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研究報告の概要

背景
パンデミック2009インフルエンザA型ウイルス(H1N1)の重症感染は、妊娠、肥満、および免疫抑制を含むリスクファクターと関連している。重症の1例で免疫グロブリンG2(IgG2)欠損が同定されたことを受けて、我々はH1N1感染患者のコホートでのIgGサブクラスのレベルを調べた。

方法
H1N1の急性で重症の感染患者(集中治療室での呼吸のサポートを必要とする感染と定義した)、中等度のH1N1感染患者(入院患者だが集中治療室へは収容されていない患者と定義した)、および健康な妊娠女性からランダムにサンプリングした被験者を対照として、患者および対照の血清IgGおよびIgGサブクラスのレベルを含む特性を調べた。

結果
H1N1感染した39例の患者(重症感染が19例、そのうち7例が妊娠中；中等度感染が20例でそのうち2例が妊娠中)のうちで、低アルブミン血症(P<0.001)、貧血(P<0.001)、および総IgG(P=0.01)、IgG1(P=0.022)、IgG2(19例中15例 vs. 20例中5例；P=0.001；平均値±標準偏差[SD]，1.8±1.7 g/L vs. 3.4±1.4 g/L；P=0.003)が低レベルであったことは、統計学的に有意に重症H1N1感染と関連していたが、多変量解析で統計学的に有意であったのは低アルブミン血症(P=0.02)と平均のIgG2レベルが低値であったこと(P=0.043)のみであった。IgG2欠損患者で生存していた15例(79%)のフォローアップを急性期の最初の検体採取後、平均(±SD)で90±23日目(範囲は38-126日目)に行ったところ、低アルブミン血症は大多数の症例で解消していたが、15例中11例(73%)の患者ではIgG2欠損はそのままであった。対照の健康な妊娠女性17例では、10例で軽度のIgG1および/またはIgG2レベルの低値が認められたが、H1N1感染のあった妊娠患者ではIgG2レベルが有意に低かった(P=0.001)。

結論
重症H1N1感染はIgG2の欠損と関連し、それは患者の多くで持続性となるものと考えられる。IgG2レベルの妊娠に関連した低下が、妊娠女性の全てとは言えないまでもいくらかの比率でH1N1感染の重症度が増加することを説明するものかもしれない。H1N1感染の発症機序におけるIgG2欠損の役割を知るにはさらに研究が必要であるが、それはこのことが治療上意義を有する可能性があるからである。

使用上の注意記載状況・その他参考事項等

代表としてノイアート静注用500単位の記載を示す。
2. 重要な基本的注意
(1)本剤の原材料となる献血者の血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体、抗HTLV-I抗体陰性で、かつALT(GPT)値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、Cohnの低温エタノール分画で得た画分から人アンチトロンビンIIIを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びウイルス除去膜によるろ過処理を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見	今後の対応
パンデミック2009インフルエンザA型ウイルス(H1N1)重症感染と血清中のIgG2低値は関連しているとの報告である。 インフルエンザA(H1N1)はオルソミクソウイルス科に属し、ビリオンは球形で、直径80～120nmの脂質エンベロープを有する比較的大きなRNAウイルスである。万一、インフルエンザA(H1N1)が原料血漿に混入したとしてもBVDをモデルウイルスとしたウイルスバリデーション試験成績から、製造工程にて十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないものと考えるので、特段の措置はとらない。

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BENESIS 2010-004

Association between Severe Pandemic 2009 Influenza A (H1N1) Virus Infection and Immunoglobulin G_2 Subclass Deficiency

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Background. Severe pandemic 2009 influenza A virus (H1N1) infection is associated with risk factors that include pregnancy, obesity, and immunosuppression. After identification of immunoglobulin G_2 (IgG_2) deficiency in 1 severe case, we assessed IgG subclass levels in a cohort of patients with H1N1 infection.

Methods. Patient features, including levels of serum IgG and IgG subclasses, were assessed in patients with acute severe H1N1 infection (defined as infection requiring respiratory support in an intensive care unit), patients with moderate H1N1 infection (defined as inpatients not hospitalized in an intensive care unit), and a random sample of healthy pregnant women.

Results. Among the 39 patients with H1N1 infection (19 with severe infection, 7 of whom were pregnant; 20 with moderate infection, 2 of whom were pregnant), hypoabuminemia ($P < .001$), anemia ($P < .001$), and low levels of total IgG ($P = .01$), IgG_1 ($P = .022$), and IgG_2 (15 of 19 vs 5 of 20; $P = .001$; mean value $\pm$ standard deviation [SD], 1.8 ± 1.7 g/L vs 3.4 ± 1.4 g/L; $P = .003$) were all statistically significantly associated with severe H1N1 infection, but only hypoalbuminemia ($P = .02$) and low mean IgG_2 levels ($P = .043$) remained significant after multivariate analysis. Follow-up of 15 (79%) surviving IgG_2-deficient patients at a mean ($\pm$SD) of 90 ± 23 days (R, 38–126) after the initial acute specimen was obtained found that hypoalbuminemia had resolved in most cases, but 11 (73%) of 15 patients remained IgG_2 deficient. Among 17 healthy pregnant control subjects, mildly low IgG_1 and/or IgG_2 levels were noted in 10, but pregnant patients with H1N1 infection had significantly lower levels of IgG_2 ($P = .001$).

Conclusions. Severe H1N1 infection is associated with IgG_2 deficiency, which appears to persist in a majority of patients. Pregnancy-related reductions in IgG_2 level may explain the increased severity of H1N1 infection in some but not all pregnant patients. The role of IgG_2 deficiency in the pathogenesis of H1N1 infection requires further investigation, because it may have therapeutic implications.

Since the onset of the current novel influenza A (H1N1) virus pandemic, it has been recognized that certain risk factors, such as pregnancy, obesity, and immunosuppression, are associated with severe disease [1, 2]. In Victoria, Australia, which was one of the key regions for the H1N1 pandemic in the Southern Hemisphere [3, 4], such risk factors have been frequently observed in our sickest patients, but the explanation for this association has remained elusive [5].

We identified immunoglobulin G_2 (IgG_2) subclass deficiency in 1 young pregnant patient who had an unusual presentation with severe H1N1 infection that required intensive care unit (ICU) admission. Because of this observation, we systematically assessed total IgG and IgG subclasses in all patients with H1N1 infection requiring ICU care (many of whom were pregnant) and

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compared these results with those obtained from all inpatients with less severe H1N1 infection (ie, those patients who did not require ICU admission), as well as a random sample of healthy pregnant women who presented for routine antenatal care.

METHODS

The study was initially undertaken at Austin Health (AH), a tertiary university teaching hospital in Melbourne, Australia. After the observation of IgG_2 deficiency in a patient with H1N1 infection, all patients with polymerase chain reaction (PCR)–proven H1N1 infection who were sufficiently unwell to require admission to AH underwent routine hematological and biochemical assessment, had their serum immunoglobulin levels and subclasses determined, and were reviewed for their clinical features, demographic characteristics, and treatment outcome. Acute-phase serum samples were either assessed prospectively or were retrieved from storage for analysis; patients for whom there were no appropriate stored serum samples were noted but not included in the study. Because of the potential therapeutic implications of our initial findings, and after discussions with the Department of Human Health Victoria, we subsequently broadened recruitment to 2 other hospitals in Victoria (Royal Melbourne Hospital [RMH] and Bendigo Health [BH]), which were actively managing patients with severe H1N1 infection and had ICU admission criteria that were similar to those at AH, to obtain similar acute-phase serum specimens and clinical details.

The following definitions were used for the study: patients with severe H1N1 infection were defined as those with confirmed H1N1 infection who required admission to the ICU for respiratory (invasive or noninvasive mechanical ventilation) and/or vasopressor support, whereas patients with moderate H1N1 infection were defined as those who required hospital inpatient (but not ICU) care. Community-acquired pneumonia was defined according to the Infectious Diseases Society of America guidelines [6].

The clinical and laboratory features of patients with severe H1N1 infection at the 3 recruitment sites (AH, RMH, and BH) were compared with those of patients with moderate H1N1 infection (AH). All patients who were found to be IgG subclass deficient during their acute illness were followed up to obtain convalescent immunoglobulin and IgG subclass levels to assess whether the identified deficiency was transitory or persistent.

Because a large number of our patients with severe H1N1 infection were pregnant, we investigated the immunological status of a random sample of healthy pregnant women to compare these results with those observed among pregnant women with moderate and severe H1N1 infection. Thus, we obtained serum samples from 15–20 healthy pregnant women who had antenatal outpatient visits at the Mercy Hospital for Women (Melbourne, Australia) on 19 or 20 July 2009.

All data were summarized and analyzed according to H1N1 infection severity (severe vs moderate), presence of pregnancy, and, if the patient was pregnant, presence of H1N1 illness (patients with H1N1 infection vs healthy control subjects). Ethics committee approval was obtained at all 4 participating centers that undertook the study.

Laboratory assays. The presence of H1N1 infection was confirmed by strain-specific PCR at the Victorian Infectious Diseases Reference Laboratory and World Health Organization Influenza Reference Laboratory (Melbourne, Australia) using standard H1N1 assays.

Serum immunoglobulins (IgG, IgM, and IgA) were assessed using both a Beckman IMMAGE 800 analyzer (Beckman Coulter) and an Abbott Architect *ci*8200 analyzer (Abbott Laboratories, Abbott Park) in accordance with the manufacturers' instructions. Similarly, immunoglobulin subclasses (IgG_1, IgG_2, IgG_3, and IgG_4) were measured using Binding Site Human IgG Subclass kits on a Beckman IMMAGE 800 analyzer in accordance with the manufacturer's instructions. The reference ranges for normal adults according to the manufacturer were as follows: total IgG, 7.0–16.5 g/L; IgG_1, 3.8–9.3 g/L; IgG_2, 2.4–7.0 g/L; IgG_3, 0.22–1.76 g/L; IgG_4, 0.04–0.86 g/L. Routine hematological and biochemical analyses were performed in the Pathology Departments at contributing hospitals.

Statistical analysis. Univariate analysis was undertaken using Fisher's exact test, Student's t test, or the Wilcoxon rank-sum test (as appropriate) with Stata software, version 8.2 (Stata Corporation), to identify features associated with H1N1 infection severity. Variables that were potentially associated ($P < .2$) on univariate analysis were included in a multivariate analysis to identify features statistically associated with severe H1N1 infection. Similarly, a univariate analysis of the clinical and laboratory features of healthy vs H1N1-infected pregnant participants was undertaken to assess for any associations with the presence of H1N1 infection. A P value of $\leq .05$ was considered to be statistically significant.

RESULTS

Severe versus moderate H1N1 infection. A total of 47 patients with acute H1N1 infection (19 with severe infection and 28 with moderate infection) were assessed from 30 May through 16 August 2009. Appropriate serum specimens were available for 39 patients (19 with severe infection and 20 with moderate infection), and results are shown in Table 1. Among the 8 patients for whom no serum samples were available, no special features were noted to explain the lack of stored serum samples.

Patient demographic data and comorbidities for the 39 participants were similar between the severe and moderate H1N1

Table 1. Comparison of Results for Immunoglobulin (Ig) Levels for Patients with Severe versus Moderate H1N1 Infection

Variable	Severe H1N1 infection (n = 19)	Moderate H1N1 infection (n = 20)	P
Age, mean years ± SD (range)	36 ± 19 (16–79)	41 ± 16 (19–76)	.32
Male sex	7	11	.34
Pregnant[a]	7	2	.065
Comorbidity			
Hematological malignancy[b]	1	2	>.99
Solid-organ transplantation	0	2	.49
Asthma (requiring inhaled corticosteroids only)	3[c]	6[d]	.45
Obesity	1[c]	3[d]	.60
Diabetes mellitus	3[c]	5[d]	.70
Influenza-related myocarditis	1	0	...
Pneumonia present[e]	16	4	<.001
ICU management[f]			
Endotracheal intubation/ventilation alone	12	...	...
Endotracheal intubation/ventilation plus ECMO	2	...	...
Noninvasive ventilation/high-flow oxygen	5	...	...
Mortality	2	0	.23
Laboratory results			
Hemoglobin level, mean g/L (±SD)	104 ± 23	133 ± 21	<.001
Leukocyte count, mean cells × 10^9/L (±SD)	10.4 ± 10.5	8.7 ± 8.3	.56
Lymphocyte count, mean cells × 10^9/L (±SD)	0.94 ± 0.5	3.0 ± 8.8	.31
Renal impairment (creatine level >110 μmol/L)	4	3	.70
Abnormal liver function	16	11	.08
Serum albumin level, mean g/L ± SD (range)[g]	23 ± 5 (16–34)	35 ± 5 (23–42)	<.001
Immunoglobulin data			
Mean day (±SD) of H1N1 illness when serum immunoglobulins assessed (range)	6.2 ± 2.4 (3–11)	6.9 ± 6.1 (1–23)	.67
Low IgA	3[h]	2[h]	.66
Low IgM	2[h]	4[h]	.66
Low total IgG	12[i]	4	.01
Total IgG levels, mean g/L (±SD)	7.2 ± 5.5	9.7 ± 2.4	.069
Patients with low IgG_1	11	4	.022
IgG_1 levels, mean g/L (±SD)	4.2 ± 3.9	5.2 ± 1.9	.31
Patients with low IgG_2	15[i]	5	.001
IgG_2 levels, mean g/L (±SD)	1.8 ± 1.7	3.4 ± 1.4	.003

NOTE. Data are no. of patients, unless otherwise indicated. Severe H1N1 infection was defined as requiring intensive care unit (ICU) admission and respiratory support. Moderate H1N1 infection was defined as requiring hospital admission but not ICU admission. ECMO, extra-corporeal membrane oxygenation; SD, standard deviation.

