Original Article

DOI: 10.3947/ic.2010.42.6.391
Infect Chemother 2010;42(6):391-396

Infection & Chemotherapy

마우스모델에서 흡연이 인플루엔자 감염증과 백신 반응에 미치는 영향

이진수[1] · 기세윤[2] · 황인숙[3] · 박신구[4] · 김루시아[5] · 정문현[1] · 정희진[3] · 김우주[3]
인하대학교 의과대학 내과학교실[1], 건국대학교 의과대학 내과학교실[2], 고려대학교 의과대학 내과학교실[3], 인하대학교 의과대학 산업의학교실[4], 인하대학교 의과대학 병리학교실[5]

Effect of Smoking on Influenza Illness and Vaccine-induced Immune Response in Mice

Background: Since cigarette smoking is an important risk factor for respiratory infections and alters the immune response, the severity of influenza illness and the immunogenicity of influenza vaccination may differ between cigarette smokers and non-smokers. This study investigated the effect of cigarette smoke exposure on the severity of influenza illness and vaccine-induced antibody production in mice.
Materials and Methods: Cigarette smoke exposed (CE) and non-cigarette smoke exposed (NCE) mice were infected with mouse-adapted influenza A/PR8/8/34 (H1N1). Influenza virus was quantified in bronchoalveolar lavage fluid by real-time polymerase chain reaction and the lung pathology was examined to investigate the influence of smoke exposure on the severity of illness. To assess immunogenicity, hemagglutination inhibition antibodies were measured in pre- and post-influenza vaccination blood samples from CE and NCE mice.
Results: Influenza viral proliferation was higher and inflammatory changes such as macrophage infiltration in the alveolar space and necrotizing bronchitis were more pronounced in CE mice, compared with controls. Vaccine-induced immunogenicity was achieved in both CE and NCE mice.
Conclusions: Cigarette smoke exposure enhanced influenza viral replication and the inflammatory changes associated with influenza illness, but had no significant effect on vaccine-induced immunogenicity.

Key Words: Immunogenicity, Inflammation, Influenza, Vaccine, Smoking

Jin-Soo Lee[1], Sae-Yun Ki[2], In-Sook Hwang[3], Shin-Goo Park[4], Lucia Kim[5], Moon-Hyun Chung[1], Hee-Jin Cheong[3], and Woo-Joo Kim[3]

Departments of [1]Internal Medicine, [4]Occupational and Environmental Medicine, and [5]Pathology, Inha University College of Medicine, Incheon; [2]Department of Internal Medicine, Konkuk University College of Medicine, Chungju; [3]Department of Internal Medicine, Korea University College of Medicine, Seoul, Korea

Submitted: July 26, 2010
Revised: October 20, 2010
Accepted: October 20, 2010
Correspondence to Hee-Jin Cheong M.D.
Department of Internal Medicine, Korea University College of Medicine, Guro Hospital, 97, Gurodong-gil, Guro-gu, Seoul 152-703, Korea
Tel: +82-2-2626-1114, Fax: +82-2-866-1643
E-mail: heejinmd@medimail.co.kr

www.icjournal.org

Introducion

The influenza virus causes seasonal epidemics and pandemics through continuous antigenic changes [1]. In particular, influenza causes serious morbidity and mortality in the elderly and those with chronic medical illnesses [2]. Cigarette smoking is a well-known risk factor for cancer, cardiovascular disease, and chronic obstructive pulmonary disease, and has been linked to a range of respiratory tract and other systemic infections [3]. Cigarette smoking

also affects the immune response by altering structural and immunologic defenses [4].

For these reasons, both the severity of influenza illness and the immunogenicity of influenza vaccination in cigarette smokers might be different from non-smokers. However, studies evaluating the effect of cigarette smoking on influenza have yielded inconsistent results [5-7]. Given that cigarette smoking exacerbates influenza-related morbidity [3] influenza vaccination for smokers might be an efficient preventive strategy. This study investigated the effect of cigarette smoke exposure on the severity of influenza illness and vaccine-induced antibody production in mice.

Materials and Methods

1. Animals

Female BALB/c mice aged 4-6 weeks were obtained from Orient Bio NHP Inc. (Sungnam, Korea). The experiments described in this paper were conducted in accordance with Inha University guidelines on animal use and care. Mice were housed at 23℃ (±3℃) with humidity of 50% (±10%), a 12-hour light-dark cycle, a noise level of 40-50 phon, and unlimited access to food and water.

