資料4－2

インフルエンザが輸血により伝播する可能性についての文献調査

関連各種論文等(要約)一覧表

血液事業部会運営委員会委員 山口照英

①	N Engl J Med. 1963. 31; 269:964-6	Human Influenza Infection with Proved Viremia, Report of a Case	発症後4日の血液からインフルエンザウイルス(A型Type2)が分離された症例報告。
②	Trans Assoc Phys. 1966: 79: 376-377	Viremia in Asian Influenza	原文取り寄せ中
③	British Medical Journal 1969:4, 208-209	Proved viraemia in Asian influenza (Hong Kong variant) during incubation period	21例のインフルエンザ様症状の患者うち、12例の咽頭ぬぐい液からウイルスを検出。その他潜伏期間中にあった1例より咽頭ぬぐい液及び血液よりウイルスを検出。
④	Can Med Assoc J. 1976 September 4; 115(5): 435-437	Postsplenectomy sepsis due to influenza viremia and pneumococcemia	原文取り寄せ中
⑤	J Hyg Epidemiol Microbiol Immunol. 1979;23(1):35-41	Investigation of the incidence of influenza A viraemia caused by virus strains circulating among children in 1968 - 1977	原文取り寄せ中
⑥	Clin Infect Dis. 1997 Apr;24(4):736-737	Use of the polymerase chain reaction for demonstration of influenza virus dissemination in children	インフルエンザ患者14名の有症状時の血液を調べたところ、いずれからもウイルスは検出されなかった。
⑦	Journal of Medical Virology 58:420-425 (1999)	Detection of Influenza Virus RNA by Reverse Transcription-PCR and Proinflammatory Cytokines in Influenza-Virus-Associated Encephalopathy	インフルエンザ脳症の小児患者でのウイルス同定調査結果。咽頭スワブで100%(9/9)、血漿で0%(0/11)、PMBC(末梢血単核球)で11%(1/9)、赤血球で0%(0／9)、脳脊髄液で9%(1／11)であった。インフルエンザ脳症を起こしていないコントロール群では、咽頭スワブで100%(29/29)であったが、血漿、末梢血単核球、赤血球のいずれからも同定されなかった(0/29)。
⑧	WHO, 19 May 2006	Maintaining a Safe and Adequate Blood Supply in the Event of Pandemic Influenza: Guidelines for National Blood Transfusion Services	インフルエンザへの血液を介しての感染のリスクは極めて低い。これまで、輸血を介してインフルエンザに感染したという報告はなく、呼吸器疾患ウイルスが輸血を介して感染することは、ウイルス量が極端に多い場合を除き、起こりそうにない。重要なことは、(パンデミック下では)血液を通じて感染するリスクは、呼吸器を通じて感染するリスクより、よほど低いことである。
⑨	Transfusion, 47, 1071-1079 (2007)	Planning for pandemic influenza: effect of a pandemic on the supply and demand for blood products in the United States	鳥インフルエンザウイルスのパンデミック対応。1918年のパンデミックインフルエンザであるいわゆる「スペインかぜ」についての検証を行っている。パンデミックにより、血液製剤の採血、製造、輸送に大きな影響が起こりうる。血液サービスで働く従業者も大きく減少する可能性がある。インフルエンザウイルスが輸血によって伝播したという報告は無い。また、一般にインフルエンザを発症していても血液からウイルスが検出されることは無い。しかし、高病原性鳥インフルエンザH5N1のベトナム株やインドネシア株では感染した子供の血清中や血漿中にウイルスが存在するという報告がある。しかし、インフルエンザウイルスのウインドウ期はきわめて短いと想定されることから、歴史的にインフルエンザウイルスが輸血により感染する可能性は低いとされてきているが、H5N1の場合には伝播の懸念が否定できない。

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⑩	Transfusion, 47, 1080-1088 (2007)	Influenza viremia and the potential for blood-borne transfusion	鳥インフルエンザウイルスのパンデミックが起こった場合を想定し、輸血によるウイルス伝播の可能性について考察した。これまで輸血によるインフルエンザウイルスの伝播について報告された事例は無い。インフルエンザウイルス血症は極めてまれにしか起きないこと、及び無症候の献血者からしか採血されないことを考えると、血液によってウイルスが伝播する可能性はきわめて低いと想定される。仮に輸血によりインフルエンザウイルスの伝播が起こるとすると、輸血を受けた免疫抑制状態の患者では重症化や致死率が上昇する可能性はある。ウイルス血症に関するデータは殆ど無く、1960-1970年代の古いデータである。殆どのデータが発症後にサンプリングされた検体でのデータである。インフルエンザ脳症を起こしている患者の血液や脳髄液にはウイルスが検出されることはまれなのに、インフルエンザ脳症患者では全身にウイルスが広がることが示唆されている。このことは、脳神経症状の発症には脳髄液でのウイルスの存在は必要がないこと、換言すれば、ウイルス血症や脳脊髄液でのウイルスの出現の前に、インフルエンザ脳症が発症しうることを意味しているかもしれない。
⑪	Vaccine, 26, D59-D66 (2008)	Pathology of human influenza revisited	H5N1は肺や気管支上皮に感染しやすく、そのために感染部位から拡散しやすい性質を持つ。季節性インフルエンザと異なり、H5N1はウイルス血症及び呼吸器系外へ感染が広がる可能性が高い。H5N1がウイルス血症を起こす可能性としては2つのルートが考えられる。一つには、肺胞へ感染したウイルスが組織破壊を起こした際に、血管バリアーが壊れウイルスが血中にもれてしまう可能性。もう一つの可能性として、増殖したH5N1が積極的に血液中に入っていく可能性が考えられる。これまでインフルエンザウイルスがウイルス血症を起こしたという報告(1-4)もあるが、逆には発症前には血液中にウイルスを検出できないとする報告(5-7)もある。季節性インフルエンザウイルスに関してはウイルス血症を起こす可能性は低く、万が一起こしたとしても極めて短い期間であろう。H5N1では16人中9人がウイルス血症を起こしたとう報告がある。
⑫	FDA (Nov 2009)	Guidance for Industry Recommendations for the Assessment of Blood Donor Suitability, Blood Product Safety, and Preservation of the Blood Supply in Response to Pandemic (H1N1) 2009 Virus DRAFT GUIDANCE	2009H1N1インフルエンザウイルスによるウイルス血症については、限られた情報しか得られていないが、米国その他の地域において、輸血により季節性インフルエンザに感染した事例は報告されておらず、同様に輸血により2009H1N1インフルエンザに感染した事例は報告されていない。現時点において、2009H1N1インフルエンザに感染した無症候状態の者の血液や血清から2009H1N1インフルエンザウイルスは分離されていないが、研究は継続中である。輸血による2009H1N1インフルエンザ感染の可能性は不明のままである。

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MEDICAL INTELLIGENCE

HUMAN INFLUENZA INFECTION WITH PROVED VIREMIA*

Report of a Case

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ALTHOUGH there is some indirect evidence in the medical literature that viremia may occur during human influenza infections[1,2] the isolation of this virus from a patient's blood has not, to the best of my knowledge, been reported. The present communication describes the isolation of influenza virus Group A, Type 2, from both the blood and throat secretions of a patient with clinical manifestations of influenza.