[a] Of the 7 pregnant women with severe H1N1 infection, 2 had mild asthma (not using inhaled corticosteroids), whereas 1 pregnant woman with moderate H1N1 infection had both type 2 diabetes mellitus and obesity.

[b] One patient in each group had chronic lymphocytic leukemia.

[c] One patient had obesity and diabetes, and 1 patient had asthma and diabetes. All 3 patients had type 2 diabetes.

[d] One patient had asthma, obesity, and diabetes; 2 patients had obesity and diabetes; 3 patients had asthma and diabetes; 1 patient had obesity and asthma. Two of 5 patients had type 1 diabetes, and 3 of 5 patients had type 2 diabetes.

[e] Community-acquired pneumonia was defined according to Infectious Diseases Society of America guidelines [6].

[f] Among patients who required endotracheal intubation/ventilation alone, ECMO, and noninvasive ventilation/high-flow oxygen, pregnancy was present in 4, 1, and 2 patients, respectively.

[g] Serum albumin level on same day that immunoglobulin levels were measured.

[h] Deficiencies in IgM and IgA were all mild.

[i] An additional patient who was 16 years and 11 months of age was not reported to have deficient immunoglobulin levels, because her immunoglobulin levels were within the pediatric range; however, these values would have been considered to be deficient if the adult (defined as ≥17 years of age) normal range values had been used.

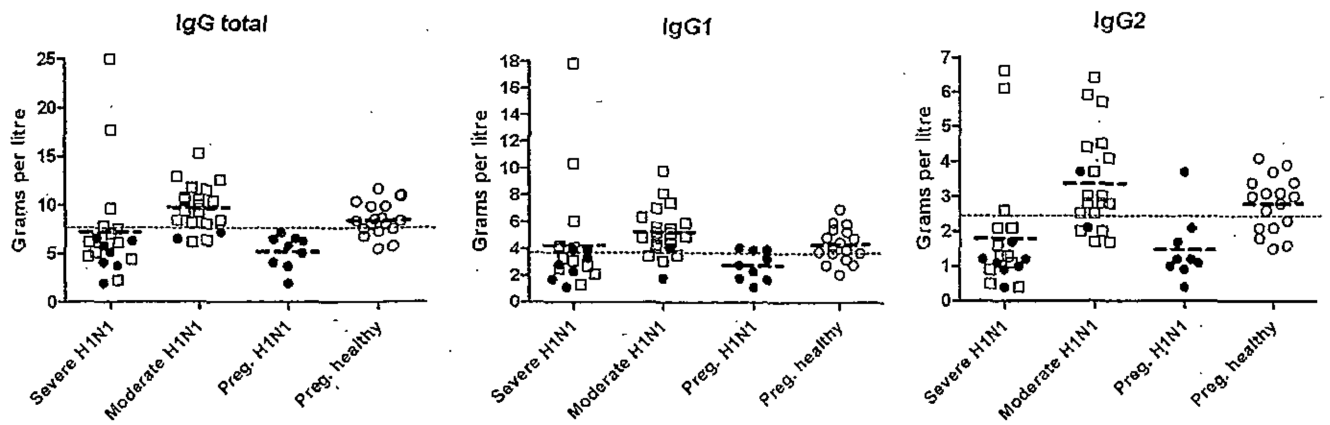

Figure 1. Serum immunoglobulin G (IgG) (total), IgG_1, and IgG_2 levels for patients with acute H1N1 infection stratified according to disease severity (severe vs moderate) and compared with healthy pregnant (Preg) patients. Data are shown for pregnant patients with H1N1 infection (●), nonpregnant patients with H1N1 infection (□), and healthy pregnant control patients (○). *Dashed line,* mean value of each grouping; *dotted line,* lower limit of normal adult range for the relevant immunoglobulin.

infection groups, except that pregnancy was more common among patients in the severe H1N1 infection group (7 of 19 vs 2 of 20); however, this difference did not achieve statistical significance ($P = .065$; Table 1).

Hypoalbuminemia and anemia were more common among patients with severe H1N1 infection ($P < .001$ for both; Table 1). Similarly, the presence of severe H1N1 infection was significantly associated with low levels of total IgG (12 of 19 vs 4 of 20 patients; $P = .01$), IgG_1 (11 of 19 vs 4 of 20 patients; $P = .022$) and IgG_2 (15 of 19 vs 5 of 20 patients; $P = .001$; Table 1 and Figure 1), compared with patients with moderate H1N1 infection. Furthermore, 1 patient with severe H1N1 infection (patient A) was a pregnant woman at 21 weeks gestation (age, 16 years and 11 months) who had an IgG_2 level of 1.1 g/L, which was reported as normal on the basis of the IgG_2 reference ranges used for children (age ≤16 years: 0.6–5.0 g/L) but would have been considered to be deficient if the adult reference ranges (age ≥17 years: 2.4–7.0 g/L) had been applied.

Assessment of the mean (± standard deviation [SD]) concentrations of total IgG and IgG subclasses demonstrated that patients with severe H1N1 infection had significantly lower levels of IgG_2 (and therefore lower levels of total IgG) than did patients with moderate H1N1 infection (Table 1). However, the mean (±SD) levels of IgG_1 (4.2 ± 3.9 vs 5.2 ± 1.9 g/L; $P = .31$), IgG_3 (0.50 ± 0.28 vs 0.77 ± 0.55 g/L; $P = .07$) and IgG_4 (0.28 ± 0.43 vs 0.24 ± 0.24; $P = .68$) were not significantly different between patients with severe and patients with moderate H1N1 infection (Figure 1).

The association between pregnancy, hypoalbuminemia, anemia, and low levels of IgG_2 with severe H1N1 infection were assessed in a multivariate model. The results are shown in Table 2. Abnormal liver function test results were not included in this analysis, because they were correlated with hypoalbuminemia ($P = .024$). After this analysis, only low mean serum concentrations of IgG_2 and albumin remained statistically significantly associated with severe H1N1 infection, compared with moderate H1N1 infection ($P = .043$ and $P = .02$, respectively; Table 2).

Among the 21 patients identified as IgG_2 deficient during the acute stage of H1N1 infection (16 with severe infection, including patient A; 5 with moderate infection), convalescent serum samples was obtained from 15 patients (71%; 11 with severe infection, 6 of whom were pregnant; 4 with moderate infection, 1 of whom was pregnant) a mean (±SD) of 90 ± 23 days (range, 38–126 days) after the initial acute-phase specimen was obtained. Convalescent-phase serum samples were not available for 6 patients, because 2 had died, 3 were not contactable, and 1 refused testing. Serum IgG_2 results are shown in Figure 2. Among the 11 patients with previous severe H1N1 infection, serum IgG_2 levels remained in the deficient range for 8 (73%; 3 postpartum, one pregnant, and 4 nonpregnant; Figure 2). Two of the 3 patients with severe H1N1 infection with normal convalescent serum IgG_2 levels were postpartum women; 1 of these 2 women had received intravenous pooled immunoglobulin as a component of her therapy for severe

Table 2. Multivariate Analysis of Features Potentially Associated with Severe versus Moderate H1N1 Infection

Variable	Odds ratio (95% confidence interval)	P
Pregnancy	8.9 (0.32–248.2)	.20
Mean hemoglobin per g/L	1.01 (0.94–1.08)	.80
Mean serum albumin per g/L	1.6 (1.08–2.3)	.02
Mean immunoglobulin G_2 level per g/L	2.25 (1.03–4.92)	.043

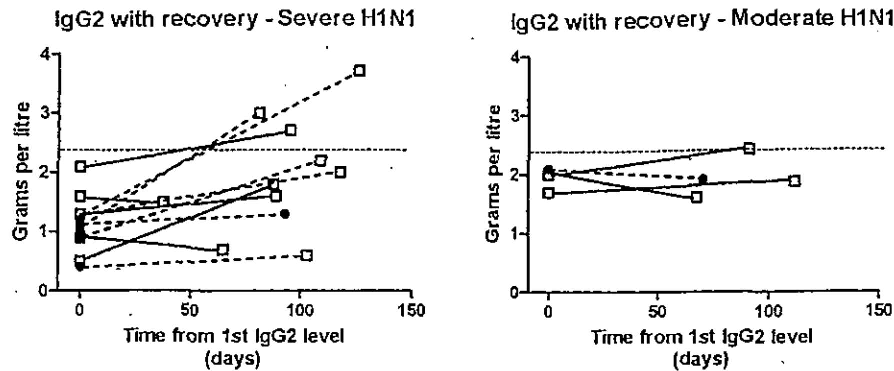

Figure 2. Comparison of serum immunoglobulin G subclass 2 (IgG_2) levels among patients with IgG_2 deficiency during severe H1N1 infection and with recovery (nonpregnant and pregnant women). Data are shown for pregnant patients with H1N1 infection (●) and nonpregnant patients with H1N1 infection (□). *Dashed line,* pregnant patient at time of initial IgG_2 sample; *dotted line,* lower limit of normal adult range for IgG_2.

H1N1 infection, but this was 77 days before testing of convalescent-phase serum samples. Notably, the only patient with severe H1N1 infection with normal convalescent-phase IgG_2 levels who was nonpregnant was only mildly deficient during the acute phase of illness (acute-phase IgG_2 level, 2.1 g/L; convalescent-phase IgG_2 level, 2.6 g/L; normal range, ≥2.4 g/L). Of the 4 patients with moderate H1N1 infection who were assessed at follow-up, 3 remained IgG_2 deficient, including 1 woman who was still pregnant at this time (Figure 2).

Persistence of immunoglobulin deficiency was less prominent for non-IgG_2 subclasses. Among the 8 patients with severe H1N1 infection who were initially deficient in IgG_1, 6 had normal IgG_1 levels on testing of convalescent-phase serum samples (data not shown). Similarly, hypoalbuminemia had resolved in most patients (9 of 14 assessable patients); however, of the other 5 patients, 2 remained pregnant at the time of follow-up.

Immunoglobulin levels and pregnancy. A total of 9 patients with H1N1 infection were pregnant (23%; Table 1). Serum immunoglobulin levels for these patients were compared with levels for 17 healthy pregnant control subjects, and results are shown in Figure 1 and Table 3. The healthy pregnant women were slightly older than those with H1N1 infection, but both groups were similar with regard to mean gestation period (Table 3). Among the 17 healthy patients, 10 had mildly low IgG_1 and/or IgG_2 levels, compared with the standard reference range for nonpregnant women (IgG_1 alone, 4 patients; IgG_2 alone, 4 patients; IgG_1 and IgG_2, 2 patients). However, pregnant women with H1N1 infection had significantly lower mean levels of total IgG ($P<.001$), IgG_1 ($P = .005$), and IgG_2 ($P = .001$) than did the 17 control subjects (Table 3 and Figure 1).

Table 3. Comparison of Results for Pregnant Women with H1N1 Infection versus Healthy Control Subjects

Variable	Patients with H1N1 infection[a] (n = 9)	Healthy control subjects[b] (n = 17)	P
Age, mean years ± SD (range)	24 ± 6.2 (16–37)	30 ± 3.9 (20–36)	.008
Gestation, mean weeks ± SD (range)	32 ± 6.0 (21–38)	35 ± 2.9 (29–40)	.16
Low total IgG	7[c]	3	.009
Total IgG level, mean g/L (±SD)	5.2 ± 1.7	8.5 ± 1.7	<.001
Low IgG_1	6	6	.22
Mean (±SD) IgG_1 level, mean g/L (±SD)	2.8 ± 1.1	4.4 ± 1.3	.005
Low IgG_2	7[c]	6	.097
IgG_2 level, mean g/L (±SD)	1.5 ± 1.0	2.8 ± 0.8	.001

NOTE. Data are no. of patients, unless otherwise indicated. IgG, immunoglobulin G.

[a] Including 7 patients with severe H1N1 infection and 2 patients with moderate H1N1 infection.

[b] Two healthy pregnant patients had gestational diabetes.

[c] An additional patient who was 16 years and 11 months of age was not reported to have deficient immunoglobulin levels, because her immunoglobulin levels were within the pediatric range; however, these values would have been considered to be deficient if the adult (defined as ≥17 years of age) normal range values had been used.