2. Overview of treatment

Twenty mice were assigned to cigarette smoke exposed (CE, n=10) group or a non-cigarette smoke exposed (NCE; n=10) control group (Fig. 1). Within each group, mice were allocated to influenza virus challenge on day 21, followed by bronchoalveolar lavage (BAL) and histopathology (five CE and five NCE mice) on day 24, or to influenza vaccination on days 14 and 28, followed by hemagglutination inhibition (HI) testing on day 42 (five CE and five NCE mice). Details of each treatment are given below.

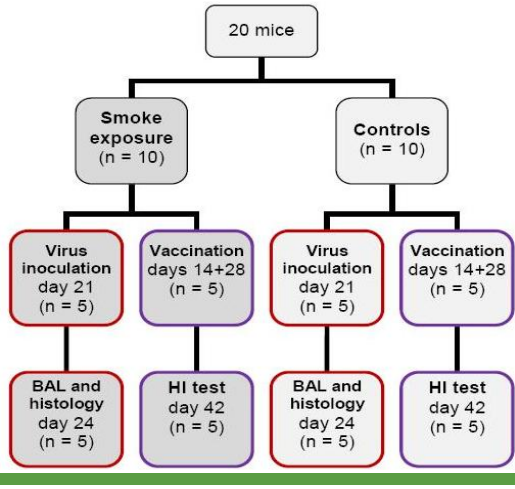

Figure 1. Treatment allocation.

3. Smoke exposure protocol

In a plexiglas chamber designed for cigarette smoke exposure, ten mice were daily exposed to the fume of smoke coming out of five cigarettes; five mice inoculated with influenza virus were exposed to the smoke for 21 days and the remaining five mice vaccinated against influenza were exposed for 42 days. Mice were placed in the cabinet for 25-30 minutes per day, which was the duration of smoke exposure, and then returned to their usual cage.

4. Influenza virus inoculation

After 21 days of cigarette smoke exposure, five of ten mice in the CE group, together with five NCE controls, were anesthetized with isoflurane and inoculated intranasally with 10^8-10^9 pfu/mL of the mouse-adapted influenza virus strain was kindly presented by Chang-Seun Song, Avian Disease Laboratory, College of Veterinary Medicine, Konkuk University.

5. Lung histology and bronchoalveolar lavage

Three days after influenza virus inoculation (day 24), mice were sacrificed and processed for histopathology and BAL as described previously [8]. Left lung lobes were fixed with 10% neutral buffered formalin and stained with hematoxylin and eosin (H&E) for histopathologic examination using an Olympus BX51 research microscope (Olympus, Tokyo, Japan) and ProgRes C14 camera (Jenoptik, Jena, Germany). Two left lung lobes from each group were used for microarray analysis (data not shown), thus six lungs were available for histopathologic evaluation. Right lung lobes were lavaged and after centrifuging, the BAL supernatant was used for measuring the influenza virus titer.

6. Influenza virus titration

Influenza virus titers in BAL fluid were assayed using real-time polymerase chain reaction (PCR) [9]. mRNA and cDNA were isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and the Omniscript RT Kit (Qiagen, Hilden, Germany), respectively. The primers for PCR amplification were designed for the influenza virus M protein: forward, CAT GGA ATG GAT AAA GAC AGA CC; reverse, CCA TT TAG GGC ATT TTG GAC A.

7. Influenza vaccination

After 2 weeks of cigarette smoke exposure (day 14), five mice

each from the CE and NCE groups were vaccinated with 100 μL of purified split vaccine (A/Fujian/411/2002 (H3N2)-like, A/New Caledonia/20/99 (H1N1)-like, B/Shanghai/361/2002-like) intramuscularly into the thigh muscle. A second dose of vaccine was administered on day 28. Smoke exposure was maintained for 2 more weeks in the CE group.

8. Antibody response

Serum was collected prior to the vaccination and 2 weeks after the second vaccine dose (day 42), and stored at -20°C. HI testing was performed using a standard microtiter assay as described previously [10]. The geometric mean titer (GMT) was determined in pre- and post-vaccination samples. The seroconversion factor was defined as the ratio of the post-vaccination GMT to the pre-vaccination GMT.