CASE REPORT

Three days before admission a 40-year-old physician noted the onset of severe headache and generalized malaise. He did not believe that he was febrile. This continued until the day before admission, when he noted shaking chills, and the temperature rose to 104°F. At that time he felt confused and somewhat restless. On the morning of admission to the Peter Bent Brigham Hospital he had several bouts of shaking chills followed by fever.

At 32 years of age an episode of fever accompanied by chest and arm pain had resulted in hospitalization and a diagnosis of idiopathic pericarditis. At the time of discharge from the hospital the electrocardiogram had returned to normal. No immunization against influenza had been taken at any time.

Physical examination revealed no abnormalities other than an enlarged thyroid gland. Throughout the hospital course the lungs were clear to percussion and auscultation, and the heart sounds were normal, without any murmur or rub.

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The white-cell count was 10,650, with 60 per cent neutrophils, 23 per cent band forms, 15 per cent lymphocytes, 1 per cent monocytes and 1 per cent basophils. The hematocrit was 51.5 per cent, and the corrected erythrocyte sedimentation rate 4 mm. per hour. Throat culture grew alpha-hemolytic streptococci and *Diplococcus pneumoniae*. Sputum culture revealed alpha-hemolytic streptococci, *D. pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*. Febrile agglutination tests, including typhoid O and H, paratyphoid A and B, *Brucella abortus* and *Proteus X-19*, were negative.

The temperature on admission was 99.4°F., and on the following day the patient experienced shaking chills and the temperature rose to 103.6°F. On the 3d and 4th days the temperature did not rise over 100.2°F., but on the 5th day he again had a shaking chill accompanied by a rise in temperature to 102.8°F. From the 6th day on, he had only low-grade fever and noted a gradual diminution of the marked malaise. He became completely afebrile on the 9th hospital day. An electrocardiogram obtained on the 3d hospital day showed flattened T waves in Lead V_4. A chest film on the 4th day revealed that the heart was somewhat enlarged in transverse diameter as compared with films taken after the previous bout of pericarditis. Fluoroscopy on the 6th day showed enlargement of the left ventricle. Five days later an electrocardiogram revealed a normal T wave, and a chest film showed normal cardiac size and shape.

On the 2d hospital day specimens were obtained for attempts at viral isolation. These included clotted blood, a throat wash and a stool specimen that was made up as a 10 per cent suspension in tissue-culture medium.

Aliquots of each of these specimens (0.2 ml.) were inoculated into both primary grivet monkey-kidney and human-amnion cell cultures as well as into the amniotic sac of 7-day-old embryonated hens' eggs. Inoculated cultures and uninoculated controls were tested for hemadsorption 8 or 10 days after inoculation. The amniotic fluid of inoculated and control eggs was harvested on the 5th to the 7th day after inoculation and tested for hemagglutinating factors. Table 1 summarizes the results of the laboratory tests involved in the original isolation, passage and reisolation of the agent.

Hemagglutination-inhibition tests with guinea-pig red cells were used as a means of identifying the agent present in amniotic fluid that had been inoculated with passage material. Tests in accordance with the hemagglutination-inhibition technic recommended by the Committee on Standard Serological Procedures in Influenza Studies[4] revealed no inhibition with antibody to prototype influenza A (PR-8) and B (Arizona) strains. Antiserum to influenza Group A, Type 2 (Asian), inhibited hemagglutination by both the blood and the throat agent in dilutions up through 1:180. Subsequently, the hemagglutinating agent thus identified was reisolated in eggs from both blood and throat specimens (Table 1).

Using blood obtained on admission as an acute-phase and blood obtained 2 weeks after admission as a convalescent-phase specimen, an assay was done for hemagglutination-inhibition antibody. The blood agent, throat agent and a standard strain of influenza virus Group A, Type 2, were all used as antigens. The acute-phase blood in dilutions as low as 1:5 did not inhibit hemagglutination with any of the 3 agents, whereas the convalescent-phase serum inhibited hemagglutination by blood, throat and standard antigen against influenza virus Group A, Type 2, in dilutions through 1:80.

TABLE 1. *Summary of Attempts to Isolate Influenza Virus from Various Materials.*

MATERIAL	RESULTS OF 1ST ATTEMPT*			RESULTS OF 2D ATTEMPT						
	ORIGINAL INOCULATION			1ST PASSAGE			ORIGINAL INOCULATION		1ST PASSAGE		
	human amnion	*grivet monkey kidney*	*egg†*	*human amnion*	*grivet monkey kidney*	*egg†*	*grivet monkey kidney*	*egg†*	*grivet monkey kidney*	*egg†*	*identification‡*
Throat	–	–	+	–	+	+	–	+	+	+	Influenza virus Group A, Type 2
Blood	–	+	+	–	+	+	+	+	+	+	Influenza virus Group A, Type 2
Stool	–	–	–	–	–	–	–	–	–	–	
Controls	–	–	–	–	–	–	–	–	–	–	

*Virus recovered.

†7-day-old embryonated hens' eggs, inoculated intra-amniotically.

‡By hemagglutination-inhibition tests.

An attempt was made to determine the amount of virus present in both the blood and throat specimens. Serial half-log dilutions were made of both specimens and then inoculated into eggs. Only the undiluted specimens were positive.

DISCUSSION

Repeated isolation of influenza virus Group A, Type 2, from a specimen of this patient's blood gives clear evidence that on occasion viremia may occur in influenza caused by this agent. A report of the detection of influenza virus from the liver, spleen, kidney, heart and lymph nodes of patients who died during the outbreak of Asian influenza in 1957[3] strongly suggests that the virus might enter the circulation during the course of the disease. The report of isolation from human urine by another investigator[2] affords additional evidence that viremia may occur. Hamre, Appel and Loosli[4] have shown that viremia may be established in mice after intranasal inoculation of influenza virus Group A (PR-8). A low titer of virus was sporadically demonstrable in the blood only of mice that had a high viral concentration in their lungs. These investigators suggested that viremia in mice might arise as a result of a spillover from the pulmonary focus. If one accepts such a mechanism in human influenza infection, it seems logical to look for viremia at the peak of pulmonary infection rather than at an earlier stage.

Loosli and his co-workers[5] have shown that in mice given airborne influenza infection, both pneumonia and antibody to the agent develop. When mice are given influenza antibody intraperitoneally at the time of viral inoculation pneumonia but not active immunity develops. These results are interpreted by Hamre, Appel and Loosli[4] as indicating a need for generalized spread of virus to antibody-forming sites before active immunity can occur. If this assumption is correct and if it also applies to human influenza infections one can hypothesize that viremia of at least some degree occurs in all patients with influenza infection in whom antibody to the agent develops.

To my knowledge there have been no previous reports of the isolation of influenza virus from the blood of patients. Two papers[6,7] have noted unsuccessful attempts at such isolation. The possibility also exists that there are many unpublished accounts of other unsuccessful attempts at such isolation. One unpublished study by Gresser and Dull[8] includes 9 patients with the clinical signs and symptoms of influenza, with isolation of influenza virus from the throat washings of 7 and without isolation of the virus from any of the blood specimens when the washed leukocyte fractions were tested. It is difficult to account for the differences between the present case and the previous cases studied. As previously suggested,[7] the viremia in influenza may be quite transient, and by chance the present specimen was obtained at the proper time. Another unlikely possibility is that the present patient had some immunologic defect. However, both this patient and those studied by others[7] had no detectable hemagglutination-inhibiting antibody at the time blood was drawn for viral studies. Furthermore, medical history, antibody response to the agent isolated and serum electrophoretic pattern give no indication of any abnormalities of the present patient's immune mechanism. Minuse and his associates[7] suggest that nonspecific inhibitors in the patients' blood may have accounted for their failure to demonstrate influenza virus in blood specimens. The possible lack of such inhibitors was not investigated in the present patient.