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DISCUSSION

Although a number of authors have described the clinical features of H1N1 infection [7–9], including those of pregnancy as a risk factor for severe H1N1 infection [10], this is, to our knowledge, the first report to identify a potential association between H1N1 disease severity and the presence of immunoglobulin subclass deficiency. Patients with severe H1N1 infection were significantly more likely to be deficient in IgG_2 than were patients with moderate H1N1 infection ($P = .001$); IgG_2 deficiency was not necessarily noticeable if only total IgG levels were assessed. Furthermore, our findings suggest that, for the majority of such patients (11 of 15 patients; 73%), IgG_2 deficiency persists after recovery from H1N1 infection, regardless of whether the illness was associated with possible risk factors, such as pregnancy. Low IgG_2 levels are therefore less likely to be simply related to a severe inflammatory response, as is sometimes noted for acute-phase reactants, such as albumin, creatine kinase, and lactate dehydrogenase [8, 11].

IgG subclass deficiency is usually asymptomatic, and low levels of 1 or more IgG subclasses can be found in 2%–20% of healthy individuals [12, 13]. If symptomatic, patients with IgG subclass deficiency tend to have recurrent sinopulmonary bacterial infections [13]. However, to our knowledge, IgG subclass deficiency has not been studied in detail in humans with influenza infection, although in mouse models, anti-influenza antibody (and specifically IgG) has a key role in virus control in the lower respiratory tract, compared with the upper respiratory tract [14, 15]. In humans, Logtenberg et al [16] described a single patient with severe transitory hypogammaglobulinemia associated with acute influenza A virus infection. However, in this case, all immunoglobulin classes (IgG, IgM, and IgA) were affected. Other than this report, we can find no other association between influenza and immunoglobulin deficiency.

Thus, it is uncertain whether we have simply identified a cohort of patients with H1N1 infection with underlying unrecognized IgG_2 deficiency, or whether there is an interaction between the H1N1 virus and the host that leads to such deficiency. Given that the half-life of IgG_2 is ~3 weeks [17], a potent and specific interaction between H1N1 virus and host B cells would need to occur to lead to such a precipitous decrease in serum IgG_2. Bone marrow apoptosis of B cells by influenza virus has been demonstrated in mice [18], but how this relates to disease in humans remains unclear. However, the fact that the IgG_2 deficiency that we identified appears to persist in most cases long after disease resolution (convalescent serum samples were collected a mean ($\pm$SD) of 90 $\pm$ 23 days after the acute phase of illness) suggests the possibility of potential long-term implications for these patients and that follow-up of moderate and severe cases of H1N1 infection may be warranted.

Because of our findings, we hypothesize that IgG_2 deficiency may be associated with an inability to mount an early effective immune response to influenza and may therefore be linked to severe disease. Furthermore, if the IgG_2 deficiency that we observed is long-lasting or permanent, will this affect the patients' likely response to influenza vaccination? Response to influenza vaccination is measured by specific neutralization assays, rather than by total immunoglobulin concentrations, and it is not known whether response to influenza vaccination by individuals who are IgG_2 subclass deficient is diminished.

Pregnancy is a known risk factor for increased severity of both seasonal and pandemic influenza infections [19–23], which is thought to be attributable to pregnancy-related physiologic and immunologic changes, such as decreased lung capacity and increased cardiovascular demand, as well as a shift away from cell-mediated immunity to humoral immunity [24]. Our finding that a substantial number (10 of 17) of our healthy pregnant cohort had mildly low IgG_1 and/or IgG_2 levels is consistent with the known decrease in immunoglobulin levels that occurs during normal pregnancy and resolves after delivery [25, 26]. Low IgG_2 levels in pregnant women could therefore potentially explain why pregnancy appears to be a risk factor for severe H1N1 infection [2–4]. However, this alone does not appear to explain the significantly lower levels of IgG_2 observed among pregnant patients with H1N1 infection, compared with levels among our healthy pregnant control subjects ($P = .001$), nor the fact that IgG_2 deficiency persisted postpartum in some women with severe H1N1 infection.

Although IgG_2 deficiency appears to be associated with H1N1 infection severity, it remains uncertain whether administration of immunoglobulin to patients who are IgG_2 deficient is likely to be therapeutically beneficial. We administered pooled immunoglobulin to some of our patients with severe H1N1 infection who had IgG_2 deficiency, but our observations were uncontrolled. Nevertheless, convalescent blood products were administered during the Spanish influenza pandemic with a reduction in mortality [27], and more recently, convalescent-phase plasma samples obtained from a patient who recovered from H5N1 influenza infection was used successfully [28]. Further investigation of the use of convalescent-phase blood products in severe pandemic H1N1 infection is needed.

Our study has a number of important limitations, including being of relatively limited size and lacking suitable specimens to analyze patient cellular immunity or to assess influenza virus neutralization, and we have not compared our findings with those that might be expected among healthy nonpregnant control subjects. Furthermore, with the number of cases of H1N1 infection now decreasing in Australia, our findings need to be confirmed in other geographical locations (although the H1N1 strain circulating in Victoria appears to be the same as that isolated in the Northern Hemisphere) [4].

Nevertheless, we considered our finding of a statistically significant association between IgG_2 deficiency and H1N1 infection severity to be sufficiently notable and hypothesis-generating in terms of potential clinical therapeutic importance that prompt notification of these data to clinicians managing cases of H1N1 infection was warranted.

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一般的名称	乾燥濃縮人アンチトロンビンⅢ		研究報告の公表状況	CDC/Emerging Infections Disease/2010/03/25	公表国 日本	
販売名（企業名）	①ノイアート静注用500単位（ベネシス） ②ノイアート静注用1500単位（ベネシス） ③ノイアート（ベネシス）					

研究報告の概要

世界的に流行している(H1N1)2009 ウイルスの献血血液の輸血を介した伝播の可能性に関心が寄せられている。
日本赤十字血液センターは、献血後に世界的に流行している(H1N1)2009 ウイルス感染の可能性を示す情報のある血液製剤を止め、NAT で血液製剤のウイルス遺伝子を確認することを試みた。
2009 年 6 ～11 月の間の血液サンプルは献血から製造された血漿そして赤血球製剤から集められた、献血後情報は、献血後まもなく世界的に流行している(H1N1)2009 ウイルス感染との診断を示唆した。
ウイルス RNA は、血漿サンプルそして赤血球画分はそれぞれ QIAamp Virus Biorobot MDx kit(QIAGEN, Valencia, CA, USA)そして High Pure Viral Nucleic Acid Large Volume Kit(Roche Diagnostics, Indianapolis, IN, USA)によって抽出した。
RNA サンプルは、PRISM 7900(Applied Biosystem, Foster City, CA, USA)を用いてインフルエンザ A 型の赤血球凝集素(HA)とマトリックス(M)の遺伝子をリアルタイム逆転写-PCR(RT-PCR)にかけた。
HA の RT-PCR は世界的に流行している(H1N1)2009 ウイルスに特異的であったが、M の PT-PCR は世界的に流行している(H1N1)2009 ウイルスと季節性インフルエンザ A ウイルスの両方検出できるように設計された。
プローブそしてプライマーの配列は日本の国立感染症研究所によって開発されたプロトコルに従って合成された。
献血血液サンプルを用いての試験前に、NAT システムの感度はスパイク実験によって確認した。
NAT は献血後 7 日以内にインフルエンザの症状を示した 96 人のドナーから 96 の血漿と 67 の赤血球サンプルを用いて実施された。
20 人のドナーについては、世界的に流行している(H1N1)2009 は献血後 1 日以内に、そして他の 20 人については献血後 2 日以内に診断された。
世界的に流行している(H1N1)2009 ウイルスはどの試験サンプルからも検出されなかった、しかし外部陽性コントロールでは一貫して検出された。
これらの結果は、世界的に流行している(H1N1)2009 ウイルスによるウイルス血症は、あるとしても非常に低く現行の NAT では見逃されているかもしれないこと、あるいはウイルス血症の期間がウイルス血症を確認するにはあまりにも短いことを示唆している。
輸血による世界的に流行しているインフルエンザの伝播のリスクは低いようであるが、にもかかわらず、さらにこのリスクを解明する調査が必要である。

使用上の注意記載状況・その他参考事項等

代表としてノイアート静注用 500 単位の記載を示す。
2. 重要な基本的注意
(1) 本剤の原材料となる献血者の血液については、HBs 抗原、抗 HCV 抗体、抗 HIV-1 抗体、抗 HIV-2 抗体、抗 HTLV-I 抗体陰性で、かつ ALT(GPT)値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV 及び HCV について核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該 NAT の検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、Cohn の低温エタノール分画で得た画分から人アンチトロンビン III を濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において 60℃、10 時間の液状加熱処理及びウイルス除去膜によるろ過処理を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見	今後の対応
日本赤十字血液センターの献血後にインフルエンザの症状を示したドナーにおける、NAT によるパンデミック A(H1N1)2009 インフルエンザウイルスの調査報告である。 インフルエンザA(H1N1)はオルソミクソウイルス科に属し、ビリオンは球形で、直径80～120nmの脂質エンベロープを有する比較的大きなRNAウイルスである。万一、インフルエンザA(H1N1)が原料血漿に混入したとしてもBVDをモデルウイルスとしたウイルスバリデーション試験成績から、製造工程にて十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないものと考えるので、特段の措置はとらない。

NO

Risk for Transmission of Pandemic (H1N1) 2009 Virus by Blood Transfusion

To the Editor: Influenza A pandemic (H1N1) 2009 virus emerged in early 2009 in Mexico and has since spread worldwide. In Japan, the first outbreak of the novel influenza was reported in May 2009 (*1*) and became pandemic in November. Although no cases of transfusion-transmitted influenza have been published, evidence exists of brief viremia before onset of symptoms (*2,3*). The possibility of transmission of this virus through transfusion of donated blood is of concern. The Japanese Red Cross Blood Centers have intercepted blood products with accompanying postdonation information indicating possible pandemic (H1N1) 2009 infection and attempted to identify the viral genome in those products by using nucleic acid amplification technology (NAT).

During June–November 2009, blood samples were collected from plasma and erythrocyte products that had been processed from donations; postdonation information indicated diagnosis of pandemic (H1N1) 2009 infection soon after donation. Viral RNA was extracted from plasma samples and erythrocyte fractions by using a QIAamp Virus Biorobot MDx kit (QIAGEN, Valencia, CA, USA) and a High Pure Viral Nucleic Acid Large Volume kit (Roche Diagnostics, Indianapolis, IN, USA), respectively. RNA samples were subjected to real-time reverse transcription–PCR (RT-PCR) of hemagglutinin (HA) and matrix (M) genes of influenza A by using PRISM 7900 (Applied Biosystems, Foster City, CA, USA). The RT-PCR of HA was specific for pandemic (H1N1) 2009 virus, whereas the RT-PCR of M was designed to detect both pandemic (H1N1) 2009 and seasonal influenza A viruses. The sequences of probes and primers were synthesized according to the protocols developed by the Japanese National Institute of Infectious Diseases (*4*). Either 200 μL of a plasma sample or 100 μL of packed erythrocytes was used for each test, and the test was performed 2× for each gene in each sample. Before the investigation using donated blood samples, the sensitivity of the NAT system was checked by spiking experiments. Viral particles of pandemic (H1N1) 2009 virus (A/California/04/2009 [H1N1]), donated by the National Institute of Infectious Diseases, were spiked into plasma and erythrocyte samples from healthy volunteers. Viral RNA was detected in the plasma samples spiked with viral particles corresponding to 300 genome equivalents/mL and in the packed erythrocyte samples spiked with viral particles corresponding to 3,000 genome equivalents/mL.

NAT was conducted by using 96 plasma and 67 erythrocyte samples obtained from 96 blood donors who had symptoms of influenza within 7 days postdonation. For 20 donors, pandemic (H1N1) 2009 was diagnosed within 1 day postdonation and, for another 20, within 2 days postdonation (Figure). Pandemic (H1N1) 2009 virus was not found in any of the samples tested, but it was consistently detected in the external positive control. These results suggest that the viremia with pandemic (H1N1) 2009 virus, if any, is very low and can be missed by current NAT or that the viremic period is too brief to identify viremia. Although the risk for transmission of pandemic influenza by transfusion seems to be low, further investigation is needed to elucidate this risk.