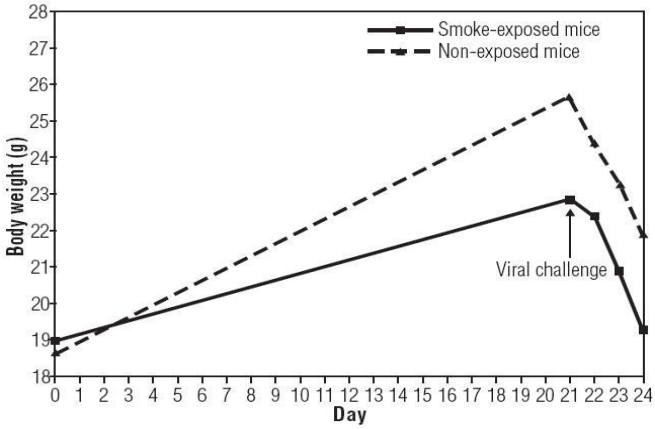

Figure 2. Mean body weight before and after influenza virus challenge in cigarette smoke exposed and non-cigarette smoke exposed mice.

Table 1. Pathologic findings in Lung Tissues after Influenza Virus Infection in Cigarette Smoke-exposed and Non-cigarette Smoke Exposed Mice

	NCE mice			CE mice		
	# 1	# 2	# 3	# 1	# 2	# 3
Inflammatory changes ≥ 1/3 of total lung area	–	–	–	+	+	+
Pigment-laden macrophages in alveolar spaces	–	–	–	+	+	+
Necrotizing bronchitis	+	+	+	+	+	+
Disordered bronchial epithelial proliferation	–	–	–	+	+	+
Mild cytologic atypia[a]	+	–	–	+	+	+

[a]Mild cytologic atypia was defined as slight nuclear enlargement with hyperchromasia, vesicular chromatin pattern and prominent nucleoli.
NCE, non-cigarette smoke exposed; CE, cigarette smoke exposed; +, present; –, absent.

9. Statistical analysis

Statistical analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, USA). The Mann-Whitney U test was used for between-group comparisons (CE vs. NCE) of viral titers in BAL fluid, HI titers following vaccination, and body weight change after influenza viral challenge. A *P*-value less than 0.05 was considered statistically significant.

Results

1. Body weight change

The mean body weight at baseline was similar in mice allocated to CE group and NCE group (18.7-19.0 g), but CE mice showed a less weight increase over the following 3 weeks: mean increase of 20.2% compared with 37.0% in NCE controls (Fig. 2). Prior to influenza virus inoculation on day 21, CE mice had a lower mean body weight than NCE mice (22.9 g vs. 25.7 g), probably reflecting an inhibitory effect of cigarette smoke exposure on growth (Fig. 2). Following successful influenza virus inoculation, a similar reduction in body weight was observed in both CE and NCE mice. The mean body weight loss at 3 days after influenza virus infection (day 24) was 3.52±0.19 g (15.4%) and 3.75±0.44 g (14.6%) in CE and NCE mice, respectively (*P*=0.754).

2. Influenza virus titer in BAL fluid and lung pathology

The mean influenza virus titer in BAL fluid was significantly

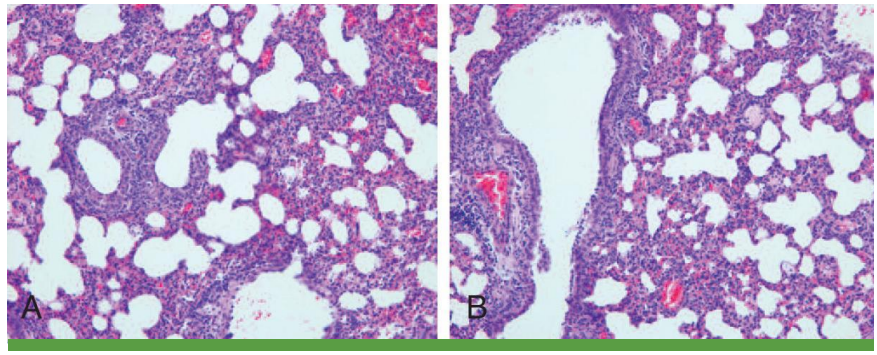

Figure 3. A representative lung histology following influenza virus infection after cigarette smoke exposure. (A) Necrotizing bronchitis has occurred after cigarette smoke exposure (H&E, ×200). (B) Neutrophils and lymphocytes are seen in the peribronchial area and alveolar spaces (H&E, ×200).

