that for the cattle eating $C_3$ rations reported in this study. However, many cattle in the NTZ receive food rations of corn and sorghum, both $C_4$ plants. For instance, approximately 25 percent of a steer's diet in the United States is corn and sorghum (*11*). For Europe and the Soviet Union, the amount is less, only about 10 percent. Nomadic herds do not get special rations. The total corn and sorghum fed in the United States, the Soviet Union, and Europe, if distributed throughout the entire cattle population of the NTZ ($660 \times 10^6$) (*12*), would be equivalent to 8 percent of the total bovine diet. Livestock fed on diets containing a significant amount of grain produce perhaps 10 percent less $CH_4$ than livestock eating only hay or alfalfa (*13*). However, for the present study such a difference is immaterial. Thus, carbon in the $CH_4$ from NTZ cattle should be perhaps 1 per mil heavier than the carbon in $CH_4$ from cattle eating only $C_3$ plants.

Another $560 \times 10^6$ cattle live in the tropics and South Temperate Zone (STZ) (*12*). Although the distribution of $C_3$ and $C_4$ plants in their diet is not known, many of the forage crops and pasture grasses they eat use the $C_4$ metabolic pathway (*10*). Thus, the $CH_4$ from these cattle should have a $\delta(^{13}C/^{12}C)$ value similar to, or lighter than, the $CH_4$ from corn-fed cattle.

Sheep, the only other significant source of ruminant methane, produce about 6 percent as much $CH_4$ as cattle (*12, 14*). Their geographic distribution is approximately the same as that of cattle ($570 \times 10^6$ in the NTZ and $470 \times 10^6$ in the tropics and STZ) (*12*) and their grazing diet is similar. Only the isotopic composition of $CH_4$ from two wethers fed $C_3$ plants was measured in Illinois (−70.2 per mil and −67.0 per mil, respectively). If sheep eating $C_4$ plants give off $CH_4$ that is 14 per mil heavier in $^{13}C$ (as in the case of cattle), the worldwide average $\delta(^{13}C/^{12}C)$ value for ovine methane would be −63 per mil or lighter. [It is interesting that the methane released by marshes and lakes, apparently a very different environment, has an average $\delta(^{13}C/^{12}C)$ of −65 per mil (*15*).]

The published value for the average $\delta(^{13}C/^{12}C)$ of atmospheric $CH_4$ is −41 per mil (*15*), and the measured carbon kinetic isotope effect in the reaction of $CH_4$ with OH, its principal tropospheric sink, is 1.003 (*16*). Therefore, the calculated average $\delta(^{13}C/^{12}C)$ value for all sources of tropospheric $CH_4$ is −44 per mil. None of the ruminant $CH_4$ carbon isotopic ratios reported here match the calculated source average. Evidently, then, ruminant $CH_4$ is quite different isotopically from the average source of atmospheric $CH_4$.

According to Ehhalt and Schmidt (*14*) and Baker-Blocker *et al.* (*17*), cattle and sheep combined generate $1 \times 10^{14}$ g of $CH_4$ per year (at least 67 percent of all herbivorous production), which is 10 to 20 percent of the annual global release of $CH_4$ to the atmosphere. Consequently, other significant sources of $CH_4$ enriched in $^{13}C$ compared to ruminant $CH_4$ account for the average $\delta(^{13}C/^{12}C)$ of all sources. Such sources are unknown (*14*).