SUMMARY

Influenza virus Group A, Type 2, was isolated and reisolated from both the throat washings and blood specimens of a forty-year-old physician hospitalized with shaking chills and fever. A significant rise in hemagglutination-inhibiting antibody was demonstrated both to the agent isolated from the patient and to the standard influenza antigen. Although the report of isolation of influenza virus at autopsy from many of the organs of influenza patients gives evidence of a viremia phase in human influenza, the present study is believed to be the first report of a direct isolation of influenza virus from a patient's blood.

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BRIEF RECORDING

Hemolytic Reaction after Novobiocin Therapy

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A SIX-year-old girl was seen at the United States Army Hospital, Fort Rucker, Alabama, with a chief complaint of mild jaundice and dark urine present for one day. The child had previously been seen by a civilian physician, five days before admission, because of sore throat and fever, with a maximum temperature of 105°F. At that time she had been thought to have pharyngitis, and novobiocin, 30 mg. per kilogram of body weight per day, was started by mouth. The fever subsided and the patient improved. However, on the day of admission she was noted to be mildly jaundiced and had been passing dark-brown urine.

Physical examination disclosed icteric sclerae and pale mucous membranes and conjunctivas. The throat was red, but no exudate was present. The remainder of the physical examination was negative.

The initial impression was that of hepatitis. A blood specimen revealed marked hemolysis on three different occasions, and a hemolytic reaction was suspected. The initial white-cell count was 4800, with a normal differential. The hemoglobin was 7.0 gm. per 100 ml., and the reticulocyte count 1.1 per cent. The blood urea nitrogen was 24.2 mg. per 100 ml. The remainder of the blood chemical findings, including the antistreptolysin-O titer, were within normal limits. A red-cell fragility test showed hemolysis at 0.50 per cent and ending at 0.00 per cent. A tourniquet test was negative. The platelet count was normal. Blood cultures were negative at ten days. During the first twenty-four hours in the hospital the hemoglobin dropped to 4 gm. per 100 ml. The urine was within normal limits except for a trace of bile and a positive test for hemoglobin. The direct and indirect Coombs tests were positive. The blood was Type O+, and two transfusions of this type of blood were given. Prednisone (Meticorten), 40 mg. per day, was started. After the two blood transfusions the hemoglobin rose to 10.2 gm. per 100 ml. The reticulocyte counts increased steadily from 1.1 per cent to a high of 10.2 per cent just before discharge on the fifteenth hospital day. The hemoglobin rose slowly from 10.2 gm. per 100 ml. after transfusions to a discharge level of 13 gm.

The urine cleared within two days after transfusion and institution of cortisone therapy, and the patient became essentially asymptomatic. She was discharged on the fifteenth hospital day with a final diagnosis of acquired hemolytic anemia.

It is possible that the hemolysis resulted from sepsis, but this is unlikely in view of the normal white-cell counts and the absence of fever during the hospital stay. This hemolytic reaction could also have been of the idiopathic variety, but novobiocin remains strongly suspected as the etiologic agent.

The patient has been seen on several follow-up visits, and the hemoglobin is holding steady at 13.5 gm. per 100 ml. The Coombs tests, direct and indirect, have returned to negative, and she is doing well.

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BY THE LONDON POST

Lord Nuffield — Pharmacy in Britain — Holiday Reading

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THE story of the life of Lord Nuffield, who died in August at the age of eighty-six, is like a fairy tale. Born in 1877, William Morris was educated in local schools until the age of sixteen, when, having shown some mechanical aptitude, he was sent to work in a bicycle shop in Oxford. Within a year he had borrowed £4, with which he opened a shop on his own account. He started by repairing bicycles, then he sold and raced them, and later he produced a model of his own. In his first six years of bicycle manufacturing he accumulated £2,000 of capital, and in another ten years, by the age of thirty-three, he had doubled that amount. By 1911 there were some 50,000 private motorists in Britain, and in the following year the Morris car appeared. During World War I the Morris works were turned over to war work, but at the end of hostilities motorcar production was started in earnest. In 1922 nearly 7000 cars were sold, and by 1925 the annual figure had risen to over 52,000.

His business success thus assured, he began to direct his attention to giving financial support to advance the study and practice of medicine, which, in fact, had secretly been his own first choice of career.

Proved Viraemia in Asian Influenza (Hong Kong Variant) During Incubation Period

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British Medical Journal, 1969, 4, 208–209

Summary: During an outbreak of influenza specimens were obtained from 21 patients with influenza-like illnesses and from 29 healthy subjects in close contact with the patients. Throat washings from 12 of the patients were positive for influenza virus but virus was not detected from the blood specimens. One healthy contact became ill 12 hours after the specimens were obtained, and the virus was isolated from his blood and throat washings. The remaining contacts showed no clinical illness ; but the virus was isolated from the throat washings of four of them, with no viral isolation from the blood specimens.

Introduction

The occurrence of viraemia in influenza infection has been suspected after recovery of the virus from extrapulmonary tissues of man and animals (Hamre *et al.*, 1956 ; Kaji *et al.*, 1959 ; Oseasohn *et al.*, 1959). One of us (K. N.) reported isolation of Asian influenza virus from blood and throat-washing specimens of a physician suffering from an influenza-like illness (Naficy, 1963). Our further attempts to detect influenza virus from 18 proved cases of influenza were unsuccessful (K. Naficy, unpublished data). Stanley and Jackson (1966) showed that viraemia in influenza occurred in their human volunteers only during the first three days of the incubation period. We here report the successful isolation of Asian influenza virus, Hong Kong variant, from blood and throat-washing specimens of a patient who was in the incubation period, and an unsuccessful attempt to find viraemia in the same person and 21 other patients while demonstrating the clinical manifestations of influenza-illness.

Materials and Methods

Subjects.—In mid-December 1968 we were informed of an outbreak of influenza illness among prisoners of the Tehran Ghasr Prison. The outbreak had apparently been present for a few weeks, during which period more than 200 prisoners had contracted the disease. Specimens were obtained from 21 patients in the first 24 hours of their illness, as well as from 29 healthy individuals who denied having had influenza-like symptoms in the two weeks prior to our visit. Both groups of prisoners gave informed consent to these procedures.

Specimens.—Throat washings and clotted and heparinized blood were obtained from all subjects. Sera from clotted blood were stored at −20° C. before use for serological tests ; throat washings and heparinized blood specimens were either inoculated within a few hours of collection or stored at −70° C. before inoculation. Second blood specimens were obtained three weeks later from only nine subjects.

Viral Isolations.—Each specimen was inoculated in a volume of 0·1 ml. into the amniotic sac of three 10-day-old embryonated hen's eggs, and incubated at 35°C. for 40 hours, then left at 4° C. overnight before harvesting the amniotic fluid. The fluid was tested for haemagglutinating activity ; if positive, passage was carried out allantoically, otherwise at least one blind passage was performed amniotically.

Haemagglutination and Haemagglutination Inhibition Tests.—These tests were carried out according to the standard technique modified for microtitration by four haemagglutination units of antigen and chick red cells.

