techniques included sections of lung tissue from normal *A. agrarius* and sera from persons never resident in Korea or other regions where KHF-like disease is known to occur. The specificity of reactions also was confirmed with use of the two-step inhibition method of Goldman [20]. In this procedure the direct conjugate prepared with antiserum to the KHF agent was used as the second reactant at a dilution of 1:2. In the case of convalescent-phase human sera, the end point was taken as the highest dilution of serum able to reduce specific fluorescence in *A. agrarius* lung tissues from a brilliant (+++) to a dull (+) reaction.

Criterion for immunological diagnosis of KHF in humans. Once sufficient numbers of infected *A. agrarius* lung sections were available, the diagnostic criterion established for specific diagnosis of KHF in humans was an increase in titer of fourfold or greater in two consecutively obtained sera with use of the indirect FA procedure and twofold serum dilutions.

Results

Detection of antigen in the tissues of wild rodents. Frozen tissues from 715 rodents captured during 1974–1976 in districts where cases of human KHF had occurred were examined by the indirect FA technique. Fine granular fluorescence was observed in sections of lung (figure 1) and

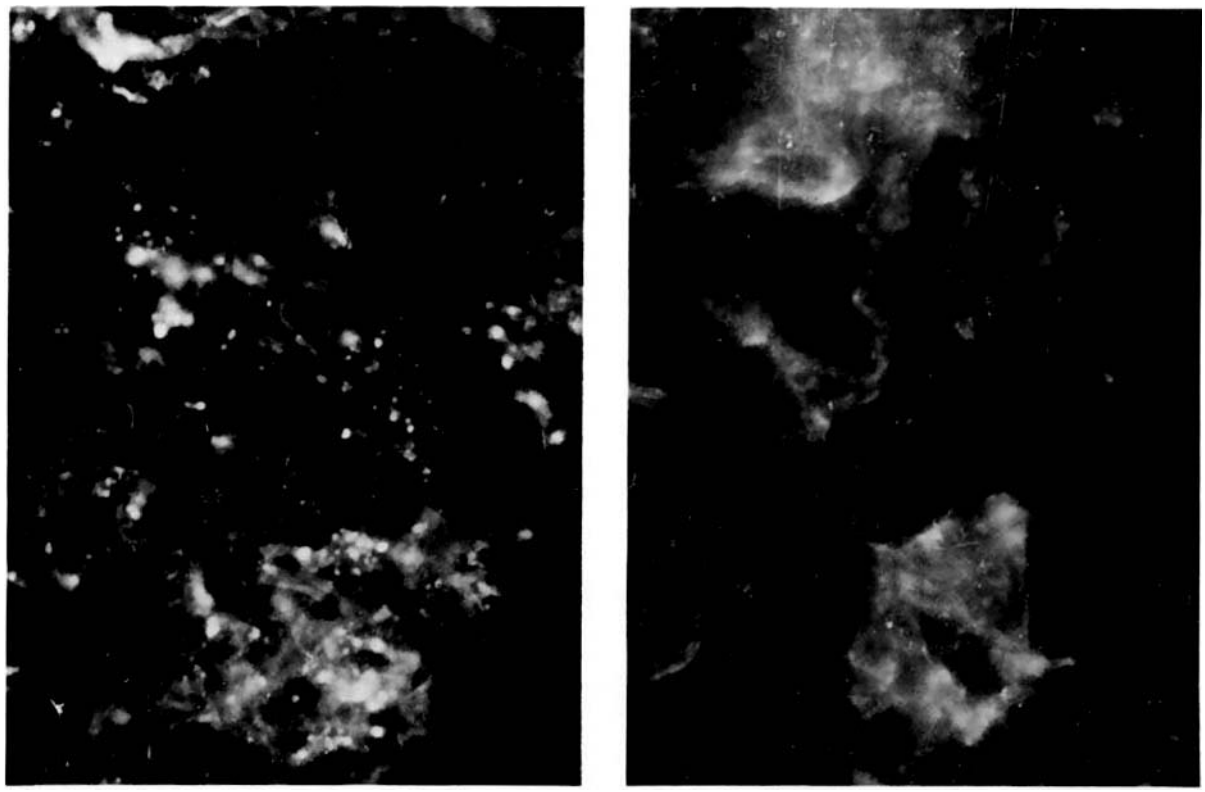

Figure 1. Fluorescent antigen specific for the agent of Korean hemorrhagic fever in the lung tissue of *Apodemus agrarius coreae*. The antiserum used in the indirect fluorescent antibody procedure was the convalescent-phase serum from patient no. 75-15-2 (×400). *Left*: Specific punctate antigen inclusions are seen in the tissue of an infected rodent. *Right*: Nonspecific diffuse fluorescence is seen in the tissue of an uninfected rodent.

renal tissue obtained from *A. agrarius coreae* when stained with convalescent-phase serum from a patient with KHF (case no. 74-74). The positive reaction was present in serum dilutions to 1:2,048, whereas serum obtained from the same patient on the third day of clinical illness was positive only to a dilution of 1:16. Sera from several persons never resident in Asia or Europe failed to react. Lung tissues from all rodents were tested with 8 antibody units of KHF-positive serum. Seventy-three (14%) of 520 *A. agrarius* were positive, but none of 195 specimens from six other species gave specific staining. The