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Dynamic changes of lymphocyte counts in adult patients with severe pandemic H1N1 influenza A

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ABSTRACT

Background: Lymphopenia has been observed in severe pandemic influenza A/H1N1 in developed countries. However, data from developing countries are rare and dynamic change of lymphocyte counts in severe pandemic influenza A/H1N1 is scarcely reported. This study aimed to observe change of lymphocyte counts in patients with severe pandemic influenza A/H1N1 and to investigate the correlation of lymphopenia and severe pandemic influenza A/H1N1.
Methods: We retrospectively analyzed the white blood cell counts and differentials and other clinical data in 21 hospitalized patients with severe pandemic influenza A/H1N1 confirmed by reverse-transcription PCR during 2009 and 2010.
Results: All patients, except two cases with bacterial co-infections, had normal or reduced white blood cell counts. Seventeen (81.0%) patients had decreased lymphocyte proportions (<20%) and counts ($<0.8 \times 10^9$/L), with the lowest value of 1.2% and 0.1×10^9/L respectively. A patient with nosocomial infection of influenza A/H1N1 showed that lymphopenia occurred on the first day of illness. Lymphocyte proportions and absolute counts returned to normal or slightly higher than normal in 16 of the 17 patients within 2–3 weeks after the disease onset.
Conclusions: Lymphopenia along with other clinical parameters may be helpful in early differential diagnosis of severe pandemic influenza A/H1N1.

Introduction

In 2009, an outbreak of a respiratory illness that was proved to be caused by a novel swine-origin influenza A/H1N1 virus occurred in Mexico; the virus caused severe pneumonia and respiratory failure with high mortality [1]. Recent studies showed that 9% of community-acquired pneumonia in Europe is associated with the infection of influenza viruses [2]. Moreover, influenza A (H1N1) pdm09 virus can cause hospital infection, resulting in death in critically ill patients admitted to the intensive care unit [3]. Influenza A/H1N1/pdm 2009 and influenza B viruses may co-infect patients during the epidemic season [4]. Thus, influenza A/H1N1 virus infection is still a significant health problem with significant morbidity and mortality.

Influenza A/H1N1 virus infection can cause a broad range of disease spectrum, from mild illness to severe pneumonia with potentially causing acute respiratory distress syndrome [5]. The management of influenza A/H1N1 patients with various severities is different; the patients with mild or moderate influenza-like illnesses are not required to be hospitalized, whereas those with severe illness or with the potentiality to develop acute respiratory distress syndrome should be hospitalized with timely administration of antiviral drugs and systemic supportive therapy. Thus, early

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differentiation of severe form of influenza A/H1N1 infection from mild or moderate infection plays a critical role in the determination of treatment strategy for influenza A/H1N1 patients.

The diagnosis of patients with influenza A/H1N1 is usually based on the detection of influenza A/H1N1 virus RNA by reverse-transcriptase polymerase chain reaction, which has been widely used for the early and rapid diagnosis [6]. However, the assay is not routinely available in health resource limited regions. Even in hospitals with routine screening for influenza A/H1N1 virus RNA, it still takes at least one or two days to get the results. Previous observations showed that white blood cell counts and differentials, a quick, routine laboratory test, may be useful to early define the severe illness caused by influenza A viruses, as lymphopenia occurred in the majority of severe form of influenza A/H1N1 infection [1,7–12]. However, these data were mainly from developed countries; whether the clinical use of lymphopenia as a clue to define severe influenza is globally applicable remains further study. In addition, data on the dynamic change of lymphocyte counts during the course of severe pandemic influenza A/H1N1 have been rarely reported.

In this retrospective study, we aimed to observe the lymphocyte counts in the longitudinal peripheral blood samples from the severe adult patients infected with pandemic influenza A/H1N1 virus in 2009 and 2010.