Reference Influenza Virus, Hong Kong Variant.—Importation of Hong Kong variant influenza virus to Iran apparently occurred during the Eighth International Congresses on Tropical Medicine and Malaria, Tehran, 7–15 September 1968. During the congresses one-third of the participants contracted the disease, and several strains were isolated from them in our laboratories (Saenz *et al.*, 1969) ; these were confirmed by Dr. Pereira of the W.H.O. World Health Influenza Centre to be Hong Kong variant A2. One strain of these isolates—designated 30T—was used as a reference antigen.

Clinical Investigation.—Owing to the absence of any medical record in the Ghasr Prison, one of us (M. K.) made a daily visit to our subjects for six days and conducted clinical follow-ups.

Results

Clinical Manifestation.—Clinical manifestation of the disease consisted of fever, headaches, and generalized symptoms such as malaise, chills, anorexia, muscular pain, cough, sore throat, and chest pain in most of our patients, lasting from one to four days. No bacterial complication, encephalitis, or myocarditis was noted. All healthy subjects remained asymptomatic during the entire period of observation except one who developed fever and generalized symptoms 12 hours after the specimens were obtained.

Viral Isolation.—Twelve out of 21 throat-washing specimens obtained from the patients were positive for influenza virus either in the original inoculation or after the first passage. No virus was detected from the blood specimens of these patients in spite of two blind passages. Haemagglutination inhibition antibody determination in paired sera of seven patients revealed eightfold or greater rise both to the isolates and the reference antigen, except in one case. Table I summarizes these results in cases with positive viral isolations. No virus was isolated from the blood specimens of 28 healthy individuals who were in close contact with the patients and remained asymptomatic

TABLE I.—*Antibody Titres to Asian Influenza (Hong Kong variant) in 12 Patients with Positive Virus*

Patient's No.	Viral Isolation		Reference Antigen		Isolate	
	Throat	Blood	Acute	Convalescent	Acute	Convalescent
1	+	−	<1:8	1:64	<1:8	1:32
2	+	−	1:32	1:1,024	1:16	1:512
3	+	−	1:64	1:1,024	1:16	1:1,024
4	+	−	<1:8	1:128	<1:8	1:64
5	+	−	1:256	1:256	<1:128	1:128
6	+	−	<1:8	1:512	<1:8	1:256
7	+	−	<1:8	1:1,024	<1:8	1:512
8	+	−	<1:8	N.T.	<1:8	N.T.
9	+	−	<1:8	N.T.	<1:8	N.T.
10	+	−	1:16	N.T.	<1:8	N.T.
11	+	−	1:256	N.T.	1:256	N.T.
12	+	−	N.T.	N.T.	N.T.	N.T.

N.T. = Not tested.

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during our six-day observation, but throat washings from four subjects were positive. One healthy subject developed clinical illness 12 hours after blood and throat-washing specimens were obtained. These were positive for influenza, and the blood isolates were sent to the World Influenza Centre, being confirmed by Dr. Pereira to be the Hong Kong variant. Reisolation of the virus from the original blood specimen was successful, but no virus was detected from the blood specimens obtained 12 and 24 hours after clinical manifestation. The paired sera of this case showed a 16-fold rise both to the blood isolate and to the reference antigen. Table II lists viral isolation and haemagglutination inhibition antibody of healthy contacts, with positive isolations.

TABLE II.—*Antibody Titres to Asian Influenza (Hong Kong variant) in 5 Healthy Contacts with Positive Virus*

Contact's No.	Viral Isolation		Reference Antigen		Isolate	
	Throat	Blood	Acute	Convalescent	Acute	Convalescent
1*	+	+	1 : 8	1 : 256	< 1 : 8	1 : 128
2	+	−	1 : 16	N.T.	< 1 : 8	N.T.
3	+	−	1 : 1,024	1 : 128	1 : 512	1 : 64
4	+	−	1 : 1,024	N.T.	1 : 512	N.T.
5	+	−	1 : 32	N.T.	1 : 16	N.T.

* Developed clinical symptoms of influenza 12 hours after obtaining specimen.

Discussion

Recovery of influenza virus from extrapulmonary tissue of man and animals was the first indication of the occurrence of viraemia during influenza infection (Hamre *et al.*, 1956 ; Kaji *et al.*, 1959 ; Oseasohn *et al.*, 1959).

Recovery of influenza virus from a patient's blood with clinical manifestations of the disease was the first report of proved viraemia in man (Naficy, 1963). Several other investigators, however, had failed to demonstrate viraemia during the clinical course of influenza infection (Kilbourne, 1959 ; Minuse *et al.*, 1962 ; K. Naficy, unpublished data). Stanley and Jackson (1966), using human volunteers, showed clearly that viraemia occurs during the incubation period and that the virus was not detected after the third day of infection. Our results demonstrate that in 12 out of 21 patients with clinical signs of influenza virus was isolated from the throat-washing specimens but none from their blood ; while in one patient—who proved to be in the incubation period at the time specimens were obtained—virus was obtained from both the blood and the throat washings. These results are in agreement with Stanley and Jackson's report and clearly explain accounts of unsuccessful attempts to demonstrate viraemia during the symptomatic phase of influenza infection.

The first successful report of the isolation of influenza virus from human blood, however, remains unexplained, since the isolation was made while the patient was symptomatic. Nevertheless, a review of the history of this patient showed that there had been two phases of clinical symptoms: (1) before admission and during the first two days of hospitalization, after which he became almost asymptomatic ; and (2) a second phase from the fifth day, when he again experienced fever and chills (Naficy, 1963). Thus it is conceivable that fever and chills on the fifth day of hospitalization marked the onset of his influenza, unrelated to his undetermined previous infection, and the specimens were obtained during the incubation period.

It should be noted that four healthy subjects from whom virus was isolated remained asymptomatic. Two of these had a high haemagglutination inhibition antibody titre (1 : 1,024) in their acute sera. Thus it seems that, in spite of high circulating antibody, local replication of the virus in the nasopharyngeal cavity takes place, and may play a part in spreading the infection.

We wish to thank Dr. Pereira of the W.H.O. World Influenza Centre for his help in confirming the Hong Kong variant of our isolates, and Dr. Jamshidy of the Ghasr Prison health centre for his co-operation.

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Medical Memoranda

Thrombotic Microangiopathy Associated with Squamous Carcinoma

British Medical Journal, 1969, 4, 209–210

The association of malignant disease with thrombophlebitis migrans is well recognized, and in some instances the lesions of the veins may be the first indication of occult malignant disease (Sproul, 1938). Such patients may also have non-bacterial thrombotic endocarditis (MacDonald and Robbins, 1957). On the other hand, disseminated arteriolar and capillary lesions occur much less frequently and do not normally give rise to clinical manifestations (McKay and Wahle, 1955 ; Azzopardi, 1966). Recently we had the opportunity of studying a patient who presented with features of thrombotic thrombocytopenic purpura, and only at necropsy did it become clear that these were associated with widespread thrombosis of small vessels and recurrent carcinoma.