percentages of *A. agrarius* positive in 1974, 1975, and 1976 were 12.2, 11.0, and 15.7, respectively. Positive rodents were detected in each of six villages with cases of KHF as seen in table 2. The seasonal distribution of rodents captured and positive reactions by indirect FA is depicted in table 3. Note that the prevalence of positive rodents also was strongly seasonal at one location, Songnaeri, where collections were made during each month. The major peak occurred from September through November, the same season during which most cases of KHF are recorded.

To establish the similarity of indirect FA re-

Table 2. Recovery of the agent of Korean hemorrhagic fever from wild rodents captured in various areas of Korea, 1974–1976.

Rodent	No. of positive rodents/no. captured						Total
	Yunchun	Songnaeri	Jihengri	Chunsuri	Kasangri	Puchun	
Apodemus agrarius	3/75	44/306	1/28	12/58	11/50	2/3	73/520
All others	0/79	0/94	0/5	0/6	0/8	0/3	0/195

Table 3. Isolation of the agent of Korean hemorrhagic fever (KHF) from wild small mammals captured in areas of Korea where KHF is endemic, by month (1974-1976).

Animal	No. of positive animals/no. tested												
	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total
Apodemus agrarius coreae	0/13	0/10	0/41	2/23	11/68	5/60	1/41	2/12	26/71	19/100	7/60	0/21	73/520
A. agrarius coreae in Songnaeri only, 1975–1976	0/8	0/2	0/21	1/9	3/16	3/26	1/23	2/12	11/28	9/26	5/21	0/21	35/213
Microtus fortis peliceus	0/10	0/10	0/12	0/3	0/29	0/6	0/8	...	0/1	0/12	0/3	0/2	0/96
Crocidura lasiura	0/1	...	0/2	0/3	0/1	0/9	0/2	...	0/1	0/19	0/13	0/1	0/52
Clethrionomys rufocanus regulus	0/5	...	0/9	0/3	0/9	0/1	...	...	...	0/2	0/1	...	0/30
Cricetulus triton nestor	...	...	0/1	...	0/1	0/3	0/1	...	0/1	0/1	...	...	0/8
Mus musculus yamashinai	0/1	...	...	...	...	...	0/1	...	0/3	0/1	0/1	...	0/7
Micromys minutus ussuricus	0/1	...	0/1	...	...	...	...	...	...	...	...	...	0/2

actions observed in tissues of *A. agrarius* from six villages during the three-year study period, titrations were carried out with the acute- and convalescent-phase sera of patient no. 74-74. Titers with the 62-day convalescent-phase serum were 1:512–1:4,096, whereas those with the three-day acute-phase serum ranged from 1:8 to 1:64. This variation corresponded in general to the amount and brilliance of fluorescence recorded in lung tissues during the original screening test.