Chieko Matsumoto, Rieko Sobata, Shigeharu Uchida, Takao Hidaka, Syunya Momose, Satoru Hino, Masahiro Satake, and Kenji Tadokoro

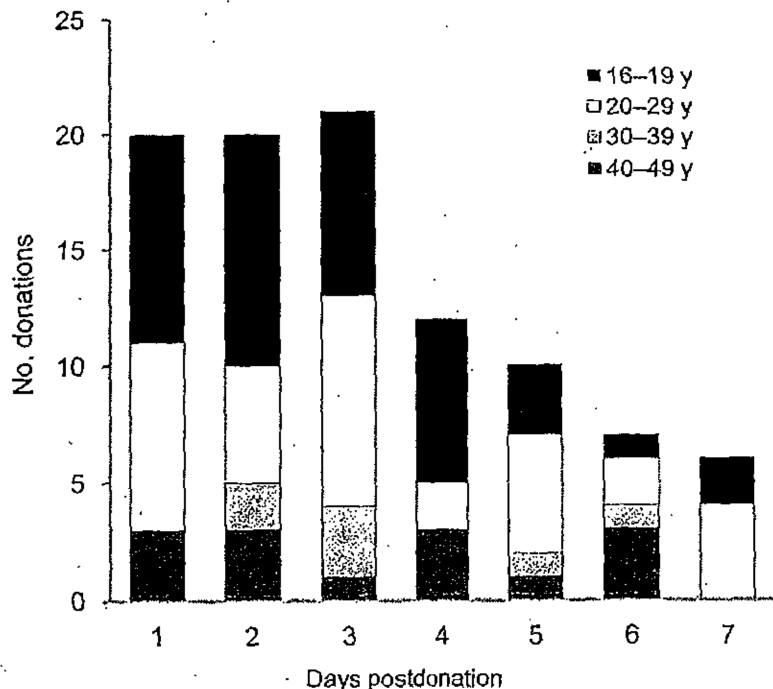

Figure. Number of blood donations from persons for whom pandemic (H1N1) 2009 infection was diagnosed postdonation and time between donation and diagnosis, by donor age, Japan.

Author affiliation: Japanese Red Cross Society Blood Service Headquarters, Tokyo, Japan

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Rapid Emergence of Oseltamivir Resistance

To the Editor: The influenza A pandemic (H1N1) 2009 virus has spread globally since it first appeared in Mexico in April 2009. This third influenza pandemic since the Spanish influenza pandemic of 1918 (*1*) has caused at least 400,000 infections within 6 months; estimated mortality rate is 1.2% (*2*). Emergence of oseltamivir resistance in the pandemic (H1N1) 2009 virus is a rising challenge to global control of the pandemic. So far, 39 oseltamivir-resistant pandemic (H1N1) 2009 viruses have been reported worldwide (*3*). Among the 32 resistant strains reported in October 2009, a total of 13 (41%) were associated with postexposure chemoprophylaxis and 16 (50%) were from samples of patients receiving oseltamivir (*3*). We report rapid emergence of resistance (H275Y mutation) in a patient, 4 days after early treatment with standard doses of oseltamivir for pandemic (H1N1) 2009 pneumonia.

On September 1, 2009, a 20-year-old man with mental retardation consulted the emergency department of Kaohsiung Veterans General Hospital after 1 day of fever, sore throat, and nonproductive cough. A rapid diagnostic antigen test (Quick Vue Influenza test; Quidel, San Diego, CA, USA) showed the man to be positive for influenza A. He was hospitalized for bilateral pneumonitis and treated with oseltamivir (75 mg 2×/day for 5 days), ampicillin/sulbactam, and erythromycin. However, a progressive increase in bilateral perihilar interstitial infiltration developed on the third day, accompanied by increasing dyspnea. Influenza A pandemic (H1N1) 2009 virus was isolated from the patient's nasopharyngeal secretions on days 1 and 4 by using MDCK cells. After DNA sequence analysis of the neuraminidase gene, the mutation of H275Y was not found in the first isolate, but sequence analysis of the second isolate detected mixed populations (C/T) in the 823-nt position of the neuraminidase gene. Only a single pattern (T) was found from the cultured viruses, indicating a mixed quasispecies of oseltamivir-resistant and -susceptible viruses emerging after 4 days of oseltamivir treatment. The oseltamivir-resistant viruses become dominant in the cell culture–propagated viruses. Chan et al. reported a similar case in which the original clinical specimens contained a mixed population of variants, and oseltamivir-resistant viruses become dominant after the passage in MDCK cells (*4*).

On his 9th day in the hospital, the patient was intubated because of acute respiratory distress syndrome (Figure) and given levofloxacin. Urine samples were negative for *Pneumococcus* and *Legionella* spp. antigens. The patient improved and was extubated on hospital day 16.

Paired serologic test results were negative for *Mycoplasma pneumoniae* and *Legionella* spp. antibody; however, immunoglobulin G for *Chlamydia pneumoniae* increased 4-fold. By 37 days after illness onset, clinical signs and symptoms resolved and bilateral lineoreticular infiltration was reduced.

On August 8, 2009, Taiwan had the most devastating typhoon (Typhoon Morakot) in 50 years. The patient reported here had stayed in a typhoon evacuation camp for 1 week before his influenza signs and symptoms developed. Although 4 sporadic cases of pandemic (H1N1) 2009 infections were reported from the same camp, none of the isolated viruses harbored the H275Y mutation in the neuraminidase gene. No evidence of virus transmission was found among healthcare personnel, family members, and camp members who had been in close contact with the patient.

Oseltamivir has been recommended by the US Centers for Disease Control and Prevention for the treatment of

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販売名（企業名）	①ノイアート静注用500単位（ベネシス） ②ノイアート静注用1500単位（ベネシス） ③ノイアート（ベネシス）					

研究報告の概要

最近現れた世界的に流行しているインフルエンザA(H1N1)2009ウイルスの感染は散発的に非常に重篤な場合があるが大多数の症例は軽症例である。ウイルス赤血球凝集素(D222G)の特異的変異は、ノルウェーでの致命的及び重篤な症例で相当な頻度で見られたが、臨床的に軽度の症例では実質的に存在しなかった。この違いは統計学的に有意であり、我々のデータは突然変異と臨床転帰の間の因果関係の可能性と整合している。
2009年に世界的に流行しているインフルエンザA(H1N1)は症例の圧倒的多数では軽度そして自己限定的疾患によって特徴づけられた。しかしながら、重篤そして致命的な症例（主にウイルス性肺炎で多い）は、そのような臨床転帰が季節性インフルエンザではあまり見られない年齢層に起こっていた。どんなウイルス及び宿主関連因子がこの二分化を決定するのかをより理解することが重要である。
我々の知る限りでは、これは重篤な臨床転帰と相関する世界的に流行しているウイルスの変化の最初の同定である。しかしながら、我々のデータがD222G突然変異と重症度の間に関係あることを統計学的に有意な支持をする一方、軽度の症例数では、非重篤症例で変異ウイルスの頻度が本当に低いのか大規模な同定が必要である。D222G突然変異ウイルスが広まっていないならば、すなわち、それは伝播性でないため、公衆衛生への影響は限定的である。しかしながら、もし大規模な曝露を通して伝播したならば、普通に伝播している異型より毒性があるかもしれない、ウイルスは重症症例の管理に関連がある可能性がある。
更に、それは現在世界的に流行しているウイルスの一般的に非常に低い毒性は固定された特徴ではない、しかも、それは個人及び集団レベルでの感染を制限するための手段を実行する際の安心感のための理由にはならない。

使用上の注意記載状況・その他参考事項等

代表としてノイアート静注用500単位の記載を示す。
2．重要な基本的注意
(1)本剤の原材料となる献血者の血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体、抗HTLV-I抗体陰性で、かつALT(GPT)値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査（NAT）を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、Cohnの低温エタノール分画で得た画分から人アンチトロンビンIIIを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びウイルス除去膜によるろ過処理を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見	今後の対応
パンデミックインフルエンザA(H1N1)の重症化にウイルス赤血球凝集素(D222G)の突然変異が関係しているとの報告である。 インフルエンザA(H1N1)はオルソミクソウイルス科に属し、ビリオンは球形で、直径80～120nmの脂質エンベロープを有する比較的大きなRNAウイルスである。万一、インフルエンザA(H1N1)が原料血漿に混入したとしてもBVDをモデルウイルスとしたウイルスバリデーション試験成績から、製造工程にて十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないものと考えるので、特段の措置はとらない。

Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010

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Infection with the recently emerged pandemic influenza A(H1N1) virus causes mild disease in the vast majority of cases, but sporadically also very severe disease. A specific mutation in the viral haemagglutinin (D222G) was found with considerable frequency in fatal and severe cases in Norway, but was virtually absent among clinically mild cases. This difference was statistically significant and our data are consistent with a possible causal relationship between this mutation and the clinical outcome.

The 2009 influenza A(H1N1) pandemic has been characterised by mild and self-limiting disease in the overwhelming majority of cases. However, severe and fatal cases, many of them with primary viral pneumonia, have been occurring in age groups where such clinical outcomes are very rarely seen in seasonal influenza [1,2]. It is important to better understand what viral and host-related factors determine this dichotomy.

Genetic characterisation of clinical specimens

As part of the intensified surveillance carried out during the current influenza pandemic, the national reference laboratory for human influenza at the Norwegian Institute of Public Health collected a large number of respiratory specimens from verified and possible cases of pandemic influenza. In the present study we analysed 61 respiratory specimens from severe and fatal cases that occurred between July and December 2009, as well as from 205 cases with mild clinical outcomes collected between May 2009 and January 2010. Genetic characterisation was performed using conventional sequencing, or with a pyrosequencing assay subsequently developed to detect the particular mutations described below and which facilitated investigation of a large number of specimens.

Here we report the occurrence of an amino acid substitution, aspartic acid to glycine in position 222 (D222G) in the HA1 subunit of the viral haemagglutinin, in clinical specimens from 11 out of 61 cases analysed in Norway with severe outcome. Such mutants were not observed in any of 205 mild cases investigated (Table), thus the frequency of this mutation was significantly higher in severe (including fatal) cases ($p<0.001$, Fisher's exact test, two-sided) than in mild cases. D222G mutants were detected throughout the sampling period, from the first recorded severe cases in July until early December. The frequency of another substitution in the same position, D222E, did not differ significantly between mild and severe cases ($p=0.772$). Yet another substitution, D222N, was observed in a very few cases ($n=4$), and at a higher rate than expected among severe cases (three of four cases, $p=0.039$). The wild type 222D was, not surprisingly, significantly less frequent in severe than in mild cases ($p<0.001$).

In several of the patients where D222G mutant viruses were found, they coexisted with wildtype 222D viruses. Further analysis of this phenomenon is ongoing.

The cases infected with the D222G-mutated virus were not epidemiologically related to each other, and the mutated viruses do not cluster together in phylogenetic analysis (data not shown).

Validity and limitations of the analysis

Cases with severe clinical outcomes were much more likely to be included in our study for several reasons: they are more likely to seek healthcare, they are more likely to be prioritised for virological testing, and their specimens are more likely to be forwarded to the national reference laboratory where they have a higher chance of being selected for detailed analysis than viruses from mild cases. Because of this, we chose to record the frequency of a given genotype in each severity group and compare it with the corresponding frequency in other severity groups. This approach is not expected to have a selection bias.

Cases were classified as mild, severe non-fatal and fatal based on the patient information that was available to us. Some seemingly mild cases may later have exacerbated to severe outcomes without our knowledge, or the presented patient information may have

been incomplete, but we think these cases must be few. On the other hand, all severe and fatal cases were confirmed as non-mild. Thus, the fact remains that only cases confirmed as severe outcomes exhibited the D222G mutation in our investigation.

The sampling period for the cases analysed spans from the initial detections of the pandemic H1N1 virus in early May 2009 until early January 2010. The first severe and fatal cases occurred in July. By the end of December, the epidemic in Norway had largely passed, and a large proportion of cases in our data set is from the peak period in October and November. At all times an effort was made to include a reasonable number of non-severe cases in our analyses, and such cases were well represented throughout the pandemic. The fractions of severe/fatal cases among all analysed cases during the two-month periods July/August (n=21), September/October (n=84), and November/December (n=149), were within the range of 23% to 26%. Severe outcomes were not recorded among the few cases in May and June (n=11) and in January (n=1). We thus do not see a trend over time in the composition of severe versus mild cases in our dataset that could lead to an artificial difference in the frequency of the D222G substitution. Furthermore, the D222G substitution was represented also among the earliest fatal and severe cases in July and August.

Specimens from both the lower and upper respiratory tract were analysed. Lower respiratory tract specimens were available from severe/fatal cases only, and in some cases they were the only materials available. However, in all cases where we had paired upper and lower airway specimens (five cases with 222D and four cases with 222G), the wildtype-versus-D222G pattern was matching between the locations. We have therefore no reason to believe that this difference in proportion of lower airway specimens distorted the analysis.

Discussion

Amino acid position 222 resides in the receptor binding site of the HA protein and may possibly influence the binding specificity and thus the cellular tropism of the virus. The corresponding difference between two viruses from the 1918 Spanish influenza pandemic correlates to a shift in receptor preference [3], which conceivably could make the virus prone to infect a wider range of cells in the lower respiratory tract [4,5]. However, the effect of a mutation depends on the molecular context and it is unclear whether the binding properties are affected likewise in the present pandemic virus as they were in the 1918 influenza virus.