Case Report

The patient was 56 years old when she was first seen in December 1963 complaining of rectal bleeding. This proved to be due to a rather poorly differentiated squamous carcinoma situated in the anal canal. Metastatic squamous carcinoma was also found in inguinal lymph nodes removed in a block dissection seven months later. After this, however, she remained well for nearly five years until bleeding occurred from the colostomy in November 1968. When admitted to hospital, after having symptoms for three days, she was severely anaemic and had a thrombocytopenia (Hb 3·7 g./100 ml., white cells 9,000/cu. mm., and platelets 65,000/cu. mm.). Blood transfusion brought some improvement in the haemoglobin level. Six days after admission, however, the platelet count was still only

Underlying diseases associated with pulmonary pseudallescheriasis include diabetes mellitus, leukemia, lymphoma, aplastic anemia, Cushing's disease, collagen-vascular diseases, and alveolar proteinosis. *P. boydii* may cause pulmonary infiltration (with or without cavitation) to occur and fungus balls to develop. However, to our knowledge, we report the first case of intrabronchial pseudallescheriasis. Moreover, we also report the first case of pseudallescheriasis in a healthy person who had no immunologic defects. Since *Pseudallescheria* species and *Aspergillus* species both produce septate hyphae and share some morphologic features, *Pseudallescheria* may be histologically misdiagnosed as *Aspergillus* in the absence of identification by culture [9, 10]. Although in our case the endobronchial biopsy findings were initially thought to be consistent with aspergillosis, the fungus was identified as *S. apiospermum* by culture.

Itraconazole therapy was administered after the fungus was identified since the MIC of this drug was lower than that of other drugs. However, the intrabronchial lesion persisted after 12 weeks of itraconazole therapy.

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Use of the Polymerase Chain Reaction for Demonstration of Influenza Virus Dissemination in Children

Most investigators believe that influenza virus does not usually induce viremia [1]. Although CNS, cardiac, and skeletal muscle complications have been described in relation to influenza, virus was successfully isolated from the blood and extrapulmonary organs in only a limited number of cases [1, 2]. We recently demonstrated with use of PCR that influenza A/PR/8 virus produces viremia in a mouse model during the acute phase of disease [3].

We searched for influenza virus in the blood and CSF of children with virologically confirmed influenza from 22 December 1994 to 26 March 1995 (table 1). Patients ranged in age from 6 months to 8 years; bronchiolitis was clinically diagnosed in four cases, bronchitis in five cases, and upper respiratory infection in six cases. No abnormal shadows were found in the lung fields on any of the children's chest roentgenograms. None of the children had a history of recurrent serious infectious diseases.

Serum hemagglutination inhibition titer of antibody to A/Kitakyushu/159/93 (H3N2) virus significantly increased (at least a fourfold increase from acute titer to convalescent titer) in 12 cases, it significantly increased to B/Mie/1/93 virus in five cases, and it significantly increased to both strains in two cases. Culture of throat swab specimens in MDCK cell suspension yielded H3N2 virus for 4 of 12 children. PCR and successive Southern hybridization were performed with primer sets for influenza A and B virus matrix gene as previously described [3, 4]. Influenza A and B viruses were detected by PCR in eight and two cases, respectively. However, blood fractions of virus could not be detected by PCR in any of the 14 cases (table 1).

Six children, including two epileptic patients with mental retardation, had convulsions during the course of our study. One child showed signs of somnolence. Because CNS infection was suspected in these cases, CSF was examined for a greater than normal number of cells and an increased protein concentration; however, pleocytosis was not detected, and the protein concentration was within normal limits. PCR was performed with these CSF samples, but they were negative for influenza A and B virus. Influenza virus was not isolated from blood samples or CSF.

This study has verified that viremia and transmission of the virus to the CNS cannot be easily detected among children infected with recent strains of influenza virus. We have previously shown that the PR8 strain of influenza A virus becomes viremic in immunocompetent mice [3]. Furthermore, we tentatively concluded that the virus enters the bloodstream through the infected alveolar septum. This hypothesis is supported by the finding that viremia does not occur when alveolitis is prevented by previous intraperitoneal administration of the antiserum to the virus. The fact that it was difficult to detect viremia among the children in our study might support this hypothesis since none of our patients had obvious pneumonia on the basis of chest roentgenogram findings.

In addition, we could not find any direct evidence that influenza virus invades the CNS of these infected children. Rantala et al. described the successful isolation of influenza B virus from the CSF of a child with febrile convulsions [2]. It might be possible that a certain strain of influenza virus induces systemic dissemina-

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Detection of Influenza Virus RNA by Reverse Transcription-PCR and Proinflammatory Cytokines in Influenza-Virus-Associated Encephalopathy

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Eleven children with acute encephalopathy associated with an influenza virus infection were treated during the 1997–1998 influenza season. Reverse transcription-polymerase chain reaction (RT-PCR) assay was used to detect the viral genome in peripheral blood and cerebrospinal fluid (CSF) samples. The results were compared with those of control influenza patients without neurological complications. Viral RNA was detected only in the peripheral blood mononuclear cells of one patient with influenza-virus-associated encephalopathy (1 of 9; 11%) and in the CSF of another patient (1 of 11; 9%). RT-PCR was negative in the blood of all the controls, but the percentage of RT-PCR-positive samples in the two groups was not significantly different. Cytokines and soluble cytokine receptors in plasma and CSF were then quantified using an enzyme-linked immunosorbent assay. The CSF concentrations of soluble tumor necrosis factor receptor-1 were elevated in two patients and interleukin-6 (IL-6) was elevated in one patient with influenza-virus-associated encephalopathy. On the other hand, the plasma concentrations of IL-6 were elevated in four of nine patients. The number of encephalopathy patients who had elevated plasma concentrations of IL-6 100 pg/ml was significantly higher than that of controls ($P = .01$). In conclusion, the infrequent detection of the viral genome in the CSF and blood showed that direct invasion of the virus into the central nervous system was an uncommon event. Proinflammatory cytokines and soluble cytokine receptors may mediate the disease. The high plasma concentration of IL-6 could be an indicator of the progression to encephalopathy. ***J. Med. Virol. 58:420–425, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: influenza virus; encephalopathy; RT-PCR; interleukin 6

INTRODUCTION

Infection with influenza viruses can produce a spectrum of clinical responses ranging from a febrile upper respiratory illness to central nervous system (CNS) involvement with significant mortality. After the first human influenza virus was isolated in 1933, several examples of influenza-associated encephalopathy have been reported. Two specific types of acute encephalopathy are reported to accompany influenza infection: Reye syndrome and influenza-associated encephalopathy. Reye syndrome, which is a neurologic and metabolic disease with hepatic dysfunction and fatty accumulation in the viscera, often follows viral infections and the use of salicylate [Balistreri, 1996].

Influenza-associated encephalopathy, which occurs at the height of illness and may be fatal, has been described by many investigators [Dunbar et al., 1958; Flewett and Hoult, 1958; McConkey et al., 1958; Delorme and Middleton, 1979; Protheroe and Mellor, 1991; Murphy and Webster, 1996]. The cerebrospinal fluid (CSF) is usually normal, the brain shows severe congestion at autopsy, and histological changes are minimal [Murphy and Webster, 1996]. The pathogenesis of this CNS syndrome is, however, unclear. In regards to the viral pathogenesis, one explanation is that CNS complications may be caused by hematogenous transmission of the virus to the CNS, although the existence of viremia is disputed and isolation of the in-

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Table 1. Use of PCR for detection of Viremia in children with virologically confirmed influenza.