Serial passage, titration, and distribution of the antigen of the KHF agent in tissues of A. agrarius. A 5% lung suspension of strain 76-118, first-passage *A. agrarius* tissue was prepared and inoculated by intrapulmonary and sc routes into 50 wild-caught *A. agrarius*. Lung tissues from animals sacrificed nine to 69 days after inoculation were examined (table 4). Because the mice used for this experiment were from the Korean mainland, we were unable to exclude the possibility that some of them were naturally infected. Nevertheless, positive reactions were seen significantly more often between 13 and 27 days after inoculation (67%) than during earlier (25%) or later periods (30%). These data suggest that the agent multiplied in the inoculated *A. agrarius* and that infection, as measured by immunofluorescence, was not a lifelong chronic phenomenon.

Serial passages of lung tissue suspensions were next made in *A. agrarius* at intervals of about 20 days. As shown in table 5, infection of all mice was not achieved until the seventh passage. By this time, the material contained $10^{4.2}$ 50% *A. agrarius* indirect FA infectious units. Lungs from

Table 4. Detection of immunofluorescent antigen in the lungs of *Apodemus agrarius coreae* on indicated day after inoculation with strain 76-118 of the agent of Korean hemorrhagic fever.

Days after inoculation	No. of positive animals/no. tested
9	0/4
10	1/4
12	2/4
13	3/4
14	2/4
20	3/4
23	1/3
27	7/13
33	2/4
69	1/6

NOTE. Animals were inoculated with 0.1 ml of a 5% lung suspension of strain 76-118 first-passage *A. agrarius* tissue by the sc route and 0.05 ml by the intrapulmonary route.

Table 5. Propagation of the agent of Korean hemorrhagic fever (strain 76-118) in *Apodemus agrarius coreae.*

Passage no.	Total days in *A. agrarius coreae*	Cumulative $\log_{10}$ dilution of original inoculum	No. of animals infected/no. tested	50% infectious doses/0.1 ml
1	20	1.0	10/16	...
2	47	2.0	7/13	...
3	70	3.0	14/32	...
4	91	4.0	7/28	...
5	111	5.0	23/34	...
6	131	6.0	3/4	...
7	151	8.0	12/12	$10^{4.2}$
8	171	12.0	9/9	$10^{5.3}$

a positive rodent receiving a 10^{-4} dilution of this material were used to make an eighth passage. This material had a titer of $10^{-5.3}$ and represented a cumulative dilution of $10^{-17.3}$ of the original inoculum.

Table 6 presents results of indirect FA distribution studies among *A. agrarius* inoculated by different routes with unpassaged lung suspension of strain 76-118. Intrapulmonary and sc inoculation were superior to ip and nasal-oral routes, and maximal fluorescence was observed in the lungs after incubation for 20 days. No antigen was ever detected in spleen. As indicated in table 7, similar variation in distribution and intensity of staining was observed in the tissues of six wild *A. agrarius* captured during 1976. Neither wild-caught nor inoculated *A. agrarius* ever displayed overt signs of disease. Histological examination of infected rodent tissues is incomplete but does not show striking inflammatory lesions.

All attempts to establish the agent of KHF in hosts other than *A. agrarius* have been unsuccessful. Various species of laboratory animals as well as several types of cell cultures failed to evince specific indirect FA staining when inoculated with fifth-passage lung suspension from strain 76-118, which infected 23 of 34 *A. agrarius*. Animals tested included suckling white mice and weaned mice, hamsters, guinea pigs, rats, and rabbits. The continuous cell lines that were employed were African green monkey kidney (Vero), dog embryo (R-1247), porcine kidney (PS), human embryo lung (WI-38), mink kidney (Mu 1 Lu), and Chinese hamster lung (De-de). Primary cell cultures prepared from rhesus monkey kidney, duck, and chicken embryo, rat liver, and human embryonic liver also failed to develop CPE or specific immunofluorescence when inoculated with infectious *A. agrarius* lung suspension.