Our observations are consistent with an epidemiological pattern where the D222G substitution is absent or infrequent in circulating viruses, with the mutation arising sporadically in single cases where it may have contributed to severity of infection. This may aid in filling some knowledge gaps identified in a recent preliminary review of this and other mutations in the pandemic virus [6]. The correlation between presence of the D222G substitution and a severe clinical outcome may reflect an increase in pathogenicity caused by the mutation, possibly related to a change in cellular tropism rendering the virus more pneumotropic. Conversely, it is possible that the likelihood of such mutations arising is higher in patients who fail to fight off the virus rapidly and have virus already colonising the lower respiratory tract. These two possibilities are not mutually exclusive. A large proportion of the fatal and severe cases had underlying risk conditions. However, some of the D222G cases manifested themselves as a rapid unexpected deterioration after a period of mild symptoms in previously healthy subjects, and we consider it likely that there is a causal relationship between the occurrence of the D222G mutation in this virus and severe disease.

It should be borne in mind, however, that the majority of severe and fatal cases investigated did not carry the D222G substitution and, clearly, this mutation is not required for a severe outcome.

Conclusions

To our knowledge, this is the first identification of a change in the pandemic virus that correlates with a severe clinical outcome. However, whereas our data lend statistically significant support to an association between the D222G mutation and severity, the number

TABLE

Pandemic influenza A(H1N1) viruses characterised for amino acid position 222 of the haemagglutinin HA1 domain, by clinical outcome, Norway, May 2009–January 2010 (n=266)

HA1 position 222 genotype[b]	Clinical outcome[a] Mild (n=205)	Severe (n=34)	Fatal (n=27)	Severe plus fatal (n=61)	All cases (n=266)
222D (wt)	92% (189)	82% (28)	59% (16)	72% (44)	88% (233)
222G	0% (0)	8.8% (3)	30% (8)	18% (11)	4.1% (11)
222E	7.3% (15)	2.9% (1)	7.4% (2)	4.9% (3)	6.8% (18)
222N	0.5% (1)	5.9% (2)	3.7% (1)	4.9% (3)	1.5% (4)
Total	100%	100%	100 %	100 %	100 %

[a] Clinical outcome based on patient information, assigned into categories by a medical specialist according to WHO guidance criteria [1].
[b] Percentage of genotype within each clinical category is given, with number of cases per category in parentheses.

of mild cases would need to be larger to determine whether mutant viruses are indeed circulating at a very low frequency also in non-severe cases. Provided that D222G mutant viruses are not circulating, i.e. that they are less transmissible, the immediate public health impact of this finding is limited. However, it may have implications for the management of severe cases where the virus, if transmitted through massive exposure, may be more virulent than the commonly circulating variant. Furthermore, it may serve as a reminder that the generally very low virulence of the current pandemic virus is not a fixed characteristic, and that there is no reason for complacency in carrying out measures that limit infection with this virus at individual and population level.

Further virological, clinical and epidemiological investigations are needed to ascertain the role of this and other mutations that may alter the virulence and transmissibility of the pandemic influenza A(H1N1) virus.

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医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	総合機構処理欄
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販売名(企業名)	赤十字アルブミン20(日本赤十字社) 赤十字アルブミン25(日本赤十字社) 赤十字アルブミン20%静注4g/20mL(日本赤十字社) 赤十字アルブミン20%静注10g/50mL(日本赤十字社) 赤十字アルブミン25%静注12.5g/50mL(日本赤十字社)					

研究報告の概要

○感染者におけるトリインフルエンザH5N1ウイルスの感染および複製
本試験では、感染者の組織中のH5N1型ウイルスの分布を理解し、H5N1型ウイルスが肺以外の組織で複製できるかどうかを調べるため、剖検を実施した。
肺のウイルス量が脾臓より多く、心臓、肝臓、腎臓、大腸、小腸または脳ではウイルスが検出されないことを認めた。具体的には、左肺(7.1 log 10 copies/mL)の方が右肺(5.7log 10 copies/mL)よりウイルス量が多かったため、左肺病変の方が病理学的損傷がひどく、肺組織中はプラス鎖・マイナス鎖ウイルスRNAの双方が存在した。
しかし、脾臓にはH5N1型ウイルス量は少なく(3.8 log 10 copies/mL)、プラス鎖RNAは存在しなかった。この結果は、H5N1型ウイルス複製が主に肺で起こり、肺の損傷程度は肺中ウイルス量と相関が高いことを示している。脾臓中の低いウイルス量は、循環血液、その他の状況によって起こったことが考えられる。

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使用上の注意記載状況・その他参考事項等

赤十字アルブミン20
赤十字アルブミン25
赤十字アルブミン20%静注4g/20mL
赤十字アルブミン20%静注10g/50mL
赤十字アルブミン25%静注12.5g/50mL

血液を原料とすることに由来する感染症伝播等

報告企業の意見	今後の対応
H5N1型鳥インフルエンザウイルスの複製が主に肺で起こり、脾臓中の低いウイルス量は、循環血液、その他の状況によって起こったことが考えられるとの報告である。 インフルエンザは毎年流行をみる最もポピュラーな疾患であるが、本剤によるいかなるインフルエンザウイルス感染の報告はない。本剤の製造工程には、平成11年8月30日付医薬発第1047号に沿ったウイルス・プロセスバリデーションによって検証された2つの異なるウイルス除去・不活化工程が含まれているため、本剤の安全性は確保されていると考える。	日本赤十字社では家禽に高病原性トリインフルエンザの流行が認められた場合、当該飼養農場の関係者や防疫作業従事者の献血制限を行っている。新型インフルエンザが流行した場合、献血者減少につながることも予想される。今後も引き続き情報の収集に努める。

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Infection and replication of avian influenza H5N1 virus in an infected human

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Abstract The highly pathogenic avian influenza H5N1 viruses usually cause severe diseases and high mortality in infected humans. However, the tissue tropism and underlying pathogenesis of H5N1 virus infection in humans have not been clearly elucidated yet. In this study, an autopsy was conducted to better understand H5N1 virus distributions in tissues of infected humans, and whether H5N1 virus can replicate in extrapulmonary tissues. We found that the lungs had the higher viral load than the spleen, whereas no detectable viruses in tissues of heart, liver, kidney, large intestine, small intestine, or brain. Specifically, the viral load was higher in the left lung (7.1 log10 copies per ml) in relation to the right lung (5.7 log10 copies per ml), resulting in more severe pathological damage in the left lung, and lung tissues contained both positive- and negative-stranded viral RNA. However, there existed a low level of H5N1 viruses in the spleen (3.8 log10 copies per ml), with the absence of positive-stranded viral RNA. Our results indicate that replication of H5N1 viruses mainly occurs in the lungs, and the degree of lung damage is highly correlated with the viral load in the lungs. The low-load viruses in the spleen might be introduced through blood circulation or other ways.

Keywords Influenza virus · H5N1 · Replication · Viral load · Tissue distribution

Jing-Jiao Zhou and Dan-Yun Fang equally contributed to this work.

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Introduction

The highly pathogenic avian influenza H5N1 viruses could replicate systemically in a variety of organs in poultry, such as respiratory tract, intestine, and spleen, affecting these organs, even the central nervous system [1–4]. H5N1 viruses could also cause disseminated infections in either naturally or experimentally infected mammalian species, i.e., tiger, leopard, and ferret [5–10]. However, evidence for extrapulmonary replication of H5N1 viruses in humans, in general, has been lacking at present. Some studies had found H5N1 viruses exclusively in respiratory tract (mainly in lung) [11, 12]. Other studies had found the presence of H5N1 viruses in many extrapulmonary organs, such as intestine, liver, and brain, which indicated that virus dissemination seems to occur in some humans through blood circulation or other ways [13–17]. In March 2006, the first case of avian influenza H5N1 virus infection was identified in Guangdong province of China. An autopsy was then conducted to detect the virus distribution and load, which, we hoped, would provide some insights into H5N1 infection and replication in both pulmonary and extrapulmonary organs.

Materials and methods

Patient and virologic diagnosis

A thirty-six-year-old male patient, who had a 4-day history of discomfort of fever, throat pain, and dry cough, was admitted to hospital on February 26, 2006. A chest radiograph obtained on admission showed evidence of left lower pneumonia. His condition was rapidly deteriorated, featured by consecutive high body temperature and

dysfunctions of multiple systems, including respiratory system, circulatory system, central nervous system, liver, kidney, and gastrointestinal system. Chest radiograph revealed a massive consolidation shadow in both lungs on February 28, which, however, was much more severe in the left lung. Despite active treatment, the patient finally died from adult respiratory distress syndrome (ARDS) complicated with multiple organ failure (MOF) on March 2. None of the anti-influenza drugs, such as oseltamivir or amantadine, were used in the treatment.

This patient had been to the market where live chickens were slaughtered for sale 1 week prior to onset of symptoms, so the patient's tracheal aspirates were detected for H5N1 viral RNA using H5N1 real-time RT-PCR Kit (PG Biotech, China) on March 1. The full-length gene segments of hemagglutinin (HA) and neuraminidase (NA) were amplified by using one-step RT-PCR Kit (Qiagen, Germany) with the specific primer pairs (HA-F 5′-AGCAAA AGCAGGGGTTCAAT-3′, HA-R 5′-AGTAGAAACAAG GGTGTTTT-3′; NA-F 5′-AGCAAAAGCAGGAGTTCAA A-3′, NA-R 5′-AGTAGAAACAAGGAGTTTTTTT-3′), the reaction was subjected to a pre-cycle condition consisting of 30 min at 50°C(for reverse transcription), 15 min at 95°C followed by 25 circles of amplification. Each cycles consisted of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min 45 s. The amplification ended with a final extension at 72°C for 10 min. The PCR products were purified and cloned into the pGEM-T vector (Promega, USA). The positive clones were sequenced with T7 and SP6 primers by a 3730 automated DNA sequencer (ABI, USA).

Analysis of viral load and replication in autopsy specimens

On March 4, 2006, an autopsy was carried out in Zhongshan School of Medicine, Sun Yat-sen University. Tissues of the left lung, right lung, brain, heart, spleen, liver, kidney, large intestine, and small intestine were obtained, respectively. Some specimens were used for pathological analysis, and the remaining was stored at −80°C in small pieces for future study.

The obtained tissues were minced on ice with presence of culture medium, which were then centrifuged at the speed of 1,500 rpm for 15 min at 4°C. Supernatant was collected and added into lysis buffer of QIAamp Viral RNA Kit (Qiagen, Germany). RNA was then extracted according to the manufacturer protocol. Viral RNA was detected using H5N1 real-time RT-PCR Kit (PG Biotech, China) on ABI 7000 Real-Time PCR System (ABI, USA). Standard curve was used in the quantitative analysis of H5N1 RNA isolated from the autopsy tissues. In our study, the preparation of reagents, nucleic acids extraction, and nucleic acid amplification were performed in three physically separated laboratories.

To analyse viral replication in autopsy tissues, strand-specific RT-PCR was performed with H5 specific primer pairs H5F (5′-GCCATTCCACAACATACACCC-3′, 943–963) and H5R (5′-CTCCCCTGCTCATTGCTATG-3′, 1158–1139). Briefly, two-step reactions were used. First, RT reaction was done in the presence of the primer H5F or H5R. cDNA products then underwent PCR with H5F and H5R. The amplified fragment was about 216 bp and detected by agarose gel electrophoresis.

Results

Real-time RT-PCR had revealed H5N1 viral RNA in the patient's tracheal aspirates. HA and NA gene sequences amplified were the most related to those of avian influenza H5N1 viruses, Duck/Guangxi/5165/05 and Duck/Hunan/1265/05 (99.5 and 99.1% homologous, respectively). Therefore the patient was identified as avian influenza H5N1 virus infected.

Real-time RT-PCR had detected H5N1 viral RNA in the lungs and spleen, whereas there was no detectable viral RNA in tissues of heart, liver, kidney, large intestine, small intestine, or brain. Specifically, the viral load was higher in the left lung (7.1 log10 copies per ml) in relation to the right lung (5.7 log10 copies per ml); and there existed a lower level of H5N1 viruses in the spleen (3.8 log10 copies per ml) (Fig. 1). To confirm a successful H5N1 viral RNA isolation from the autopsy tissues, GAPDH mRNA amplified using RT-PCR served as the internal reference in our study (data not shown). At the same time negative controls did not produce H5 genes, which suggests there is no cross contamination in RT-PCR amplification.

To further elucidate whether H5N1 viral RNA in the lungs and spleen was H5N1 genome RNA, or alternatively, was replicated by H5N1 viruses, we performed strand-specific RT-PCR amplification. Our results indicated that negative- and positive-stranded RNA were detectable in both the left and the right lung, but there was only negative-stranded RNA in the spleen (Fig. 2). An independent duplication RT-PCR was performed under the same condition to confirm the result.