Age	Date of onset of influenza	Clinical sign; diagnosis	Temperature		Serum HAI antibody titer to influenza virus			Virus isolation		Sample (d)*	Results of PCR				
			Conv. Acute	°C No. of days	H1N1	H3N2	B	Conv. Acute	Throat		Throat	PBMC	RBC	Plasma	CSF
6 mo	12/22/94	FC; bronchiolitis	40.0	6	<4 <4	<4 256	<4 <4	32 32	ND	2	ND	ND	ND	ND	–
8 y†	1/8/95	Convulsion; bronchitis	40.4	5	128 128	<4 1,024	32 32	0	–	–‡	–	–	–	ND	
2 y	1/11/95	Bronchitis	40.2	7	<4 <4	<4 128	32 64	1	–	A§	–	–	–	ND	
3 y	1/11/95	Bronchitis	41.5	5	<4 <4	<4 128	32 32	1	ND	ND	–	–	–	ND	
3 y	1/12/95	FC; URI	39.5	7	<4 <4	<4 128	16 16	1	–	A§	–	–	–	–	
2 y	1/12/95	Bronchitis; URI	39.8	5	8 8	<4 64	8 8	0	H3N2	A§	–	–	–	–	
1 y, 5 mo	1/25/95	FC; URI	40.3	6	<4 <4	<4 128	<4 <4	0	H3N2	A§	–	–	–	–	
9 mo	2/1/95	URI	40.1	11	<4 <4	<4 256	8 8	1	H3N2	A§	–	–	–	–	
1 y	2/5/95	FC; bronchitis	39.0	3	<4 <4	<4 128	32 128	1	ND	ND	–	–	–	–	
5 y	2/5/95	Convulsion; bronchitis	39.2	5	<4 <4	<4 128	<4 128	1	–	A§	–	–	–	ND	
1 y, 4 mo	2/9/95	Bronchiolitis	39.7	7	<4 <4	<4 128	<4 <4	5	H3N2	A§	–	–	–	ND	
1 y, 4 mo	2/9/95	Bronchiolitis	38.5	8	<4 <4	<4 512	<4 <4	5	–	A§	–	–	–	ND	
4 y	2/13/95	URI	40.0	5	<4 <4	256 256	16 2,048	4	–	B§	–	–	–	ND	
5 y	3/5/95	URI	40.0	2	<4 <4	256 256	32 128	1	–	B§	–	–	–	ND	
1 y, 7 mo	3/26/95	Bronchiolitis	39.6	7	<4 <4	<4 <4	<4 32	2	–	–	–	–	–	ND	

NOTE. Conv = convalescent; FC = febrile convulsion; HAI = hemagglutination-inhibiting; ND = not done; PBMC = peripheral blood mononuclear cells; URI = upper respiratory infection.

* No. of days after the onset of illness.
† This patient had a history of intractable epilepsy and mental retardation.
‡ Negative for both influenza A and B viruses.
§ Positive for influenza A virus–specific sequences.
‖ Positive for influenza B virus–specific sequences.

tion if it is pneumotropic enough to cause pneumonia. Host factors should also be considered when investigating virus spread in immunocompromised individuals because one might expect them to have more serious illnesses [5].

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TABLE I. Clinical Features of Patients With Influenza-Virus-Associated Encephalopathy

Patient no.	Age (years)/ Sex	GCS	Convulsion	Cerebrospinal fluid		Serum			Mortality and morbidity
				Cell count (/μl)	Protein (mg/dl)	AST (IU/L)	ALT (IU/L)	NH_3 (μg/dl)	
1	2/F	11	Yes	2	24	92	24	18	Recovery
2	2/M	12	Yes	15	27	49	15	34	Recovery
3	2/M	13	Yes	0	13	26586	13879	74	Recovery
4	2/M	12	Yes	6	21	56	41	53	Recovery
5	3/M	3	Yes	0	57	18088	10472	50	Sequelae
6	5/M	3	Yes	6	20	1276	1667	37	Sequelae
7	6/F	3	Yes	NA	NA	32	14	NA	Recovery
8	6/F	11	No	0	10	39	17	NA	Sequelae
9	11/M	11	No	NA	NA	200	72	NA	Sequelae
10	11/M	11	Yes	3	23	35	13	21	Recovery
11	13/F	3	Yes	NA	NA	10510	3160	NA	Death

GCS, Glasgow Coma Scale; AST, aspartate aminotransferase; ALT, alanine aminotransferase; F, female; M, male; NA, not applicable.

fluenza virus from CSF is rare [Stanley and Jackson, 1969; Lehmann and Gust, 1971; Mori et al., 1997; Tsuruoka et al., 1997].

In the 1997–1998 flu season, 11 children with acute influenza-virus-associated encephalopathy were treated. Reverse transcription-polymerase chain reaction (RT-PCR) assay was used to detect the viral genome in peripheral blood and CSF samples. Several cytokines and soluble cytokine receptors were quantified in samples from encephalopathy patients. The presence of tumor necrosis factor-α (TNF-α), soluble tumor necrosis factor receptor 1 (sTNF-R1), interleukin-1β (IL-1β), and IL-6 in CSF samples is important for predicting the clinical outcome and diagnosing encephalitis/encephalopathy [Ichiyama et al., 1996a, 1998]. However, little is known about the levels of these cytokines in plasma and CSF from patients with influenza-virus-associated encephalopathy. Study of the dynamics of these cytokines may improve understanding of the mechanisms of influenza-virus-associated encephalopathy.

MATERIALS AND METHODS

Patients and Controls

Eleven consecutive patients, aged 2–13 years (7 boys, 4 girls; mean age: 5.7 years), who were diagnosed with influenza-virus-associated encephalopathy between January and February 1998, were investigated. The clinical data for these patients are summarized in Table I. The level of consciousness was assessed using the Glasgow Coma Scale [Teasdale and Jennett, 1974; Reilly et al., 1988]. Influenza-virus-associated encephalopathy was defined as follows: (1) The patient had a preceding upper respiratory tract infection and an altered level of consciousness that could not be explained by other identifiable causes. (2) Reye syndrome according to the case definition of the Center for Disease Control and Prevention (U.S.A.) [Center for Infectious Diseases, 1991] was excluded. (3) Influenza virus RNA was detected in throat swabs with the RT-PCR assay. The serum hemagglutinin inhibition titer of antibody to H3N2 virus increased significantly in all 9 patients in which it was measured, at least fourfold from acute to convalescent titers.

Twenty-nine control patients aged 1–15 years (13 boys, 16 girls; mean age: 3.8 years) with influenza virus infections without any neurological complications were also studied. In all the control patients, the diagnosis of an influenza virus infection was also confirmed by the detection of viral RNA in throat swabs.

Samples

Peripheral blood samples from the patients and controls were collected in standard blood tubes containing ethylenediamine tetraacetic acid (EDTA). Plasma, peripheral blood mononuclear cell (PBMC), and erythrocyte fractions were isolated from 1 ml of whole blood by Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) density centrifugation at 400 × *g* for 30 min at room temperature. The PBMC and erythrocyte fractions were washed twice with phosphate-buffered saline (PBS), resuspended in 200 μl of PBS, and stored at −70°C until use. CSF was obtained from patients with influenza-virus-associated encephalopathy and stored at −70°C.