In an effort to obtain a "clean" supply of *A.*

Table 6. Distribution of fluorescent antigen in the tissues of *Apodemus agrarius coreae* on indicated day after inoculation.

Route of inoculation, tissue	Days after inoculation* 9	13	20	27	33
Intrapulmonary					
Lung	–	+++	++++	++	+++
Kidney	–	–	++	–	++
Liver	...	...	++	–	–
Parotid glands	...	...	+	–	–
Submaxillary gland	...	...	–	–	+
Bladder	...	...	+	–	+
Spleen	–	–	–	–	–
Subcutaneous					
Lung	–	+++	++++	++++	++++
Kidney	–	–	++	++	++
Liver	...	–	+	++	+
Parotid glands	...	...	–	–	+
Submaxillary gland	...	–	–	+	–
Spleen	–	–	–	–	–
Intraperitoneal					
Lung	–	–	++++	–	–
Kidney	–	–	+	–	–
Liver	...	...	–	–	...
Parotid glands	...	...	–	...	...
Spleen	...	...	–	–	–
Nasal and oral					
Lung	–	+	+++	–	–
Kidney	–	...	–	–	–
Parotid glands	–	–	–	–	...
Liver	...	...	–	–	...
Spleen	...	–	–	–	–

NOTE. The inoculum consisted of 0.1 ml of the supernatant of 5% unpassaged lung and kidney suspensions from antigen-positive, wild *A. agrarius coreae* (strain 76-118).

*The fluorescent reaction was graded as negative (–) or positive (ranging in degree from dull [+] to brilliant [++++]), according to the intensity and, particularly, the extent of the reaction in tissue.

Table 7. Distribution of the agent of Korean hemorrhagic fever (KHF), by the indirect fluorescent antibody test, in the tissues of *Apodemus agrarius coreae* captured in 1976 in areas of Korea where KHF is endemic.

Tissue	Rodent no. R-76-66 (Yunchun, May 6)	R-76-79 (Songnaeri, May 13)	R-76-81 (Songnaeri, May 13)	R-76-98 (Songnaeri, June 2)	R-76-100 (Songnaeri, June 2)	R-76-118 (Songnaeri, June 23)
Lung	++++	++	++++	++	+++	+++
Kidney	++	+	++	–	+	++
Parotid glands	++	+	+	+	+	+
Bladder	+	–	+	–	–	+
Liver	++	–	+	–	–	+
Submaxillary gland	+	–	+	–	–	–
Spleen	–	–	–	–	–	–

NOTE. The areas in which the rodents were captured and dates of capture are given in parentheses. See table 6 for definition of symbols representing the reaction.

agrarius, rodents of the subspecies *jejudoica* resident on Jeju Island were captured. No case of KHF has ever been reported on this island, which has a population of 366,000. Lung tissues from 30 of these rodents were examined and found negative by the indirect FA test when tested against convalescent-phase sera from patients with KHF. Unpassaged lung suspension of strain 76-236 was inoculated into seven *A. agrarius jejudoica* from Jeju Island and six *A. agrarius coreae* from the mainland. When examined after incubation for 18 days, lungs of all seven *A. agrarius jejudoica* were positive for indirect FA, whereas only four *A. agrarius coreae* gave positive reactions. The number and intensity of fluorescent-positive cells in the rodents from the island also were greater than in the mainland mice. Whether this is a host difference in susceptibility to the agent or reflects specific, naturally induced immunity in mainland *A. agrarius* remains to be determined.

Detection of the KHF agent and KHF-specific antibodies in human sera. Acute- and convalescent-phase serum samples were obtained from more than 100 hospitalized cases of suspected KHF during 1976. Only 11 of those patients who were subsequently shown to have diagnostic increases in indirect FA titer had no antibody in their first serum specimen. The interval from onset of symptoms to collection of these 11 sera was two to six days. All sera were stored at −70 C for one to 12 weeks and then inoculated by intrapulmonary and sc routes into adult *A. agrarius.* Cases no. 76-109 and 76-243 were positive; four of eight and one of four *A. agrarius,* respectively, had specific pulmonary fluorescence when tested 18–20 days after inoculation.