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### References and Notes

1. In $\delta(^{12}C/^{13}C)$, $\delta = \{[(^{13}C/^{12}C)_x/(^{13}C/^{12}C)_{std}] - 1\} \times 1000$, where $x$ is the sample and std is the standard, Pee Dee belemnite is the standard.
2. The first product of photosynthesis in $C_3$ plants is a 3-carbon acid, and in $C_4$ plants, a 4-carbon acid.
3. M. D. Hatch and C. R. Slack, *Annu. Rev. Plant Physiol.* **21**, 141 (1970).
4. J. H. Troughton, K. A. Card, C. H. Hendy, *Carnegie Inst. Washington Yearb.* **73**, 768 (1974).
5. M. M. Bender, *Phytochemistry* **10**, 1239 (1971).
6. B. N. Smith and S. Epstein, *Plant Physiol.* **47**, 380 (1971).
7. L. M. Gaines, J. M. Hayes, R. P. Gunsalus, *Geochim. Cosmochim. Acta* **42**, 1295 (1978); G. Fuchs, R. Thauer, M. Ziegler, W. Stichler, *Arch. Microbiol.* **120**, 135 (1979).
8. D. J. Minson, M. M. Ludlow, J. H. Troughton, *Nature (London)* **256**, 602 (1975).
9. S. B. Cohen, Ed., *Oxford World Atlas* (Oxford Univ. Press, Oxford, 1973), p. 100.
10. J. Goudriaan and G. L. Ajtay, in *The Global Carbon Cycle*, B. Bolin, E. T. Degers, S. Kempe, P. Ketner, Eds. (Wiley, New York, 1979), pp. 240 and 245.
11. L. Berger, private communication.
12. *United Nations Statistical Yearbook* (1977), pp. 115–125.
13. C. E. Coppock, W. P. Flatt, L. A. Moore, W. E. Stewart, *J. Dairy Sci.* **47**, 1359 (1964).
14. D. H. Ehhalt and U. Schmidt, *Pure Appl. Geophys.* **116**, 452 (1978).
15. D. H. Ehhalt and A. Volz, in *Microbial Production and Utilization of Gases ($H_2$, $CH_4$, CO)* (Akademie der Wissenschaften zu Göttingen, Germany, 1976), pp. 23–33.
16. F. Rust and C. M. Stevens, *Int. J. Chem. Kinet.* **12**, 371 (1980).
17. A. Baker-Blocker, T. M. Donahue, K. H. Mancy, *Tellus* **29**, 3 (1977).
18. I thank C. Boldt, C. Lansdorf, Jr., and R. Mool for permitting the collection of air samples from their barns; also M. Bryant and J. Rogers of the University of Illinois at Urbana for helping in the collection of the rumen samples. I thank C. Stevens of Argonne National Laboratory for very helpful discussion and criticism. This work was performed under the auspices of the Office of Basic Energy Sciences, Division of Chemical Sciences, U.S. Department of Energy.

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## Korean Hemorrhagic Fever: Propagation of the Etiologic Agent in a Cell Line of Human Origin

Abstract. *The etiologic agent of Korean hemorrhagic fever has been propagated in a human cultured cell line derived from a carcinoma of the lung. The cells, described as type II, alveolar epithelial, support replication of the agent and successive passages. Antigen of the Korean hemorrhagic fever agent is readily detected in infected cells by means of direct or indirect fluorescent antibody techniques. Previous attempts to propagate this agent in vitro had been unsuccessful.*

Korean hemorrhagic fever (KHF), which is presumed to be of viral origin, is one member of a group of similar hemorrhagic fevers with renal syndrome that occur throughout large portions of the world from Japan in the East, throughout Soviet Russia, to Sweden in the West (*1–4*).

H. W. Lee and co-workers reported recently the isolation of the KHF etiologic agent from a rodent, *Apodemus agrarius coreae* (*1*). Specific antigen for KHF was detected in various tissues of this rodent, by using an indirect fluorescent antibody (IFA) technique (*5*) and samples of serum from human patients recovering from KHF. Isolation of the KHF agent had been attempted by several groups of investigators since 1952 (*1, 2, 6–8*). Attempts to propagate the agent in cell culture have also been made, without success (*1, 3, 8, 9*). However, we now report the successful propagation of the etiologic agent of KHF in an in vitro substrate, a human cell line. This line, designated A-549 and described as type II, alveolar epithelial cells, was derived from a carcinoma of the lung (*10, 11*).