Materials and methods

Patients and definition of severe H1N1 influenza A

In total, 21 adult patients who were admitted in Nanjing Drum Tower Hospital and confirmed by the positive results of pandemic influenza A/H1N1 RNA detected by the reverse-transcriptase polymerase chain reaction were included in this analysis. The reverse-transcriptase polymerase chain reaction was performed by extraction of total RNA from throat swab samples and reverse transcription to cDNA, followed by amplification of pandemic influenza A/H1N1 RNA as described elsewhere [13].

The severe form of pandemic influenza A/H1N1 virus infection was defined based on the criteria set by the Ministry of Health of the People's Republic of China [14]. Briefly, based on the positive results in the amplification of pandemic influenza A/H1N1 RNA, a patient who met at least one of the following features: (1) sustained high fever (>39 °C) more than 3 days, accompanied severe toxemia, (2) severe cough accompanied purulent and/or bloody sputum, with or without chest pain, (3) increasing respiratory frequency, dyspnea and cyanosis, (4) lethargy, agitation, and/or convulsion, (5) severe vomiting, diarrhea, and dehydration, (6) pneumonia confirmed by chest X-ray or CT scanning, (7) rapid elevation of creatine kinase, creatine kinase isoenzyme and other myocardial enzymes, (8) respiratory failure, (9) septic shock, (10) multiple organ dysfunction, and (11) other severe clinical conditions requiring intensive care treatment, was preliminarily defined with severe H1N1 influenza. The final diagnosis was established after the exclusion of other known causes for above conditions.

This study was approved by the institutional ethics review committee of Nanjing Drum Tower Hospital and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from each patient.

Routine white blood cell counts and definition of lymphopenia

White blood cell counts and differentials were routinely, automatically performed. Briefly, the peripheral blood samples collected by venipuncture in BD Vacutainer® EDTA tubes were loaded on the Sysmex XE-5000 Automated Hematology System (Kobe, Japan), which utilizes the power of fluorescent flow cytometry and hydrodynamic focusing technologies based on a unique diode laser bench and can measure and differentiate cell types in whole blood and other body fluids with high sensitivity and accuracy. Since this study included pregnant women and the total white blood cell counts are usually increased because of the elevation of neutrophils during pregnancy, leading to the relatively decreased proportion of lymphocytes. Therefore, lymphopenia was defined when absolute lymphocyte counts were $<0.8 \times 10^9$/L, which was set up by the calculation of $20\% \times (4 \times 10^9)$/L, in which 20% represents the lower normal limit of lymphocyte proportion of the whole white blood cells and (4×10^9)/L represents the normal lower limit of white blood cell counts.

Data collection

All clinically relevant data, including patient characteristics, pre-existing medical conditions, onset of initial symptoms and signs, laboratory and chest image findings at admission and during the disease courses, and clinical outcomes were retrieved from the information system of Nanjing Drum Town Hospital.

Statistical analyses

Continuous variables were represented as median (min–max), and categorical variables were represented as number and percentage. All analyses were performed with statistical analysis system software, version 9.4 (SAS Institute Inc. Cary, USA).

Results

Since only pneumonic patients who were suspected of having influenza A/H1N1 were hospitalized during the pandemic influenza A/H1N1 season in 2009 and 2010, we only included 21 adult patients with severe influenza A/H1N1 virus infection in the analysis, but no mildly or moderately ill patients were available for analysis. Of them, 9 were male and 12 were female. The average age was 38.4 years old (17–81 years). All 21 patients were confirmed to have pneumonia by computerized tomography (CT) or X-ray chest radiograph. The general characteristics and dynamic changes of white blood cell counts during the clinical course are presented in Table 1. All patients had pneumonia, with 17 (81.0%) in both lungs and only 4 (19.0%) other in either of left or right lung. Of the 21 patients, 19 (90.5%) showed normal or relatively lower white blood cell counts, and two other patients were confirmed to be co-infected with bacteria, patient no. 1 with *Staphylococcus aureus* and patient no. 12 with methicillin-resistant *S. aureus*. Of all 21 participants, 20 recovered after antiviral therapy (oseltamivir) and systemic supportive treatments, and patient no. 12 died on day 46 after the illness onset because of complicated multiple organ failures.