Most patients with KHF had serum antibodies to the agent in *A. agrarius* lung tissue by the end of the first week of symptoms. Nevertheless, increases in antibody titer in paired acute- and convalescent-phase sera were detected in 113 of 116 cases that displayed the classical clinical phases of fever and toxemia, hypotension, and diuresis (table 8). Among 34 patients without hypotensive or diuretic manifestations, only 11 were diagnosed as having KHF by the indirect FA test.

The evolution of indirect FA during the 60-day period from the onset of disease is shown in figure 2. The highest titers were observed at two to three weeks, followed by a slow decline. Blocking tests for indirect FA specificity were done in five of these cases. Results are given in figure 3. Although titers were considerably lower than those measured by the indirect FA test, temporal

Table 8. Detection of immunofluorescent antibodies to the agent of Korean hemorrhagic fever (KHF) in the sera of humans with KHF in Korea.

Group	No. positive/no. tested (%)
"Typical" cases of KHF	113/116 (97.4)
"Mild" cases of KHF	11/34 (32.4)
Adult blood donors in Seoul	3/252 (1.2)
Outpatients of all ages in Seoul and Chunchon	2/205 (1.0)
Military personnel	4/378 (1.1)

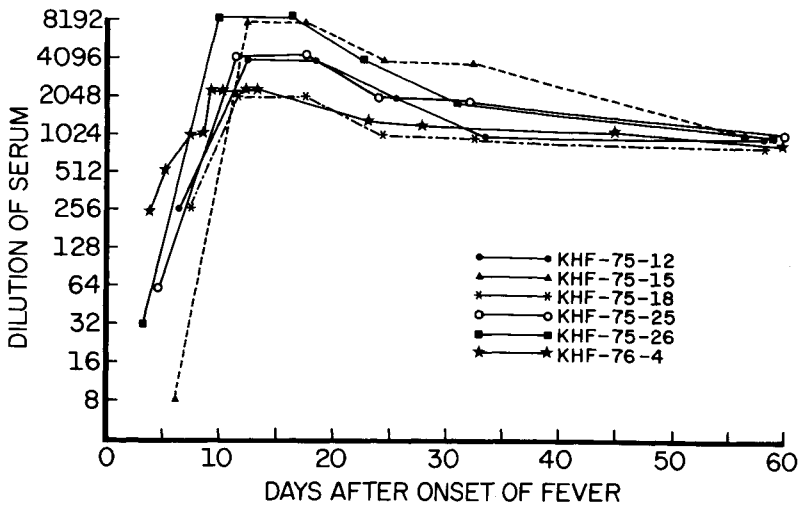

Figure 2. Titers of indirect fluorescent antibody to the agent of Korean hemorrhagic fever (KHF) during a period of 60 days in six patients with KHF.

patterns of evolution of antibody corresponded closely to those observed in the original tests.

Antibodies to the agent in *A. agrarius* lung tissue persisted in patients for several years following clinical KHF. Measurements made in one patient during a six-year period are depicted in figure 4. Antibodies also were present in each of 13 sera obtained from patients with KHF three to 14 years after acute illness. The geometric mean titer of these sera was 1:147.

Limited surveys for antibodies to the KHF agent were carried out with use of serum specimens obtained from persons resident in the area of Seoul. As seen in table 8, antibody prevalence among adult blood donors, outpatients, and Korean soldiers was about 1%. The outpatient group consisted of 114 males and 91 females; 21–42 individuals in each of seven age deciles were tested. Data on history of disease compatible with KHF were available only for the soldiers. Three of the antibody-positive persons had been hospitalized for this disease in the past. No such history was elicited from two other persons who had positive indirect FA tests.