The starting infectious material for this study was a pooled suspension (10 percent) of lung tissue from several *A. a. coreae* killed 21 days after they were inoculated with pooled tissue from several *Apodemus* infected with fourth-passage KHF strain 76-118 (*1*). A $10^{-1}$ dilution of this material in medium E-199 containing 10 percent fetal calf serum (FCS) was inoculated onto monolayers of various primary and continuous avian and mammalian cells prepared on 12-mm glass cover slips in 24-well plates (*12*). The cover slip monolayers were maintained with medium E-199 with 5 percent FCS which was changed at 3- to 5-day intervals as required. Inoculated cultures were examined daily for cytopathogenic effect (CPE). On day 8 after inoculation and at 2-day intervals thereafter through day 24, two to three cover slips were removed, fixed in 100 percent cold acetone, and examined by the IFA tech-


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nique for the presence of KHF-related antigen (*1*). In addition, occasional cover slips were stained at various intervals with acridine orange or May-Grünwald stain and examined for the presence of viral inclusions. The different cell cultures used in this and earlier studies are shown in Table 1.

The only cultured cells in which we could reproducibly demonstrate specific fluorescence or other evidence of infection were the A-549 cells. These cells were first recognized as positive on day 12 after inoculation, with the appearance of a single fluorescent focus of less than a dozen cells. Fluorescence appeared as discrete pinpoint granules distributed throughout the cytoplasm. Fluorescent foci increased in number and brightness on companion cover slips throughout the remaining observation period. Subsequent passages of this agent have led to 100 percent infection of the cells with a reduction in time of the first appearance of specific fluorescence to as little as 3 days after inoculation by the sixth passage in A-549 cells. At no time during the initial or subsequent passages in A-549 cells was CPE detected or were viral inclusions found by staining with acridine orange or the May-Grünwald technique.

Numerous attempts to induce plaque formation on A-549 cells have not been rewarding. Small indistinct plaques do form under agarose overlay medium; however, their occurrence is irregular and depends on conditions that stress the cells, and the plaques have not been useful for quantitative assay. Infected A-549 cells undergo cell division in an apparently normal manner and without loss of KHF-related specific fluorescence. Infected cells remain IFA-positive for at least 100 days, the longest period of observation to date. Maximum yield of cell culture-adapted agent is obtained at 36° to 37°C with input multiplicities (infectious units per cell) that ensure infection of all cells. Peak yields that average 10 to 15 infectious units per cell are obtained 33 to 42 hours after infection with an input multiplicity of three to five infectious units per cell. Continuous harvests of spent cell culture medium have been made at 24-hour intervals after this peak through the seventh week after infection, and yields average three infectious units per cell per harvest. Input multiplicities of less than one infectious unit per cell result in sharply lower yields during the acute phase of the growth cycle; however, yields nearly comparable to those at high input multiplicity occur once persistent infection is established.

We used serologic tests to establish that the agent isolated and propagated in A-549 cells was KHF-related. In one experiment we used serum samples from 32 humans, including 13 normal (non-KHF) subjects and 19 subjects in the acute or convalescent stage of KHF. We tested these samples by the IFA technique against monolayers of infected A-549 cells. The serums from 16 of the subjects, including nine who were normal or in the acute phase of KHF and from seven convalescents, were also tested simultaneously against frozen sections of lung tissue from KHF agent-infected *Apodemus*. These 16 serum samples had identical or very similar IFA titers in the two test systems. Six of the serum samples were paired specimens (obtained at the acute and convalescent stages) from three KHF patients; all three pairs showed significant (≥ fourfold) increases in IFA titer when tested with either substrate. The 16 remaining serums, which were lyophilized samples collected from well-documented KHF convalescent patients who were studied in Korea from 1966 to 1968 (*8*) also showed positive IFA titers with the infected A-549 cells.