Table 1 shows that, of the 21 patients with severe influenza A/H1N1 virus infection, 17 (81.0%) showed reduced proportion of lymphocytes (defined by lymphocytes <20% of total white blood cells) and absolute lymphopenia (defined by lymphocytes $<0.8 \times 10^9$/L), with the lowest absolute lymphocyte count of 0.1×10^9/L. Of the four other patients (patient 5, 11, 20, and 21) who did not present lymphopenia, patient no. 21 was admitted on day 21 after the illness onset; the possibility of lymphopenia occurred in the early stage of the illness in this patient could not be excluded.

Of the 17 patients with marked lymphopenia, 16 had at least two lymphocyte counts. Table 1 shows that the lymphocyte counts were gradually increasing as the patients' conditions were improved, and most patients had normal lymphocyte counts within 2–3 weeks

Table 1
White blood cell counts at admission and during clinical course in patients with severe influenza A/H1N1 virus infection.

Patient	Sex/age (years) & other condition	Pneumonia	Days after onset	WBC[a] ($\times 10^9$/L)	N[b] (%)	L[c] (%)	L[c] ($\times 10^9$/L)
1	Female/28, gestation 30 weeks	Both lungs, ARDS	8	21.6	96.1	3.1	0.67
			9	29.7	94.6	2.9	0.86
			21	18.1	78	19.5	3.53
2	Female/39, ectopic pregnancy, nosocomial infection	Right lung	−3	14.8	83.2	11.7	1.73
			−1	5.8	55.9	28.8	1.67
			1	6.8	80.9	8.7	0.59
			4	3.2	73	16	0.51
			7	4.3	56.1	23.6	1.01
3	Female/24, gestation 31 weeks	Both lungs	7	4.5	86.4	9.8	0.44
			9	3.8	83.9	10.4	0.40
			11	3.9	69.3	19.7	0.77
			13	5.2	66.2	22.6	1.18
4	Female/27, gestation 32 weeks	Both lungs	7	3.2	83.5	12.7	0.41
			8	4.5	73.9	17.2	0.77
			11	6.6	71.5	18.6	1.23
5	Female/25, gestation 12 weeks	Both lungs	7	2.5	36	48.8	1.22
			11	7.8	70.5	23.3	1.82
6	Female/81, chronic obstructive pulmonary disease	Both lungs	4	7.3	89.3	4.1	0.30
			13	7.6	78.2	10.1	0.77
			17	7.9	72.3	16.7	1.32
7	Female/33	Left lungs	3	7.5	85.4	10.6	0.80
			4	6	86.2	9.8	0.58
			14	5.3	61.9	27.7	1.47
8	Female/17, acute granulocytic leukemia and chemotherapy	Both lungs	8	0.5	51	42.9	0.22
			9	0.4	29.7	54.1	0.22
			10	0.3	41.2	44.1	0.10
			21	0.9	40	30.6	0.28
			25	8.4	66.1	11.8	0.99
			26	5.9	37.9	30.6	1.81
9	Female/19	Both lungs	4	5	84.7	13.3	0.67
			7	2.5	51.2	39	0.98
10	Female/26	Both lungs	10	3.9	74.4	18.6	0.73
			14	3.1	69.4	19.2	0.60
11	Female/47	Left lungs	7	3.8	44.5	43.8	1.66
12	Female/27, bacterial infection and multiple organ failure, died on day 46	Both lungs	4	10.4	98.1	1.2	0.13
			6	22.6	96.8	2.8	0.63
			7	23.9	89.6	4.8	1.15
			10	31.4	90.5	4.1	1.29
13	Male/48	Both lungs	4	2.5	82.1	13.5	0.34
			8	5.7	74.5	13.8	0.79
14	Male/46	Both lungs, plural effusion	7	2.6	79.7	11.7	0.30
			10	4.5	54.9	27.3	1.23
15	Male/24	Both lungs	2	9.2	86.7	8.9	0.82
			4	4.4	41.8	36.9	1.62
16	Male/38	Both lungs	5	3.5	85.8	10.1	0.35
			7	2.3	68.2	29.9	0.69
			11	7.4	63.8	31.2	2.31
17	Male/60	Right lungs	8	4.2	90.2	6	0.25
			10	4.8	77.4	11.2	0.54
			14	6.8	77.4	14.3	0.97
18	Male/76, chronic bronchitis	Both lungs	2	6.8	90.8	4.9	0.33
			4	8	85.9	5.7	0.46
			7	3.2	73.4	14.1	0.45
			8	2.9	66.4	19.4	0.57
19	Male/31	Both lungs	6	4.1	83.2	14.3	0.59
20	Male/44	Both lungs	4	3.1	52.2	36.2	1.12
21	Male/47	Both lungs	21	4.6	16.2	53.4	2.46
			24	3.9	26.6	38.5	1.50