Discussion

In our study, a high viral load was detected only in the lungs in which both positive- and negative-stranded RNA coexisted, which was consistent with previous findings that replication of H5N1 viruses mainly occurs in the lungs of humans and mammals [18–20]. In line with the finding that

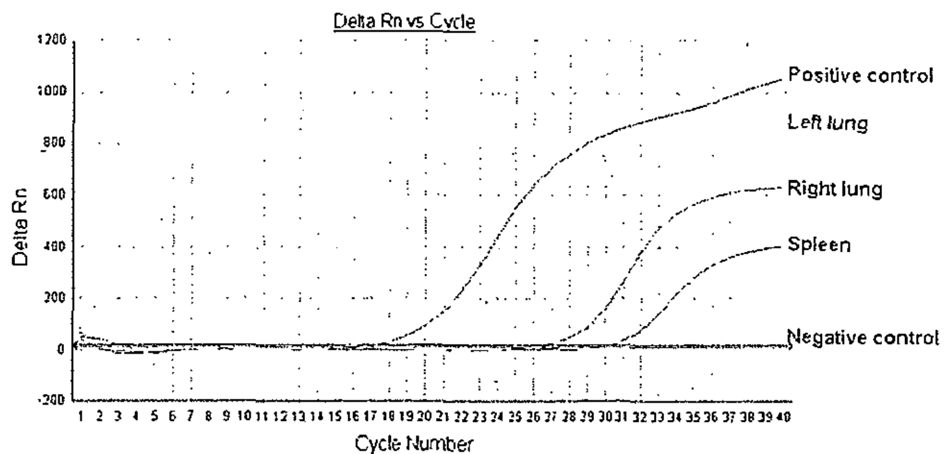

Fig. 1 Interpretation of H5N1 influenza viral RNA in autopsy tissues by single real-time RT-PCR. Different load of H5N1 influenza viral RNA existed in the left lung, right lung, and spleen

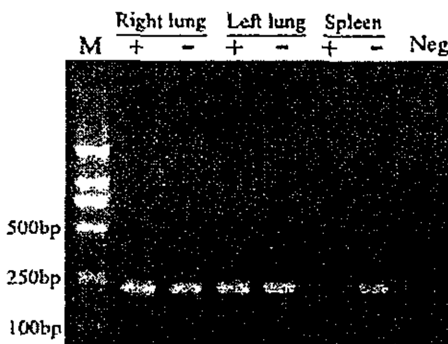

Fig. 2 Detection of positive- and negative-stranded viral RNA in the lungs and spleen by strand-specific RT-PCR. *M* DNA mark, − Negative-stranded RNA, + Positive-stranded RNA, *Neg* Negative control

the viral load was higher in the left lung in relation to the right lung, a chest radiograph obtained on admission showed evidence of left lower pneumonia, and histopathological analysis of the autopsy specimens had also suggested more severe pathological damage of the left lung, featured by more severe alveolar damage and diffuse exudation [21]. These results had demonstrated that the viral load in the lungs was related to the degree of lung damage. However, whether the observed damage was a direct result of the viral replication or a consequence of the dysfunction of cytokines and chemokines induced by these high-load viruses were still unclear.

Previous studies had shown that avian influenza H5N1 viruses could also target immune organs, in addition to the lung [22, 23]. In our study, a low viral load was detected in the spleen, but with absence of positive-stranded H5N1 viral RNA. Influenza viruses contain negative-stranded RNA, and they first replicate positive-strand RNA, which served as mRNA and the template for genome replication of progeny virus. In our study no positive-stranded viral RNA was detected, which suggested that the H5N1 virus did not replicate in the spleen, or that only little replication occurred [24]. The H5N1 viruses of low load in the spleen might be introduced through blood circulation or other ways.

When compared with that of the human- or swine-derived influenza viruses, NA activity of the avian influenza viruses is more resistant to the low pH environment in the upper digestive tract [25, 26]. Accordingly, the highly pathogenic avian influenza H5N1 viruses can replicate in human intestine, resulting in gastrointestinal symptoms, so that H5N1 viruses were detected in the intestine of infected humans [24, 27, 28]. Clinical data had suggested that the patient presented gastrointestinal symptoms in early stages of disease progression, which finally developed into gastrointestinal dysfunctions. But viral RNA was detected neither in large intestine nor small intestine in our study. Some literature suggested that antiviral drugs can lower the level of viral replication and interfere with the detection of viruses in the examined tissues [24, 29]. However, none of the anti-influenza drugs, such as oseltamivir or amantadine, were used in the treatment.

The HA cleavage site of highly pathogenic H5N1 viruses contains multiple basic amino acids, which could be hydrolyzed by a broader range of cellular proteases, so that the tissue tropism for H5N1 viruses is not restricted to the lungs, but extends to other organs, including the brain [30, 31]. A boy confirmed as H5N1 infected presented with severe diarrhea and acute encephalitis symptoms, and H5N1 virus was isolated from patient's throat, serum, feces, and the cerebrospinal fluid [32]. In addition to lung tissues, some studies had detected both positive- and negative-stranded RNA in large intestine, small intestine, and liver, suggesting the possibility of viral replication in the intestines and liver [15, 24]. Furthermore, viral gene sequences and antigen were detectable in neurons of the brain, T cells of the lymph node, and Hofbauer cells of the placenta, which was indicative of viral replication in extrapulmonary tissues [29]. The H5N1 virus obtained from the patient has multiple basic amino acids at the HA

cleavage site, which has molecular characteristics of the highly pathogenic avian influenza viruses [33]. The viral RNA was detectable in the patient's lung and spleen in our study. These findings suggested that H5N1 viruses might be transmitted to extrapulmonary tissues, causing disseminate infection. However, viral distribution and replication vary to a certain extent from individual to individual, which might be explained by tissue tropism differences of viral strains, or that viral distribution might differ in different stages of disease progression, or that different individuals reacted differently to H5N1 viruses.

The autopsy tissues of H5N1 infected cases can often not be obtained due to various reasons (e.g. religion), so reports concerning the tissue tropism and distribution of H5N1 viruses are lacking. We studied H5N1 viral load and replication in autopsy tissues, and the relationship between the viral load and tissue damage, which had significant implication for the further investigation of the tissue tropism and pathogenesis of H5N1 viruses.

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別紙様式第2−1

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日 2009年11月4日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称	別紙のとおり		研究報告の公表状況	Schweiz Arch Tierheilkd 151:433-436	公表国 スイス	
販売名(企業名)	別紙のとおり					

研究報告の概要		使用上の注意記載状況・その他参考事項等
研究報告の概要	問題点:新規なプロテアーゼ抵抗性プリオン蛋白質検出系による検査の結果、BSE罹患牛から生まれた仔牛のBSE罹患率は、非BSE罹患牛から生まれた仔牛のBSE罹患率より高率であった。 出産後にBSEを発症した母ウシから生まれ、過去の組織検査及び免疫組織検査ではBSE陰性とされた仔牛の血液検体を現在バリデーション中である新規なプロテアーゼ抵抗性蛋白質(PrP^{res})検出系で検査したところ、BSEに罹患した母ウシから生まれた仔牛のBSE罹患率が16.1%であったのに対し、BSEに罹患していない母ウシから生まれた仔牛のBSE罹患率は4.2%であり、BSE罹患牛を母ウシに持つ仔牛の方がBSE罹患率が高率であった。また、出産後1年以内にBSEと診断された母ウシから生まれた仔牛のBSE罹患率は、出産後1年以上経過してからBSEと診断された母ウシから生まれた仔牛のBSE罹患率よりも有意に高率であった。但し、検出されたPrP^{res}が感染性のプリオン蛋白質であるかは動物実験による検証が必要である。これらの結果は、神経組織学的異常が認められる前に、血液検体による感染確認の可能性を示唆すると共に、ウシにおけるBSE垂直感染の可能性が未だ払拭できないことを示している。	別紙のとおり

報告企業の意見	今後の対応
別紙のとおり	今後とも関連情報の収集に努め、本剤の安全性の確保を図っていきたい。

MedDRA/J ver.12.1

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別紙

一般的名称	①人血清アルブミン、②人血清アルブミン、③人血清アルブミン*、④人免役グロブリン、⑤人免役グロブリン、⑥人免役グロブリン、⑦乾燥ペプシン処理人免疫グロブリン、⑧乾燥ペプシン処理人免疫グロブリン、⑨乾燥スルホ化人免疫グロブリン、⑩乾燥スルホ化人免疫グロブリン、⑪乾燥スルホ化人免疫グロブリン、⑫乾燥スルホ化人免疫グロブリン、⑬乾燥スルホ化人免疫グロブリン、⑭乾燥スルホ化人免疫グロブリン*、⑮乾燥濃縮人活性化プロテインC、⑯乾燥濃縮人血液凝固第Ⅷ因子、⑰乾燥濃縮人血液凝固第Ⅷ因子、⑱乾燥濃縮人血液凝固第Ⅷ因子、⑲乾燥濃縮人血液凝固第Ⅷ因子、⑳乾燥濃縮人血液凝固第Ⅸ因子、㉑乾燥濃縮人血液凝固第Ⅸ因子、㉒乾燥濃縮人血液凝固第Ⅸ因子、㉓乾燥濃縮人血液凝固第Ⅸ因子、㉔乾燥抗破傷風人免疫グロブリン、㉕乾燥抗破傷風人免疫グロブリン、㉖抗 HBs 人免疫グロブリン、㉗抗 HBs 人免疫グロブリン、㉘トロンビン、㉙フィブリノゲン加第ⅩⅢ因子、㉚フィブリノゲン加第ⅩⅢ因子、㉛乾燥濃縮人アンチトロンビンⅢ、㉜乾燥濃縮人アンチトロンビンⅢ、㉝ヒスタミン加人免疫グロブリン製剤、㉞ヒスタミン加人免疫グロブリン製剤、㉟人血清アルブミン*、㊱人血清アルブミン*、㊲乾燥ペプシン処理人免役グロブリン*、㊳乾燥濃縮人アンチトロンビンⅢ
販売名（企業名）	①献血アルブミン 20 "化血研"、②献血アルブミン 25 "化血研"、③人血清アルブミン "化血研" *、④ "化血研" ガンマーグロブリン、⑤ガンマーグロブリン筋注 450mg/3mL「化血研」、⑥ガンマーグロブリン筋注 1500mg/10mL「化血研」、⑦献血静注グロブリン "化血研"、⑧献血グロブリン注射用 2500mg「化血研」、⑨献血ベニロン－Ⅰ、⑩献血ベニロン－Ⅰ静注用 500mg、⑪献血ベニロン－Ⅰ静注用 1000mg、⑫献血ベニロン－Ⅰ静注用 2500mg、⑬献血ベニロン－Ⅰ静注用 5000mg、⑭ベニロン*、⑮注射用アナクトC2,500 単位、⑯コンファクトF、⑰コンファクトF注射用 250、⑱コンファクトF注射用 500、⑲コンファクトF注射用 1000、⑳ノバクトM、㉑ノバクトM注射用 250、㉒ノバクトM注射用 500、㉓ノバクトM注射用 1000、㉔テタノセーラ、㉕テタノセーラ筋注用 250 単位、㉖ヘパトセーラ、㉗ヘパトセーラ筋注 200 単位/mL、㉘トロンビン "化血研"、㉙ボルヒール、㉚ボルヒール組織接着用、㉛アンスロビンP、㉜アンスロビンP500 注射用、㉝ヒスタグロビン、㉞ヒスタグロビン皮下注用、㉟アルブミン 20%化血研*、㊱アルブミン 5%化血研*、㊲静注グロブリン*、㊳アンスロビンP1500 注射用
使用上の注意記載状況・その他参考事項等	製剤①②④⑤⑥⑦⑧⑨⑩⑪⑫⑬⑮⑯⑰⑱⑲⑳㉑㉒㉓㉔㉕㉖㉗㉘㉙㉚㉛㉜㉝㉞㊳ ・重要な基本的注意 「現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病（vCJD）等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的な vCJD 等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分に行い、治療上の必要性を十分検討の上投与すること。」
報告企業の意見	異常プリオンについては、血中における存在様態等未だ不明な点が多い。今回の報告は血中の異常プリオン検出及び BSE の垂直感染の可能性を示唆するものである。 上記製剤の製造にはウシの肺臓に由来するアプロチニンを使用しているが、当所では医薬発第 1226 号（平成 12 年 12 月 12 日）等の通知に基づいて牛由来原材料に係る原材料の原産国、使用部位等の調査、確認を行い、同通知等で BSE 発生リスクが低いとされる国をウシの肺臓の原産国としている。また、「血漿分画製剤のウイルスに対する安全性確保に関するガイドライン（医薬発第 1047 号、平成 11 年 8 月 30 日）」を参考に実施したクリアランス試験により、異常プリオンのクリアランス効果を有することを確認したウイルス除去膜ろ過工程を含む工程により製造を行い、安全性確保に努めてきている。更に、これまでに上記製剤による異常プリオン感染の報告例は無い。 以上の点から、上記製剤は BSE に対する安全性を確保していると考える。

*現在製造を行っていない

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Untersuchung von BSE-Nachkommen auf Protease-resistentes Prion Protein (PrP^{res}) im Blut

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Zusammenfassung

Das Ziel der vorliegenden Arbeit war, zu untersuchen, ob im Blut von schweizerischen BSE-Nachkommen (Gruppe A) Protease-resistentes Prion Protein (PrP^{res}) vorkommt und ob sich die Häufigkeit des Vorkommens von derjenigen einer gesunden Kontrollpopulation aus dem Jahr 2006 (Gruppe B) unterscheidet. Die Gruppe A bestand aus 181 Nachkommen von an BSE erkrankten Kühen, die Gruppe B aus 240 gesunden Rindern aus einem Gebiet, in welchem in den Jahren 2001 bis 2006 keine BSE diagnostiziert worden war. Die Blutproben wurden mit einem BSE-Lebendtest (Alicon PrioTrap®) zum Nachweis von Protease-resistentem Prion Protein untersucht. Um abzuklären, ob zwischen der Zeitdifferenz von der Geburt des Nachkommens bis zur Erkrankung der Mutter an BSE eine Beziehung in Bezug auf den Nachweis von PrP^{res} beim Nachkommen bestand, wurde diese Zeitdauer bei jedem Nachkommen errechnet. Bei 29 (16.1%) von 181 untersuchten BSE-Nachkommen wurde im Blutplasma PrP^{res} nachgewiesen, 152 Tiere waren negativ. Nachkommen, die innerhalb eines Jahres vor dem Auftreten von klinischen Symptomen des Muttertieres geboren worden waren, wiesen im Blut signifikant häufiger PrP^{res} auf als Tiere, bei denen der zeitliche Abstand von der Geburt bis zur Erkrankung mehr als ein Jahr betragen hatte ($P < 0.05$). In der Kontrollgruppe wurden 10 von 240 Tieren (4.2%) positiv auf PrP^{res} getestet. Die Untersuchungen haben gezeigt, dass beim Rind im Blut Protease-resistentes Prion Protein nachgewiesen werden kann und dass dieses bei Nachkommen von BSE-Kühen häufiger vorkommt als bei Tieren aus einer gesunden Kontrollpopulation.