RT-Nested PCR

For PCR aimed at the NS gene, sense primer NS3 (GGTGATGCCCCATTCCTTGA; positions 108–127) and antisense primer NS4 (ATTTCGCCAACAATTGCTCC; positions 486–505) were used in the first round. Primers NS1 (GAGGCACTTAAAATGACCAT; positions 249–268) and NS2 (CTCTTCGGTGAAAGCCCTTAG; positions 465–485) were used in the nested PCR reaction. These oligonucleotides were designed from the highly conserved region of the influenza A/PR/8/34 NS gene sequence [Buonagurio et al., 1986].

RNA was extracted from each sample using a QIAamp viral RNA kit (QIAGEN, Hilden, Germany), using a silica-gel-based membrane that binds RNA. The RNA extracted from 200 μl of each sample was eluted in 50 μl RNase-free water. Ten microliters of this solution were used for cDNA synthesis immediately after denaturation for 2 min at 80°C. The reaction buffer (final concentrations, 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), NS3 sense primer (25 pmol), deoxynucleoside triphosphates (0.5 mM final

concentration), 200 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Rockville, MD), and dithiothreitol (50 mM final concentration) were added to a final volume of 20 μl. After incubation at 37°C for 60 min, 5 μl of this solution were added to 45 μl of PCR mixture containing NS3 and NS4 primers (25 pmol each), 1.5 U of Taq DNA polymerase (TaKaRa Taq; Takara Syuzou, Otsu, Japan), and the same reaction buffer as used in the RT reaction. Amplification was carried out in a TP-240 thermal cycler (Takara Syuzou). The PCR program consisted of a 1-min preincubation at 94°C followed by 30 cycles of 1 min at 94°C and 20 sec at 62°C. Nested PCR was performed after transferring 1 μl of the first-round PCR product into a new PCR reaction mixture containing the nested primers under the same conditions. The nested amplification product, which was expected to yield a 237 base-pair sequence, was analyzed by electrophoresis through 1.2% agarose in a Tris-acetate-EDTA gel stained with ethidium bromide. Because the sequences of the designed primers are highly conserved, both influenza A and influenza B viruses were detectable (data not shown).

Synthesis of Positive Control RNA

A first-round PCR fragment, consisting of nucleotides 108–505 of the NS gene, was cloned into the pGEM-T plasmid (Promega). RNA transcripts were synthesized from the purified recombinant plasmid with T7 RNA polymerase (the Riboprobe in vitro transcription system; Promega) and diluted serially in diethyl pyrocarbonate-treated water. Ten-fold dilutions were tested by RT-PCR, and the detection limit was established reproducibly.

Enzyme-Linked Immunosorbent Assay for Cytokines and Soluble Cytokine Receptors

The concentrations of TNF-α, sTNF-R1, IL-1β, and IL-6 were determined with commercial sandwich-type enzyme-linked immunosorbent assay (ELISA) kits (IL-1β kit, Genzyme, Cambridge, MA; TNF-α, sTNF-R1, and IL-6 kits, R&D Systems, Minneapolis, MN). These assays were carried out according to the supplier's instructions. Sample values were determined from a standard curve.

Statistical Analysis

Data were analyzed using Fisher's exact test. A level of $P < .05$ was considered significant.

RESULTS

Sensitivity of RT-PCR

To determine the sensitivity of our RT-PCR assay, dilutions of synthesized RNA transcripts of the NS gene were prepared (Materials and Methods) and used for the RT-PCR assay. A minimum of three copies per 50 μl PCR reaction mixture could be detected (Fig. 1).

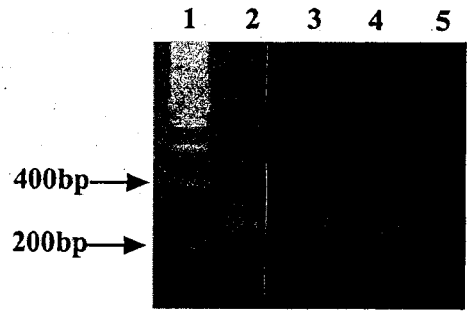

Fig. 1. Sensitivity of the reverse transcription-polymerase chain reaction (RT-PCR) in detecting influenza virus NS gene. **Lane 1**, 200 bp DNA marker ladder; lanes **2–4**, 3×10^2, 3×10^1, 3 copies of NS gene, respectively; **lane 5**, no template control.

Detection of Influenza Virus RNA

RT-PCR was carried out using blood samples (plasma, PBMC, erythrocytes) from the patients and controls, and CSF samples from the patients (Table II). Viral RNA was detected only in the PBMCs of one patient with influenza-virus-associated encephalopathy (1 [patient 9] of 9; 11%) and in the CSF of another patient (1 [patient 8] of 11; 9%). Viral RNA was not detected in plasma or erythrocytes from any of the patients. RT-PCR was also negative with all the blood samples from the controls. The percentages of RT-PCR positive blood samples in the two groups were not significantly different. The detection of viral RNA was not associated with any clinical features or the outcome, although the number of positive patients was small.

Concentrations of Cytokines and Soluble Cytokine Receptors

The levels of TNF-α, sTNF-R1, IL-1β, and IL-6 in the CSF of the patients with influenza-virus-associated encephalopathy are shown in Table III. The concentrations of TNF-α and IL-1β in the CSF were all below the detection limits. The CSF concentrations of sTNF-R1 and IL-6 were elevated in two and one patients, respectively, out of seven with influenza-virus-associated encephalopathy.

The levels of TNF-α, sTNF-R1, IL-1β, and IL-6 in the plasma of the patients with encephalopathy are shown in Table IV. The plasma TNF-α concentrations were all below the detection limits. In the nine patients with influenza-virus-associated encephalopathy, the plasma concentrations of sTNF-R1, IL-1β, and IL-6 (particularly IL-6 ≥ 100 pg/ml in three patients) were elevated in two, two, and four patients, respectively. The number of influenza-virus-associated encephalopathy patients who had elevated concentrations of IL-6 ≥ 100 pg/ml was significantly higher than that of the controls ($P = .01$) (Table V). There were no significant differences in the numbers of patients and controls with el-

TABLE II. Results of RT-PCR in Patients With Influenza-Virus-Associated Encephalopathy

Samples	Patients	Controls
Throat swab	9/9	29/29
Plasma	0/11	0/29
PBMC	1/9	0/29
Erythrocytes	0/9	0/29
CSF	1/11	ND

RT-PCR, reverse transcription-polymerase chain reaction; PBMC, peripheral blood mononuclear cells; CSF, cerebrospinal fluid; ND, not done.

TABLE III. Cerebrospinal Fluid Concentrations of TNF-α, sTNF-R1, IL-1β, and IL-6 in Patients With Influenza-Virus-Associated Encephalopathy

Patient no.	TNF-α (pg/ml)	sTNF-R1 (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
1	NA	NA	NA	NA
2	NA	NA	NA	NA
3	<15	1196	<4	324[b]
4	<15	2934	NA	<31.2
5	<15	1848	<4	<31.2
6	NA	NA	NA	NA
7	<15	555	<4	<31.2
8	<15	433	<4	<31.2
9	<15	553	<4	<31.2
10	<15	635	<4	<31.2
11	NA	NA	NA	NA
Normal range	<15	836 ± 402[a]	<4	<31.2

TNF-α, tumor necrosis factor-α; sTNF-R1, soluble tumor necrosis factor receptor 1; IL, interleukin; NA, not applicable.
[a]Mean ± SD.
[b]Underscores represent the level considered abnormal.