In a second experiment, a human KHF convalescent serum conjugated with fluorescein isothiocyanate (prepared and lyophilized at the U.S. Army 406th Medical Laboratory-Japan in 1968) produced a titer of 1:160 in the direct fluorescent antibody test with infected A-549 cells and 1:160 when tested with frozen sections of infected *Apodemus* lung. In a third experiment, several species of laboratory rodents or nonhuman primates were inoculated with either infected *Apodemus* lung suspensions or spent, cell culture media from the third or tenth passage A-549 cell culture agent. Serum samples obtained before or after inoculation were then tested in the IFA test against KHF-infected *Apodemus* lung sections or KHF-infected A-549 cells. All animals showed significant (≥ fourfold) increases in IFA titer by both test systems. Representative results with serum samples from two New Zealand White rabbits, a baboon (*Papio anubis*), and an owl monkey (*Aotus trivirgatus*) are presented in Table 2.

We conclude that the etiologic agent of KHF propagates and produces IFA detectable antigen in the A-549 human alveolar epithelial cell line. Reisolation of this agent from the fifth passage *Apodemus* (KHF strain 76-118) has been accomplished on three occasions. Characterization of the agent has not progressed as rapidly as might be hoped because of the low yield per cell, the difficulty we have experienced in separating the agent from cell debris, and the less than satisfactory assay system. However, our present results do indicate that the infec-

Table 1. The cultured cells used in this study (*13*) to isolate the etiologic agent of KHF.

| |
|---|
| Continuous cell lines |
| Human lung, alveolar epithelium (A-549) |
| Human adrenal cortex (SW-13) |
| African green monkey kidney (Vero) |
| Rhesus monkey kidney (LLC-$MK_2$) |
| African green monkey kidney (BSC-1) |
| Baby hamster kidney (BHK-21) |
| Rabbit vena cava, endothelium (REVC) |
| Dog kidney (Madin Darby) |
| Diploid cell lines |
| Human embryonic lung (WI-38) |
| Human embryonic lung (MRC-5) |
| Fetal rhesus lung (DBS-103) |
| Primary cell cultures |
| Human embryonic kidney |
| Human peripheral leukocytes |
| *Microtus montanus* whole embryo (vole) |
| Duck embryo fibroblasts |
| *Calomys callosus* kidney, lung, bladder |
| *C. callosus* alveolar and peritoneal macrophages |
| *C. callosus* peripheral leukocytes |

Table 2. Antigenic identity of the cell culture isolate to KHF agent passaged in *A. agrarius coreae*. The animals were inoculated intramuscularly with either a 15 percent suspension of lung tissue from the third passage of the KHF agent (Lee strain) in *Apodemus* or with A-549 cell culture fluids of the third or tenth cell culture passage with KHF agent, strain 76-118. The animals were bled on various days after inoculation. Serum antibody titers to the KHF agent were determined by the indirect fluorescent antibody (IFA) technique against frozen sections of infected *Apodemus* lung tissue or monolayers of infected A-549 cells. The IFA test procedure is described in (*1*).

| Animal | Inoculum | Time after inoculation (days) | IFA titer by test system | |
|---|---|---|---|---|
| | | | *A. a. coreae* | A-549 cell |
| Rabbit | *A. a. coreae* lung P-3 | 0 | < 16 | < 16 |
| | | 14 | 32,768 | 32,768 |
| | | 45 | 4,096 | 4,096 |
| Rabbit | A-549 cells P-10 | 0 | ≤ 16 | ≤ 16 |
| | | 26 | 8,192 | 4,096 |
| Owl monkey | *A. a. coreae* lung P-3 | 0 | < 16 | < 16 |
| | | 35 | 2,048 | 2,048 |
| Baboon | A-549 cells P-3 | 0 | 16 | 16 |
| | | 35 | 512 | 512 |

tious unit is lipid solvent-sensitive and acid labile ($pH \leq 5$), and that a very small proportion ($\leq 10^{-6}$) of the agent population will pass a membrane filter of 100-nm pore size (*1*). Curves showing growth of the agent after inoculation of A-549 cells with large numbers of infectious units reveal an eclipse phase of 6 to 8 hours in which less than $10^{-4}$ of the inoculum is recoverable, an observation that lends additional support to the long-standing hypothesis (*3*) that the etiologic agent of KHF is a virus.