Immunological relationship between the KHF agent and viruses associated with acute hemorrhagic fever. To date only limited studies have been completed. Antisera to the following viruses

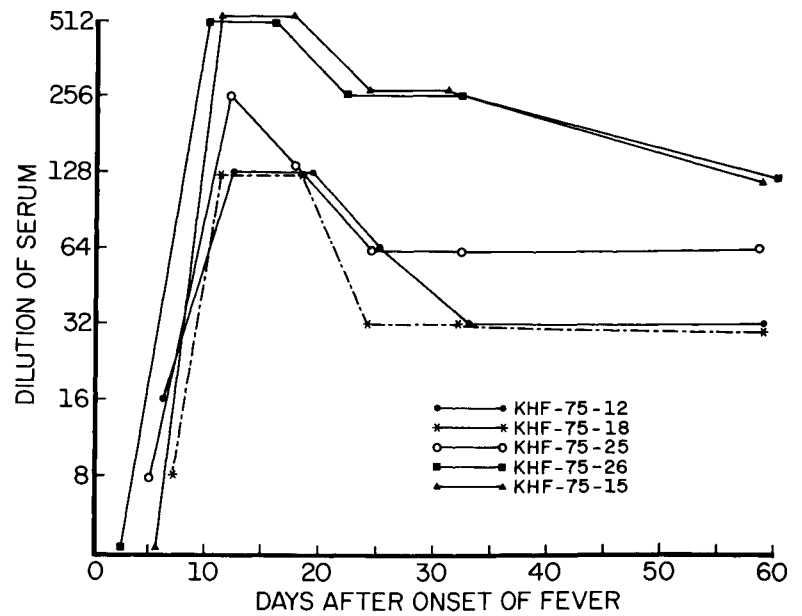

Figure 3. Titers of indirect fluorescent antibody to the agent of Korean hemorrhagic fever (KHF) during a period of 60 days in five patients with KHF, as measured by the two-step blocking test [20].

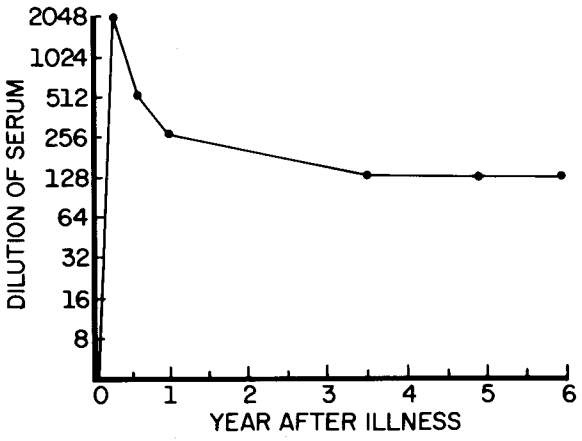

Figure 4. Persistence of indirect fluorescent antibodies to the agent of Korean hemorrhagic fever (KHF) during a period of six years in the sera of a patient with KHF.

obtained from humans, monkeys, or guinea pigs were tested by the indirect FA technique with negative results: Lassa, Machupo, lymphocytic choriomeningitis, Pichinde, and Tacaribe (all of the arenavirus group), Marburg, and Ebola viruses. Each of the antisera had homologous indirect FA titers of at least 1:128. The sixth *A. agrarius* passage of strain 76-118 was used in these tests, and a convalescent-phase serum with a titer of 1:2,048 from a patient with KHF served as a positive control.

In contrast, a definite relationship between KHF and hemorrhagic fever with renal syndrome in the Soviet Union was established. Four sera from patients with the latter disease were correctly distinguished from four other sera obtained from residents of New York State. These sera were supplied under code by Dr. Jordi Casals of the Yale Arbovirus Research Unit, New Haven, Conn. Reciprocal indirect FA titers were 128, 256, 256, and 1,024.

Discussion

These observations, although definitely preliminary, provide substantial evidence that the etiologic agent of KHF has been isolated from the wild rodent *A. agrarius coreae* and from the blood of patients with KHF. The important data may be summarized as follows. (*1*) Antigen was detected by the indirect FA procedure in lung and other tissues from *A. agrarius* but not from other species of rodents captured in KHF-endemic areas; (*2*) the antigen was demonstrated in *A. agrarius* lungs after serial passages, representing a dilution of the starting material of $>10^{-17}$; and (*3*) antigen was not found by the indirect FA test in inoculated *A. agrarius* for a period of nine days, but was then detected in an increasing proportion of animals during the next two weeks.