Schlüsselwörter: Rind, BSE-Nachkommen, Protease-resistentes Prion Protein

[1]*Die vorliegende Publikation beruht auf den Ergebnissen der Dissertation von Dr. Andreas Tschuor*

Protease-resistant prion protein (PrP^{res}) in the blood of offspring of cows that developed BSE

The goal of the present study was to investigate whether protease-resistant prion protein (PrP^{res}) occurs in plasma samples of offspring of cows that developed bovine spongiform encephalopathy (BSE; group A) and to compare the prevalence with that of a healthy control group in 2006 (Group B). Group A consisted of 181 offspring of cows that developed BSE and group B consisted of 240 healthy animals from a region in Switzerland where no cases of BSE occurred from 2001 to the end of 2006. All plasma samples were evaluated using Alicon PrioTrap®, an antemortem test for PrP^{res}. The time between birth of the offspring and onset of BSE in the dam was calculated to determine its relationship with the presence of PrP^{res} in the plasma of the offspring. From 181 offspring, 29 (16.1%) had PrP^{res}-positive plasma samples. Offspring that were born within one year of the onset of BSE in the dam had a significantly higher prevalence of PrPres-positive plasma samples than those born more than one year before the onset of BSE in the dam. Ten (4.2%) of 240 control cattle had PrP^{res}-positive plasma samples. Thus, PrP^{res} can be detected in bovine blood and occurs more frequently in the offspring of cows that develop BSE than in cattle of a healthy control population.

Keywords: cattle, offsprings of BSE cows, PrP^{res}

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Einleitung

Bei Nachkommen von britischen BSE-Kühen wurde neurohistologisch signifikant häufiger BSE diagnostiziert als bei Nachkommen von Kühen, die nicht an BSE erkrankt waren (SEAC, 1996). Das Risiko eines BSE-Nachkommens, selbst an BSE zu erkranken, wurde vom britischen Ministry of Agriculture, Fisheries and Food (MAFF) auf ca. 10% geschätzt, und es wurde proklamiert, dass eine Übertragung von BSE von der erkrankten Mutter auf das Kalb nicht ausgeschlossen werden kann (Masood, 1996). Diese Untersuchungen veranlassten die schweizerischen Behörden im Jahr 1996, alle Nachkommen von BSE-Kühen klinisch untersuchen, euthanasieren und danach auf BSE abklären zu lassen. Bei keinem Tier wurden damals Hinweise für eine BSE-Infektion gefunden (Braun et al., 1998; Fatzer et al., 1998). In der seither vergangenen Zeit haben sich viele Forschergruppen mit der Entwicklung eines Bluttests zum Nachweis von BSE beschäftigt. Einer dieser Gruppen ist es gelungen, einen Test zum Nachweis von Protease resistentem Prion Protein (PrP^{res}) in Körperflüssigkeiten zu entwickeln (Firma Allcon, unveröffentlicht). In der vorliegenden Arbeit wurde ein in der Validierung befindlicher Prototyp des ante mortem Testverfahrens zum Nachweis von BSE eingesetzt. Das Ziel der vorliegenden Arbeit war es, mit Hilfe des neuen Testverfahrens zu untersuchen, ob im Blut von schweizerischen BSE-Nachkommen Protease-resistentes Prion Protein (PrP^{res}) vorkommt und ob sich die Häufigkeit des Vorkommens von derjenigen einer gesunden Kontrollpopulation aus dem Jahr 2006 unterscheidet.

Tiere, Material und Methoden

Tiere

Blutproben von 2 Tiergruppen (A und B) wurden auf das Vorhandensein von Protease resistentem Prion Protein (PrP^{res}) im Blut untersucht. Die Gruppe A bestand aus 181 Nachkommen von an BSE erkrankten Kühen. Die Nachkommen dieser Kühe waren im Winter 1996/97 auf Anordnung des schweizerischen Bundesrats an der Klinik für Wiederkäuer untersucht und danach euthanasiert worden (Braun et al., 1998). Bei den Müttern dieser Tiere konnte BSE in allen Fällen histologisch und immunhistochemisch nachgewiesen werden. Bei den 181 Nachkommen dieser Tiere war BSE postmortal weder bei der neurohistologischen noch bei der immunhistochemischen Untersuchung gefunden worden (Fatzer et al., 1998). Für den Nachweis von Protease-resistentem Prion Protein stand von jedem Nachkommen eine Blutplasmaprobe zur Verfügung, die seit 1996/97 bei -80 °C gelagert worden war. Die Gruppe B bestand aus 240 gesunden Rindern der Schweizer Braunviehrasse im Alter von 1 bis 9 Jahren aus dem Vorderrheintal des Kantons Graubünden. Die Blutplasmaproben von diesen Tieren waren im Jahr 2006 speziell für diese Untersuchung gewonnen worden. BSE war in diesem Gebiet in den Jahren 2001 bis 2006 bei keinem einzigen Tier nachgewiesen worden.

Untersuchung der Blutproben auf PrP^{res}

Die Blutproben wurden mit dem Ante Mortem-BSE-Test untersucht. Das Testprinzip beruht darauf, dass in einem ersten Schritt die Prion Proteine PrP^{C} und PrP^{res} an ein Liganden-gekoppeltes Harz (Franscini et al., 2006) gebunden werden, welches die Prion Proteine mit hoher Affinität und Spezifität bindet. In einem zweiten Schritt wird PrP^{res} nach Behandlung der Probe mit Proteinase K wie beschrieben (McKinley 1983) im Westernblot nachgewiesen. Das normale Prion Protein, PrP^{C}, ist Protease-sensitiv und wird daher im Test nicht nachgewiesen. Ob es sich bei dem nachgewiesenen Protein tatsächlich um infektiöses Prion Protein (PrP^{Sc}) handelt, muss im Tierexperiment noch bestätigt werden.

Zeitdifferenz von der Geburt der BSE-Nachkommen zur BSE-Erkrankung der Mutter

Um abzuklären, ob zwischen der Zeitdifferenz von der Geburt des Nachkommens bis zur Erkrankung der Mutter an BSE eine Beziehung in Bezug auf den Nachweis von PrP^{res} beim Nachkommen besteht, wurde diese Zeitdauer für jeden Nachkommen errechnet.

Statistik

Die statistische Auswertung der Häufigkeiten erfolgte mit dem Programm StatView 5.0 (SAS Institut, 8602 Wangen, Schweiz).

Ergebnisse

Nachweis von PrP^{res}

Bei 29 (16.1%) der 181 untersuchten BSE-Nachkommen wurde im Blutplasma PrP^{res} nachgewiesen, 152 Tiere waren im Test negativ. In der Kontrollgruppe wurden 10 von 240 Tieren (4.2%) positiv auf PrP^{res} getestet (Differenz $P < 0.05$).

Zeitdifferenz von der Geburt der BSE-Nachkommen zum Diagnosedatum BSE beim Muttertier

41 Nachkommen waren ein Jahr, 79 Nachkommen zwei Jahre, 41 Nachkommen drei Jahre und 20 Nachkommen 4 oder mehr Jahre vor der BSE-Diagnose beim Muttertier geboren worden. Nachkommen, die innerhalb eines Jahres vor der Erkrankung des Muttertieres geboren worden waren, wiesen im Blut signifikant häufiger PrP^{res}

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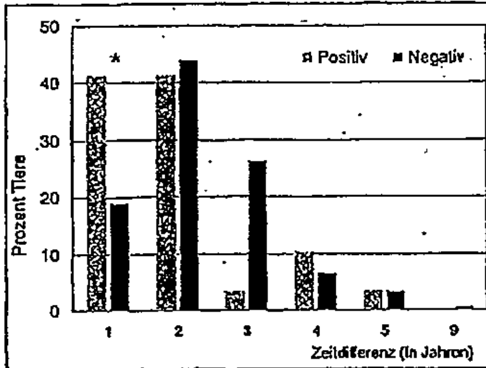

Abbildung 1: Häufigkeitsverteilung der zeitlichen Abstände von der Geburt der BSE-Nachkommen bis zur Erkrankung der Muttertiere an BSE bei Rindern mit positivem und negativem Nachweis von PrP^res im Blut (*Differenz zwischen den 2 Gruppen zum Zeitpunkt 1 $P < 0.05$).

auf als Tiere, bei denen der zeitliche Abstand von der Geburt bis zur Erkrankung mehr als ein Jahr betragen hatte ($P < 0.05$, Mann-Whitney-U-Test, Abb. 1).

Diskussion

Bei 16.1% der schweizerischen BSE-Nachkommen konnte im Blut PrP^res nachgewiesen werden, obschon diese Tiere neurohistologisch und immunhistochemisch BSE-negativ waren. Im Vergleich dazu wurde PrP^res nur bei 4.2% der gesunden Kontrollpopulation aus dem Jahr 2006 gefunden. Unsere Befunde sind ähnlich wie diejenigen der britischen Kohortenstudie. Bei dieser Studie zeigten 14% von 301 BSE-Nachkommen die für BSE charakteristischen neurohistologischen Befunde (SEAC, 1997). Die Ergebnisse können verschieden interpretiert werden. Einerseits ist es möglich, dass bei den BSE-Nachkommen deshalb mehr positive Fälle entdeckt wurden als bei den Kontrolltieren, weil die Nachkommen von ihren an BSE erkrankten Müttern infiziert wurden. Andererseits kann die niedrigere Häufigkeit bei den Kontrolltieren mit dem starken Absinken der BSE-Häufigkeit in der Schweiz erklärt werden. Die Tatsache, dass nur 16.1% der BSE-Nachkommen PrP^res positiv reagiert haben, zeigt, dass der maternalen Übertragung von BSE nur eine Nebenrolle bei der Verbreitung der BSE zukommt. Für die Bekämpfung der BSE ist es aber wichtig, dass auch dieser Infektionsweg durch die Keulung der Nachkommen von BSE-Kühen unterbrochen wird. Eine weitere Frage stellt sich, weshalb in der britischen Studie 14% der Nachkommen neurohistologische Symptome zeigten und in unserer Studie kein einziges Tier. Dieser Unterschied ist damit zu erklären, dass die von uns untersuchten Nachkommen im Alter von 3.1 ± 0.8 Jahren getötet und untersucht wurden, das heisst zu einem Zeitpunkt, wo es nur in seltensten Fällen zur BSE-Erkrankung gekommen ist. Im Gegensatz dazu wurden die britischen Tiere erst im Alter von 7 Jahren neurohistologisch untersucht. Die im Blut positive Reaktion bei gleichzeitig negativem neurohistologischem Befund weist darauf hin, dass die Infektion im Blut nachweisbar ist, bevor es zur Ausbildung neurohistologischer Veränderungen kommt.

Bei den BSE-Nachkommen mit einem einjährigen Zeitabstand zwischen ihrer Geburt und der BSE-Erkrankung der Mutter wurde signifikant häufiger PrP^res detektiert als bei den Tieren mit längerem Zeitabstand. Die Befunde decken sich mit den Befunden der MAFF-Studie, in welcher für den gleichen Zeitabstand ebenfalls eine signifikante Häufung der neurohistologisch positiven Ergebnisse festgestellt wurde (Donnelly et al., 1997). Eine mögliche Erklärung für die Befunde liegt darin, dass PrP^Sc gegen Ende der Inkubationszeit ausser im Nervengewebe auch vermehrt im Blut vorkommt und über den Blutweg den Fetus transplazentar infiziert (Aguzzi, 2006). Die maternale Transmission einer TSE konnte allerdings bisher erst bei Scrapie nachgewiesen werden, als in den plazentären Kotyledonen von präklinisch und klinisch kranken Scrapie-Schafen PrP^Sc gefunden wurde (Andreoletti et al., 2002). Die maternale Übertragung von BSE bei einer Antilope (Aldhous, 1990) und die BSE-Erkrankung einer Kuh, bei deren Mutter ebenfalls BSE diagnostiziert worden war, liessen allerdings den Verdacht aufkommen, dass eine maternale Transmission auch bei mit BSE infizierten Rindern vorkommen könnte (Aldhous, 1991).