Attempts to isolate the agent from humans and suspected rodent reservoirs in various geographic regions of the world have revealed that A-549 cells are not uniformly susceptible to all strains of the agent or related agents. On the Korean peninsula, for example, native *A. a. coreae* remains the host of choice for initial isolation of the agent. However, replication of KHF strain 76-118 in A-549 cells makes it possible to study the KHF agent in vitro and provides a means of diagnosing hemorrhagic fevers with renal syndrome in laboratories outside Korea. Although *A. a. coreae* is an adequate host for these purposes, the rodent has not been colonized and must be captured and returned to the laboratory from KHF-free areas of the Korean peninsula.

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### References and Notes

1. H. W. Lee, P. W. Lee, K. M. Johnson, *J. Infect. Dis.* **137**, 298 (1978).
2. Department of the Army Technical Bulletin, *TB MED 240* (Government Printing Office, Washington, D.C., 1953).
3. D. C. Gajdusek, *J. Pediatr.* **60**, 841 (1962).
4. J. Lähdevirta, *Ann. Clin. Res.* **3**(Suppl. 8), 1 (1971); A. Svedmyr, H. W. Lee, A. Berglund, B. Hoorn, K. Nyström, D. C. Gajdusek, *Lancet* **1979-II**, 100 (1979); H. W. Lee, P. W. Lee, J. Lähdevirta, M. Brummer-Korvenkontio, *ibid.*, p. 186; H. W. Lee, P. W. Lee, M. Tamura, T. Tamura, Y. Okuno, *Biken J.* **22**, 41 (1979).
5. The indirect fluorescent antibody test used in this study and all reagents, including the human reference positive control KHF antiserum and positive control rodent lung tissue, were described in Lee *et al.* (*1*). Samples of negative control serum were drawn from individuals who had never entered HFRS endemic areas [H. W. Lee, in *Ebola Virus Hemorrhagic Fever*, S. R. Pattyn, Ed. (Elsevier/North-Holland, Amsterdam, 1978), pp. 331-343].
6. C. F. Mayer, *Mil. Surg.* **11**, 176 (1952).
7. R. Traub, M. Hertig, W. H. Lawrence, T. T. Harriss, *Am. J. Hyg.* **59**, 291 (1954).
8. N. H. Wiebenga, A. Hubert, G. R. French, M. R. Smith, in *U.S. Army 406th Medical Laboratory Annual Report* (U.S. Army Medical Research and Development Command, Washington, D.C., 1970), pp. 1-25. The lyophilized convalescent serums were retained through the intervening years in various locations by N. H. Wiebenga who kindly made the entire collection available to us when he learned of our interest in 1976. The samples of acute-phase serum in this collection were not lyophilized and were unfortunately lost in a freezer outage. The direct conjugate was prepared by J. Thomas of Wiebenga's laboratory.
9. J. F. Metzger and D. Arbiter, in *U.S. Army 406th Medical Laboratory Annual Report* (U.S. Army Medical Research and Development Command, Washington, D.C., 1965), pp. 3-5.
10. D. J. Giard, S. A. Aaronson, G. J. Todor, P. Arnstein, J. H. Kersey, H. Dosik, W. P. Porks, *J. Natl. Cancer Inst.* **51**, 1417 (1973).
11. M. Lieber, B. Smith, A. Szakal, W. Nelson-Rees, G. Todoro, *Int. J. Cancer* **17**, 62 (1976). Evidence is presented that the A-549 cell line is of human origin and originated from the neoplastic transformation of type II alveolar cells. It is the opinion of Lieber *et al.* that the A-549 cell line originated from a single cell. The cell line, as used in our laboratory, was obtained in the 26th passage from W. A. Nelson-Rees (with support from the National Cancer Institute Viral Oncology program under the auspices of the Office of Naval Research and the Regents of the University of California). The A-549 cell line is available from the American Type Culture Collection, Rockville, Md., as accession CCL-185.
12. Tissue culture cluster-24, No. 3524, COSTAR, Cambridge, Mass.
13. Previous investigators have used other continuous and primary cell cultures, including dog embryo (R-1247), porcine kidney (PS), mink lung (MV1Lu), Chinese hamster lung (Dede), primary human embryonic lung, and primary rat liver (*1*); HEp-2, HeLa, FL, Detroit, six lines of human origin, and primary Korean hamster heart and kidney (*8*); rhesus monkey kidney (MA-104), newborn rabbit kidney (MA-111), primary hamster kidney (*5*), and rhesus monkey kidney (*1, 5, 8*).
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## Receptor for Albumin on the Liver Cell Surface May Mediate Uptake of Fatty Acids and Other Albumin-Bound Substances