Further support for the specificity of the association between the agent and human disease included diagnostic increases in indirect FA titers (several of which were confirmed with blocking tests) in sera from nearly all of the large series of "typical" KHF cases, the detection of infection in a smaller fraction of "atypical" mild cases, the low prevalence of antibodies in sera from urban Korean residents, and the identification of coded sera from persons surviving a similar clinical infection in the Soviet Union. Taken together, these data considerably extend the original work of Lee and Lee [13] who reported the relationship between *A. agrarius* antigen and antibodies in patients with KHF.

The present findings, moreover, are biologically consistent with several important clinical and epidemiological observations that have been made in past decades. First, *A. agrarius* are found in all parts of Eurasia where clinical disease similar to KHF has been reported. Indeed, Japanese investigators reported in the 1940s that a suspension of mites (*Laelaps jettmari*) taken from Manchurian *A. agrarius* and passed through bacteria-tight Chamberland and Seitz filters caused a febrile disease in a human volunteer thought to resemble epidemic hemorrhagic fever [11]. Blood obtained from this person, in turn, when inoculated into an *A. agrarius,* failed to induce acute illness in the rodent, but tissue obtained from the rodent 25 days later again produced febrile disease in another human. Further investigation of the problem by Japanese scientists was interrupted by nonscientific events that are now history.

Second, cases of this hemorrhagic fever with renal pathology occur everywhere in two seasonal peaks during late spring and autumn, times of the year when *A. agrarius* are known to exhibit peak breeding activity and highest population density. Although this behavior is by no means restricted to *A. agrarius,* this seasonal correlation

and the typical contact of rural inhabitants with environments inhabited by feral rodents have led epidemiologists to consider the striped field mouse as one of the leading reservoir candidates for the presumptive viral agent of the disease. Our own data do not provide definitive evidence of this relationship because the number of rodents captured was not based on a standardized number of trap-nights per month. Nevertheless, our impression was that *A. agrarius* were in fact more numerous in the spring and fall than at other times of the year. Rates of infection with the agent of KHF were definitely higher in Songnaeri during those seasons.

Finally, the variable incubation period of one to five weeks in KHF together with the unique "leukamoid" lymphocytosis and major renal pathology involving both glomeruli and the tubular apparatus has provoked speculation that this syndrome might have a significant immunological pathogenesis. One consequence might well be the absence of a readily detectable infectious agent in the blood or tissues of acutely ill patients. The finding of low titers of indirect FA in nearly every active-phase serum from patients with KHF is consistent with this concept as, perhaps, is the recovery of the agent itself from only two of 11 sera without such antibodies. However, we cannot regard these isolates with unequivocal security since mainland, possibly naturally infected animals were used for the assays. Thus we must regard this attractive hypothesis as still unproven.

The nature of the agent isolated in these studies awaits definitive characterization. The immunofluorescent systems using infected *A. agrarius* lung sections as antigen and human sera as the source of antibody represent an admittedly circular skeleton on which to elucidate the biology of this agent. In addition, the intensity and particularly the extent of the fluorescence found in the lung tissues do not permit easy interpretation of the reactions, as depicted in the figures. A host system that can be readily adapted to manipulation in the laboratory is needed and is being sought. *A. agrarius* has never been colonized. In the interim, animals of the subspecies *jejudoica,* which are apparently not naturally exposed to infection, will be employed in work designed to determine whether the new agent can be neutralized by sera from cases of KHF, whether it is in fact a virus, whether it is related to any known agent, and whether or not ectoparasites of *A. agrarius* are potential biological vectors of the pathogen. No immunological relationship was found between the KHF agent and certain viruses belonging to groups that cause hemorrhagic fever, particularly arenaviruses. These data do not eliminate an arenavirus etiology for KHF, but the striking pulmonary localization of KHF antigen in *A. agrarius* tissues is in contrast to that of most arenaviruses which are strongly lymphotropic in their natural, rodent reservoir species [21].

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