[a] WBC, white blood cells.
[b] N, neutrophils.
[c] L, lymphocytes.

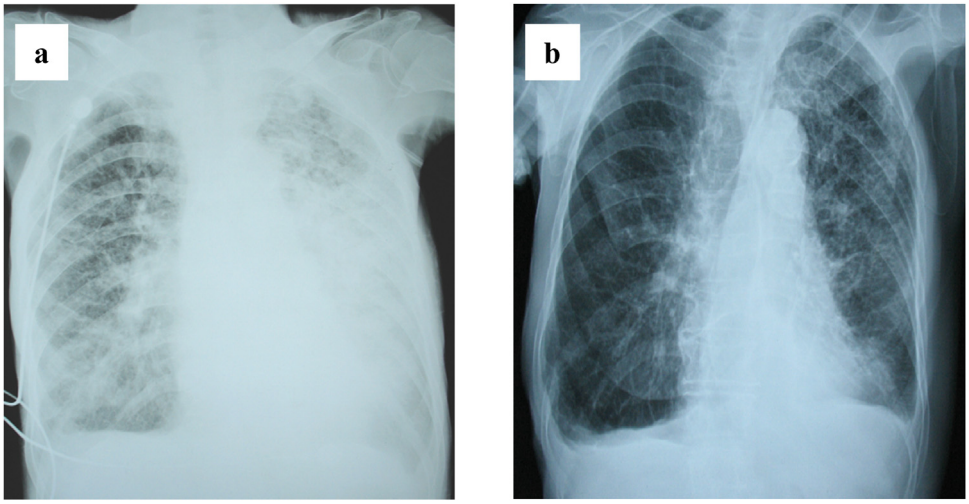

Fig. 1. The chest radiograph of patient 6 (Table 1) at different stages of illness. (a) The film was taken at lying position on day 4 of the illness. The proportion of lymphocytes to total white blood cells was 4.1% and absolute lymphocyte count was 0.3×10^9/L. (b) The film was taken at standing position on day 17. The lymphocytes were accounted for 16.7% of total white blood cells, with an absolute count of 1.32×10^9/L.

after the illness onset, demonstrating the agreement of normalization of lymphocyte counts and the improvement of the illness. Fig. 1a shows the chest X-ray radiograph on the 4th day after the onset in patient no. 6, who had a history of chronic obstructive pulmonary disease for more than 20 years (Table 1). Confluent alveolar opacities in both lungs were easily noted, particularly in the left lung. The patient had severe respiratory failure and the peripheral lymphocyte count was only 0.3×10^9/L (Table 1). After comprehensive treatment, the patient recovered. Fig. 1b shows the chest X-ray radiograph on the 17th day after the onset; the peripheral lymphocyte count had increased to 1.32×10^9/L (Table 1). Additionally, we performed the cytological examination of pleural fluid in a patient (patient 14, Table 1) who developed hydrothorax. While his peripheral lymphocyte count was only 0.30×10^9/L, lymphocytes were accounted for vast majority (78.2%) of the cells in his pleural fluid.