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Examens de descendants d'animaux BSE quant à la présence de protéines prioniques protéases résistantes (PrP^{res}) dans le sang

Le but du présent travail était d'étudier si les protéines prioniques protease résistantes (PrP^{res}) étaient présentes dans le sang de descendants d'animaux BSE (groupe A) et de voir si la fréquence de cette présence était différente de celle constatée dans une population de contrôle en 2006 (groupe B). Le groupe A se composait de 181 descendants de vaches atteintes de BSE, le groupe B de 240 bovins d'une région dans laquelle de 2001 à 2006 aucun cas de BSE n'avait été diagnostiqué. Les échantillons ont été testés avec Alicon Prio Trap ® pour mettre en évidence la protéine prionique protéase résistante (PrP^{res}). Afin de savoir s'il y avait une relation entre l'intervalle de temps séparant la naissance du veau de la maladie de la mère par rapport à la mise en évidence de PrP^{res} chez le veau, cette durée a été calculée pour chaque animal. Chez 29 (16.1 %) des 181 descendants BSE, la PrP^{res} a été trouvée dans le plasma, 152 animaux étaient négatifs. Les animaux qui étaient nés dans l'année précédant l'apparition des symptômes cliniques chez leur mère avaient de façon significative plus souvent la PrP^{res} dans le sang que les animaux chez lesquels l'intervalle entre la naissance et la maladie dépassait une année ($P < 0.05$). Dans le groupe de contrôle, 10 des 240 animaux (4.2 %) ont été positifs au PrP^{res}. Ces examens montrent que la protéine prionique protéase résistante peut être mise en évidence chez les bovins dans le sang et qu'elle est plus souvent présente chez les descendants d'animaux BSE que dans une population de contrôle saine.

Esame sanguigno della proteina prionica resistente alle proteasi (PrP^{res}) nella discendenza da mucche affette da BSE

Scopo del seguente studio è di esaminare se, nel sangue della discendenza da BSE svizzera (gruppo A), era presenta la proteina prionica resistente alle proteasi (PrP^{res}) e se si distingueva, nella sua frequenza, dalla popolazione di controllo sana del 2006. Il gruppo A era composto da 181 discendenti di mucche malate di BSE, il gruppo B era formato da 240 bovini sani provenienti da una regione nella quale dal 2001 al 2006 non sono stati diagnosticati casi di BSE. Le prove di sangue sono state analizzate con un test BSE (Alicon PrioTrap®) per la ricerca della proteina prionica resistente alle proteasi. Per chiarire se nel lasso di tempo tra la nascita della discendenza alla malattia (BSE) della madre ci sia un rapporto in relazione alla presenza di PrP^{res} nella discendenza, questa durata temporale è stata calcolata per ogni discendente. In 29 (16.1 %) dei 181 discendenti da BSE è stato rilevato nel plasma sanguigno la presenza di PrP^{res}, mentre 152 animali sono risultati negativi. I discendenti nati nell'arco di un anno dall'apparizione dei sintomi clinici della madre mostravano nel sangue una frequenza più significativa di PrP^{res} che gli animali nei quali il lasso di tempo dalla nascita fino alla malattia della madre era maggiore di un anno ($P < 0.05$). Nel gruppo di controllo 10 dei 240 animali (4.2 %) sono risultati positivi al test. Gli esami hanno rilevato che, si può ritrovare nei bovini, la proteina prionica resistente alle proteasi (PrP^{res}) e che la discendenza da mucche con BSE appare più di frequente che negli animali provenienti da una popolazione di controllo sana.

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Protease-resistant prion protein (PrPres) in the blood of offspring of cows that developed BSE

はじめに

英国においては、BSE に罹患した母ウシから生まれた仔ウシは、BSE に罹患していない母ウシから生まれた仔ウシと比較して、神経組織学的に BSE と診断される率が有意に高いとするデータが得られている（英国海綿状脳症諮問委員会 SEAC、1996）。英国農漁業食糧省（MAFF）は、BSE の母ウシから生まれた仔ウシが BSE に罹患するリスクを約10%と見積もっており、BSE に罹患した母ウシから仔ウシへの BSE 感染は排除できないとの見解をとっている（Masood, 1996）。こうしたデータを踏まえて、1996 年にスイス当局は、BSE の母ウシから生まれたすべての仔ウシに対して臨床所見検査を実施し、安楽死させ、その後に BSE の有無について検査を行った。その結果、BSE 感染の徴候を示す仔ウシは 1 頭も発見することができなかった（Braun et al., 1998; Fatzer et al., 1998）。その後は今日に至るまで、数多くの研究グループが BSE 検出用の血液検査の開発に取り組んできた。そのうちのある研究グループは、プロテアーゼ抵抗性プリオン蛋白（PrP^{res}）を体液中から検出する方法の開発に成功した（Alicon 社、未公開）。本研究の実施に際しては、この BSE 死亡前検査法の、現時点ではバリデーションの段階にあるプロトタイプを利用した。本研究は、スイスの BSE に罹患した母ウシから生まれた仔ウシの血液中からプロテアーゼ抵抗性プリオン蛋白（PrP^{res}）が検出されるかどうか、また、対照群としての 2006 年時点における正常ウシと比較して、その検出率に差が示されるかどうかについて、この新規検査法を利用して検討することを目的として実施した。

動物、材料、方法

動物

2 群（A 群と B 群）の動物から採取した血液検体を対象に、血液中からプロテアーゼ抵抗性プリオン蛋白（PrP^{res}）が検出されるか否かについて検査を実施した。BSE に罹患した母ウシから生まれた仔ウシ 181 頭を A 群とした。これらの仔ウシは 1996 年から 1997 年にかけての冬季間に、スイス連邦評議会の命令を受けて、反芻動物病院にて検査を実施した後、安楽死させた（Braun et al., 1998）。これらの仔ウシの母ウシは、組織検査の際にも免疫組織化学検査の際にもすべてが BSE 陽性であった。これに対して、181 頭の仔ウシに対する死後検査では、組織検査の際にも免疫組織化学検査の際にもすべてが BSE 陰性であった（Fatzer et al., 1998）。プロテアーゼ抵抗性プリオン蛋白（PrP^{res}）の検査用として、すべての仔ウシから血液検体を採取し、1996 年から 1997 年にかけての冬季間以降は−80℃の温度で保存しておいた。グラウビュンデン州のフォルデルラインタールで飼育された、年齢 1〜9 歳の健康なスイス褐色牛 240 頭を B 群とした。B 群のウシからは、

2006 年に本研究のために特別に血液検体を採取した。2001 年から 2006 年の期間中に、この地域に飼育されていたウシで BSF に罹患したケースは皆無であった。

血液検体に対する PrPres の検査

血液検体に対してこの新規の BSE 死亡前検査を実施した。この検査法の原理について順を追って説明すると、第一段階としてはプリオン蛋白 PrP^{C} と PrP^{res} をリガンド固定化樹脂（Franscini et al., 2006）に結合させる。このリガンド固定化樹脂は、高い親和性および特異性のもとにプリオン蛋白と結合する。第二段階としては、文献に記述の方法（McKinley 1983）に準拠して、検体に対してプロテイナーゼ K 処理を行った後に、ウェスタンブロット法によって PrP^{res} を検出する。正常プリオン蛋白 PrP^{C} はプロテアーゼに感受性を示すので、検出されることはない。検出された蛋白が実際に感染性のプリオン蛋白（PrP^{Sc}）であるかどうかについては、動物実験を実施して検証する必要がある。

母ウシの BSE 発症と BES 仔ウシ誕生との時間的な差

仔ウシが生まれてから母ウシが BSE を発症するまでの時間的長さと、仔ウシにおいて PrP^{res} が検出される率との関係を探るために、すべての仔ウシについて、上記の時間的長さの調査記録を行った。

統計処理

度数の統計評価は、Programm StatView 5.0（SAS Institut、8602 Wangen、スイス）を利用して実施した。

結果

PrPres の検出

BSE の母ウシから生まれた仔ウシ 181 頭中 29 頭（16.1%）の血液検体から PrP^{res} が検出され、残りの 152 頭は陰性であった。対照群で PrP^{res} 陽性の結果を示したのは、240 頭中 10 頭（4.2%）であった（危険率 $p>0.05$）。

仔ウシ誕生から母ウシが BSE と診断されるまでの時間的長さ

母ウシが BSE と診断された時点を基準とした場合、仔ウシ全体(181 頭)のうちの 41 頭が 1 年前、79 頭が 2 年前、41 頭が 3 年前、残りの 20 頭が 4 年またはそれ以前に生まれていた。母ウシが BSE と診断される 1 年前以内に生まれていた仔ウシでは、それ以外の時期に生まれた仔ウシと比較して、血液中から PrP^{res} が検出される率が有意に高かった（$p<0.05$、Mann-Whitney U 検定、図 1）。

考察

スイスの BSE に罹患した母ウシから生まれた仔ウシの血液検体から 16.1%の割合で PrPres が検出された。ただし、これらの仔ウシに対する神経組織検査結果や免疫組織化学検査結果はいずれも BSE 陰性であった。これに対して、対照群としての 2006 年時点における正常ウシの PrPres の検出率は 4.2%程度にすぎなかった。著者のこの所見は英国で実施されたコホート研究の結果と類似していた。英国のコホート研究の際には、BSE に罹患した母ウシから生まれた仔ウシ 301 頭のうちの 14%が、BSE に特徴的な神経組織学的所見を示した（英国海綿状脳症諮問委員会 SEAC、1997）。これらの研究結果については、様々な解釈が可能であろう。そのひとつは、BSE に罹患した母ウシから生まれた仔ウシでは、BSE の母ウシから BSE に感染してしまったために、対照群と比較して BSE の陽性率が高くなったのではないかとする考え方である。他方では、対照群において BSE 陽性率がかなり低かったのは、スイスにおける極度に低い BSE 発症率から説明することができるだろう。また、BSE の母ウシから生まれた仔ウシにおいて PrPres 陽性率が 16.1%程度にすぎなかった事実からは、BSE の伝播拡散にとって、BSE の垂直感染は副次的な意味しか持たないと考えることができるだろう。しかし、BSE との戦いにおいては、BSE に罹患した母ウシから生まれた仔ウシを殺処分することによって、こうした感染経路を絶つのも大切なことであろう。ここで疑問となるのは、英国で実施された研究の際には 14%の仔ウシにおいて神経組織検査結果が陽性であったが、本研究の際には神経組織検査結果が陽性の仔ウシが何故皆無であったかという点である。こうした相違は次のように説明できるだろう。著者の研究の際、殺処分ならびに検査の実施時の仔ウシの年齢は 3.1±0.8 歳であり、これは BSE がほとんど発症することのない年齢である。これに対して、英国で実施された研究の際は 7 歳のウシに対して神経組織検査が実施された。また神経組織検査結果が陰性であったにもかかわらず、血液中の PrPres が陽性という結果を示したことは、神経組織学的異常が開始される前に、血液検体を用いて感染の有無を確認することが可能なことを示している。

母ウシが BSE と診断される 1 年前以内に生まれていた仔ウシでは、それ以外の時期に生まれた仔ウシと比較して、血液中から PrPres が検出される率が有意に高かったが、この所見は MAFF（英国農漁業食糧省)研究の所見と一致している。ただし、MAFF 研究の際にも、同じ 1 年前以内に生まれていた仔ウシでみた場合、神経組織検査結果の陽性率が有意に高くなった（Donnelly et al., 1997）。こうした所見に対しては、PrPres は潜伏期の終わりごろになると、神経組織以外に血液中にも多く存在するようになり、血行路から胎盤を経て胎仔に移行するためとする解釈が可能かもしれない（Aguzzi, 2006）。しかし、伝達性海綿状脳症（TSE）の垂直感染が確認されているのは、現時点ではスクレイピーに限られている。つまり、無症候性または症候性スクレイピーヒツジの胎盤葉からは PrPSc が検出されている（Andreoletti et al., 2002）。しかし、母動物が BSE と診断されている場合、

アンテロープにおいてはBSEの垂直感染（Aldhous, 1990）が認められ、ウシにおいてはBSEの発症が認められることから、ウシがBSEに感染する際にも垂直感染による可能性も存在するという疑いを払拭することができない（Aldhous, 1991）。

図1：仔ウシが生まれてから母ウシがBSEを発症するまでの時間的長さと、仔ウシの血液検体におけるPrP^{res}の陽性率と陰性率との関係の度数分布（* 母ウシの発症までの時間的長さが1年の際の差、$p<0.05$）