TABLE IV. Plasma Concentrations of TNF-α, sTNF-R1, IL-1β, and IL-6 in Patients With Influenza-Virus-Associated Encephalopathy

Patient no.	TNF-α (pg/ml)	sTNF-R1 (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
1	NA	NA	NA	NA
2	NA	NA	NA	NA
3	<31.2	2232	<8	860[b]
4	<31.2	810	30.2	18.2
5	<31.2	702	<8	<12.5
6	NA	426	<8	<12.5
7	<31.2	760	<8	<12.5
8	<31.2	869	<8	100
9	<31.2	>5000	<8	1295
10	<31.2	745	<8	<12.5
11	<31.2	270	21.1	<12.5
Normal range	<15.6	1020 ± 495[a]	<4	<12.5

TNF-α, tumor necrosis factor-α; sTNF-R1, soluble tumor necrosis factor receptor 1; IL, interleukin; NA, not applicable.
[a]Mean ± SD.
[b]Underscores represent the level considered abnormal.

TABLE V. Comparison of the Percentage of Patients Exhibiting Plasma Cytokines

Cytokines (pg/ml)	Patients (%) (n = 9)	Controls (%) (n = 29)	P
TNF-α	0 (0)	0 (0)	1.00
sTNF-R1	2 (22)	1 (3)	.13
IL-1β	2 (22)	2 (7)	.23
IL-6	4 (44)	12 (41)	.58
IL-6 (≥100)	3 (30)	0 (0)	.01

TNF-α, tumor necrosis factor-α; sTNF-R1, soluble tumor necrosis factor receptor 1; IL, interleukin.

evated concentrations of TNF-α, sTNF-R1, or IL-1β (Table V).

The concentrations of cytokines and soluble cytokine receptors in the CSF and plasma were not associated with any clinical features in the encephalopathy patients. In terms of mortality and morbidity, two patients who had cytokines in both CSF and plasma recovered without sequelae (patients 3 and 4).

DISCUSSION

Viremia is unusual in influenza virus infection [Murphy and Webster, 1996], although the virus is occasionally isolated from the blood [Stanley and Jackson, 1969; Lehmann and Gust, 1971]. Even when the RT-PCR assay is used, influenza RNA is detected only occasionally in blood samples from influenza patients [Mori et al., 1997; Tsuruoka et al., 1997]. In our study, viral RNA was detected infrequently in blood from patients with encephalopathy and never in blood from the controls. Viremia may be as rare in patients with influenza-virus-associated encephalopathy as it is in patients with influenza infection. Alternatively, the virus might be present in low titers in the blood.

Human influenza A viruses are reported to be neurovirulent in mouse models. Mice infected with influenza A viruses by intracerebral inoculation developed a meningoencephalitic condition [Nakajima and Sugiura, 1980; Sugiura and Ueda, 1980, Takahashi and Yamada, 1995]. Previously, PCR assay for detection of the herpes simplex virus genome in CSF was shown to be useful for virological assessment of patients with herpes simplex virus encephalitis [Kimura et al., 1991, 1992; Ando et al., 1993]. If influenza virus replicates in the brain tissue in a similar way to herpes simplex, then RT-PCR assay should also be a useful tool for analyzing influenza-associated-encephalopathy. A recent Japanese study detected viral RNA frequently in the CSF from patients with influenza-associated-encephalopathy [Fujimoto et al., 1998]. In that study, the RT-PCR assay of five of seven patients seen in the 1996–1997 influenza season was positive. RT-PCR was not undertaken on blood samples. In the present study, we established an RT-PCR assay to detect influenza virus RNA. Using this highly sensitive method, it was found that the RT-PCR assay was positive in only 1 of 11 CSF samples from patients with influenza-virus-associated encephalopathy. This result shows that although viral replication may occur in the CNS, it is an uncommon event.

It is not known why the frequency of detection of viral RNA differed in the two studies. One possibility is that the rate of CNS invasion differs according to the epidemic virus, although we have little information regarding to the respective capacity of 1996–1997 and 1997–1998 season viruses to induce encephalopathy.

Many cytokines and soluble cytokine receptors are considered important mediators of inflammatory responses, and their levels increase in CSF or plasma during infectious inflammatory disorders of the CNS, primarily meningitis [Mustafa et al., 1989; Chavanet et al., 1992; Glimåker et al., 1993; López-Cortés et al., 1993; Aurelius et al., 1994; Ichiyama et al., 1996a, 1996b, 1997, 1998]. We also reported previously that elevation of TNF-α, IL-1β, and IL-6 in the CSF indicates acute encephalitis/encephalopathy, rather than febrile convulsions mimicking acute encephalitis/encephalopathy [Ichiyama et al., 1998]. Previous studies showed that sTNF-R1 is the natural homeostatic regulator of the action of TNF-α, and that the level of sTNF-R1 is a better indication of the true biological activity of TNF-α than the level of TNF-α itself [Duncombe and Brenner, 1988; Englemann et al., 1990]. In the present study, the CSF concentrations of sTNF-R1 and IL-6 were elevated in two and one of seven patients, respectively, with influenza-virus-associated encephalopathy. It is not clear why sTNF-R1 and IL-6 were not always detected in the CSF. The inflammation of the CNS may be mild, so that inflammatory cytokines cannot be detected. Alternatively, influenza-virus-associated encephalopathy may have a different pathogenesis. In the influenza B virus mouse model of Reye syndrome, intravenous inoculation of the virus caused a nonpermissive viral infection of vascular endothelial cells of the brain and damage to the blood–brain barrier that resulted in acute encephalopathy without inflammation [Davis et al., 1990]. In an autopsy case of human herpesvirus 6 encephalopathy, human herpesvirus 6 viral antigens were detected only in the vascular endothelium of the brain and no inflammation was observed [Ueda et al., 1996]. These observations suggest that vascular endothelial infection is part of the pathogenesis of acute encephalopathy. Toxic factors and metabolic disorders, including hereditary enzymatic deficiency, are other possibilities.

The number of influenza-virus-associated encephalopathy patients who had elevated concentrations of IL-6 ≥ 100 pg/ml in plasma was significantly higher than that in the controls in our study. Monocytes and lymphocytes produce IL-6; however, it is particularly interesting that IL-6 is also produced by the vascular endothelium. IL-6 plays an important role in host responses to infection and induces hepatic protein synthesis, including C-reactive protein and fibrinogen, during the acute phase response [Heinrich et al., 1990]. Recently, it was reported that IL-6 affected the permeability of the blood–brain barrier in rats [Saija et al., 1995; Farkas et al., 1998]. In human neonates, IL-6 is thought to play a role in hypoxic-ischemic brain damage [Martín-Ancel et al., 1997]. It is possible that the systemic reaction to IL-6 contributes to the development of the influenza-virus-associated encephalopathy. Previous studies have described how IL-6 plasma concentrations are useful in the early diagnosis of neonatal infection [Messer et al., 1996; Panero et al., 1997]. Our results suggest that IL-6 plasma concentrations might also be useful in differentiating influenza-virus-associated encephalopathy.

In conclusion, the infrequent detection of the viral genome in CSF and blood indicates that direct invasion of the influenza virus into the CNS is an uncommon event, and suggests that systemic cytokines or vascular involvement may be indirectly responsible for the encephalopathy. A high plasma concentration of IL-6 may indicate progression to encephalopathy. However, the precise mechanism of the illness remains unknown. Further studies should explore the disease mechanism and the clinical applications of these observations.

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