Discussion

The findings in this retrospective analysis indicate that, at the very early phase of the illness, the adult patients with severe influenza A/H1N1 virus infection who had no co-infection of bacteria usually have normal or mildly decreased white blood cell counts and have significant reduction in the proportion of lymphocytes and marked lymphopenia. Therefore, in managing patients with fever and flu-like symptoms, routine blood tests, including white blood cell counts and differentials, should be performed. When the absolute lymphocyte counts are significantly reduced ($<0.8 \times 10^9$/L), this should alert to the possibility of severe influenza infection and trigger the early implementation of appropriate treatment regimen, particularly in the season of influenza A. Meanwhile, further examinations including virologic tests and chest radiography should be performed in a timely manner, which will play an important role in early identifying and treating severe form of influenza A virus infection.

One of the patients, patient no. 2 (Table 1) was emergently admitted to hospital for an operation (because of the ectopic pregnancy) prior to illness onset. She had normal peripheral lymphocyte counts (1.73×10^9/L) before 3 days the onset of pandemic influenza A/H1N1 infection, although the relative percentage (11.7%) of lymphocyte was decreased (Table 1), which may be attributed to the elevation of total white blood cells (14.8×10^9/L) and increased proportion (83.2%) of neutrophile granulocyte. The white blood cell counts became normal (5.8×10^9/L) after 2 days and the proportion of lymphocytes was normal (28.8%) one day before the onset of pandemic influenza A/H1N1 infection. Suspected hospital acquired infection in this patient had us possible to closely observe the longitudinal change of lymphocyte counts. On the first day of onset of infection, the patients presented with fever and flu-like symptoms and her blood cell counts showed normal total white blood cells (6.8×10^9/L), but significant decline of the proportion (8.7%) of lymphocytes and reduced absolute lymphocyte counts (0.59×10^9/L) (Table 1). The chest X-ray radiography showed the pneumonia in the lower lobe of right lung and diagnosis of pandemic influenza A/H1N1 infection was established on the 2nd day of illness based on the positivity of pandemic influenza A/H1N1 viral RNA. The patient received quick antiviral and comprehensive supportive treatment. As the health condition improved, the lymphocyte counts increased. The dynamic changes of lymphocyte counts in this patient demonstrate that the severe lymphopenia can occur at the very early stage, almost same as the onset of symptoms. The very early occurrence of lymphopenia is also validated by the findings in two other patients (patient nos. 15 and 18), in whom the severe lymphopenia was observed on the second day of the illness (Table 1).

Lymphopenia may occur in either non-infectious or infectious diseases. The non-infectious diseases include autoimmune diseases such as systemic lupus erythematosus [15], cardiovascular diseases [16], intracerebral hemorrhage [17], or during or after chemotherapy or chemoradiation therapy [18–20], or conditions of exhaustion, after excessive X-ray irradiation, with vitamin deficiency, and in the terminal phase of uremia. Since these diseases or conditions have their own clinical manifestations and do not have fever and other toxemic symptoms, the lymphopenia in these diseases or conditions cannot be defined as that in severe influenza A/H1N1 infection. Additionally, lymphopenia may occur in elderly patients, but in the absence of any concerning symptoms, no further investigation is advised in an elderly patient with a lymphocyte count $>0.5 \times 10^9$/L [21].