Abstract. *Kinetic analysis of the uptake of carbon-14-labeled oleate in a single-pass perfusion of rat liver and saturable and specific binding of iodine-125-labeled albumin to hepatocytes in suspension suggest the existence of a receptor for albumin on the liver cell surface. The putative receptor appears to mediate uptake of albumin-bound fatty acids by the cell and may account for the efficient hepatic extraction of many other substances tightly bound to albumin.*

Long-chain fatty acids and many other substances, although tightly bound to albumin, are efficiently removed from plasma during passage through the liver. Uptake of these substances has been believed to follow their spontaneous dissociation from albumin in the bulk aqueous phase, with subsequent diffusion of free ligand to the surface of the liver cell. We now present evidence that uptake of long-chain fatty acids takes place by saturable interaction of the fatty acid–albumin complex with a receptor for albumin on the liver cell surface (*1, 2*).

Materials used in this study included oleic acid (Calbiochem), [1-$^{14}$C]oleic acid and Na$^{125}$I (New England Nuclear), bovine serum albumin (fraction V, essentially fatty acid-free), bovine γ-globulin (fraction II), human transferrin, rat serum albumin (fraction V), ovine prolactin, and ovalbumin (Sigma), and Fluosol-43 fluorocarbon emulsion (Alpha Therapeutics). Albumin, after further purification by ion-exchange chromatography, was iodinated by the chloramine-T method to a molar ratio of ~ 1.0 (*3*). Livers were from female Sprague-Dawley rats (Simonsen), aged 50 to 55 days, given free access to a standard laboratory diet. Each liver was perfused for 30 minutes with recirculating oxygenated fluorocarbon in Krebs-Henseleit buffer (Fluosol-43) containing 0.2 percent glucose (weight to volume); this was followed by a 4-minute single-pass perfusion with buffer and glucose alone to remove fluorocarbon from the system. The liver was then perfused (single pass) with a sequence of up to seven solutions containing various concentrations of albumin and [$^{14}$C]oleate in buffer at a flow rate of $3.8 \pm 0.2$ ml/min per gram of liver [mean ± standard error (S.E.)]. Steady-state uptake, achieved within 1 minute, was determined at each substrate concentration. Viability of the liver was assessed by electron microscopy, oxygen consumption, bile flow, and release of cellular enzyme markers (*4*). Fatty acids were extracted from effluent samples (*5*), and 1-ml portions of the organic phase were assayed in duplicate for radioactivity. The amount of [$^{14}$C]oleate esters in the effluent was insignificant for up to 15 minutes after the [$^{14}$C]oleate perfusion was started (*6*), but where appropriate, measurements were corrected for the presence of $^{14}$C-labeled lipids other than fatty acids. No free fatty acids other than oleate were detected in the effluent by gas-liquid chromatography. Net uptake per gram of liver was calculated as the product of the steady-state drop in the [$^{14}$C]oleate concentration across the liver and the flow rate per gram of liver


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