Lymphopenia can also be observed in other infectious diseases characterized with pneumonia or non-pneumonia. The infections characterized with non-pneumonia mainly include acquired immune deficiency syndrome caused by HIV infection, severe measles [22], West Nile encephalitis [23], Ebola virus infection [24], and disease caused by chikungunya virus [25]. The lymphopenia occurred in above diseases can be easily differentiated from that occurred in influenza A/H1N1 since these infections have

their own clinical features. On the other hand, pulmonary infections caused by other pathogens with lymphopenia may mimic the clinical presentations of H1N1 influenza. These infections include severe acute respiratory syndrome [26,27], Legionnaires disease or human parainfluenza virus type 3, metapneumovirus, and respiratory syncytial virus as well as other types of influenza A virus such as avian influenza A H5N6, H7N9 or H3N2 [28–33]. Severe acute respiratory syndrome has been eradicated even since 2004; diagnosis of severe acute respiratory syndrome is not considered when a patient is characterized by systematic toxemia and pneumonia and lymphopenia. The differentiation of influenza A/H1N1 from other pulmonary infections which mimic clinical presentations and lymphopenia can only be made by the isolation of specific pathogens by culture or molecular techniques or specific serologic assays, all of which are not easily accessible or cannot provide quick results for the differentiation at the early stage of the diseases. However, as the treatment of severe influenza A/H1N1 and pneumonia caused by avian influenza A viruses is almost same, lymphopenia in a patient with influenza like illness, in combination with the pneumonia findings of chest radiograph, can serve as a clue to early define a severe pulmonary infection that requires urgent management before the availability of results of the specific laboratory tests.

There were several limitations in this retrospective analysis. First, this study did not include laboratory-confirmed mild or moderate pandemic influenza A/H1N1 patients without pneumonia as a control group, because such patients were not hospitalized in our hospital. This had us impossible to compare the incidence of lymphopenia between the mild or moderate and severe infection, since lymphopenia can also occur in non-severe influenza A patients [1,8,9]. Second, severe influenza A/H1N1 infection has other laboratory abnormalities such as partial pressure of oxygen and carbon dioxide [9], which were not presented in details in the present study. Third, because lymphopenia can also be observed in other non-infectious and infectious diseases as mentioned above, it is impossible to set up a cutoff value to distinguish lymphopenia in severe influenza A/H1N1 infection from that in other diseases. Thus, lymphopenia cannot be used alone to define severe influenza A/H1N1 infection; it should be used in the combination of typical clinical presentations. Even though, it still has the potential to exaggerate the severity of illness. However, compared with the delayed identification of severe influenza A infection, which may delay the necessary therapies, we consider that the risk associated with delayed diagnosis and treatment exceeds that of over-diagnosis of the severe infection.

It is known that lymphocytes are the main immune cells in the elimination of viruses. In the conventional viral infection, the proportion of lymphocytes in the circulation is usually increased. However, we showed that most patients with severe pandemic influenza A/H1N1 had marked lymphopenia (Table 1). The lymphopenia might be resulted from the redistribution and migration of lymphocytes to respiratory system to combat against the virus. A report showed that infiltrated cells in the lung of a patient died of influenza A/H1N1 were predominantly lymphocytes [34]. This observation supports the hypothesis of the redistribution of lymphocytes. The recovery of lymphocytes in the circulation during the second and third weeks after the illness onset (Table 1) indicates that the lymphocytes re-enter the circulation from inflamed tissues. Additionally, lymphopenia may also be associated with apoptosis induced by viral infection [26].

In conclusion, while Jernigan et al. have proved that the usefulness of lymphopenia as diagnostic marker of pandemic influenza in adults [6], our current study presents the dynamic changes of lymphocyte counts and reveals the presence of lymphopenia at an early stage among cases with severe infection with pandemic influenza A/H1N1. Routine blood tests, including white blood cell counts and differentials, along with the help of other clinical parameters and laboratory findings, may provide clues for early identification of severe form of influenza A/H1N1 virus infection.

Authors' contributions

YC, HZ, JC, and Y-HZ conceived and designed the study. YC, HZ, PS, ZZ, and JC collected the clinical data. YC, HZ, PS, ZZ, JC, and Y-HZ analyzed the data. YC and HZ wrote the manuscript. JC and Y-HZ critically revised the manuscript. All authors have read and approved the manuscript.

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Competing interests

None declared.

Ethical approval

This study was approved by the institutional ethics review committee of Nanjing Drum Tower Hospital and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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