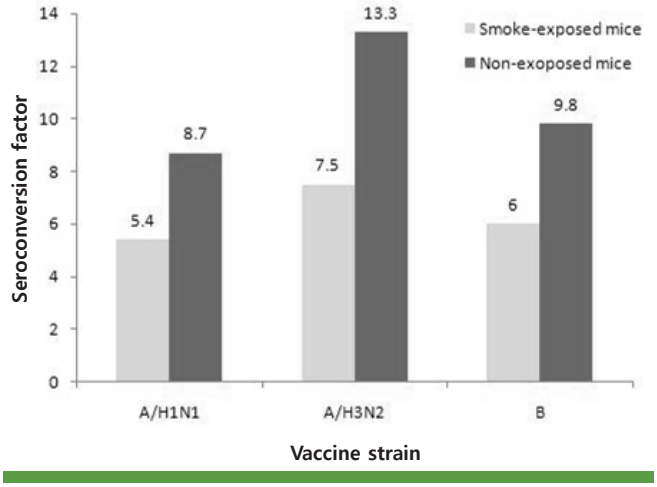

Figure 4. Seroconversion factors following influenza vaccination in cigarette smoke exposed and non-cigarette smoke exposed mice.

Table 2. Pre- and Post-influenza Vaccine Mean Antibody Titers in Cigarette Smoke Exposed and Non-cigarette Smoke Exposed Mice

Vaccine strain	NCE mice		CE mice	
	Pre-vaccination	Post-vaccination	Pre-vaccination	Post-vaccination
A/H1N1	10	88.8±1.6	10	60.6±1.5
A/H3N2	10	183.8±1.5	10	121.3±2.5
B	10	91.9±1.8	10	60.6±1.5

Values are GMT±SD.
GMT, geometric mean titer; NCE, non-cigarette smoke exposed; CE, cigarette smoke exposed.

higher in CE mice than in NCE mice (6.7×10^5 vs. 3.1×10^7 cfu/mL) (P=0.009). Influenza-infected lungs from CE mice showed more pronounced alveolar inflammation, pigment-laden macrophage infiltration in alveolar spaces, atypical cytology, and disordered epithelial proliferation than NCE mice (Fig. 3 and Table 1), but there were no emphysematous structural changes of the airways in either group.

3. Antibody response to influenza vaccination

Mean HI antibody titers were >1:60 regardless of cigarette smoke exposure (Table 2). The mean antibody titers were lower in CE mice than in NCE mice, but these differences were not statistically significant (A/H1N1, P=0.445; A/H3N2, P=0.121; B, P=0.212). The seroconversion factor for all three vaccine strains exceeded 5.0 in both CE and NCE mice, although degree of elevation of seroconversion factors were not significant in the NCE group compared with that of the CE group (A/H1N1, P=0.445; A/H3N2, P=0.121; B, P=0.212) (Fig. 4).

Discussion

Cigarette smoking appears to increase both the risk of infection and the severity of infectious diseases. However, the effect of smoking on infectious diseases has received little attention from physicians, and the precise mechanisms underlying these effects are not fully understood [2]. The objective of this study was to investigate the influence of cigarette smoking on the severity of influenza illness and the immune response to influenza vaccination in mice.

Several studies have suggested that smokers are vulnerable to influenza. Finklea et al. reported that cough, sputum, dyspnea, and other respiratory symptoms caused by influenza were more frequent and more severe in cigarette smokers than in non-smokers [5]. Among young men in the Israeli military, influenza was also more common and more severe in smokers; 50.6% of smokers lost work days, as compared with 30.1% of non-smokers [11]. Similarly, in female military recruits, the risk of influenza illness was greater in smokers than in non-smokers (60.0% vs. 41.6%) [12]. Thus, cigarette smoking itself should be considered a risk factor for influenza illness.

The effects of cigarette smoking are due to a combination of alterations in the structure of the respiratory tract and immunologic mechanisms [3]. These structural changes, which include peribronchial inflammation and fibrosis, impaired mucociliary clearance, and disruption of the respiratory epithelium are thought to increase vulnerability to respiratory tract infections and exacerbate inflammation [13]. Leuchtenberger et al. found that influenza-induced inflammatory changes of the respiratory tract, such as necrotizing bronchitis and atypical proliferation, were worsened in mice exposed to cigarette smoke [14]. In our study, CE mice showed more severe inflammatory changes of the lung and greater influenza viral proliferation following influenza infection, compared with NCE mice. No emphysematous morphologic changes were observed in either group. However, these mice were exposed to the smoke from 5 cigarettes daily for a total of 3 weeks, which is equivalent to subacute exposure and may have been insufficient to initiate emphysematous structural changes. Nonetheless, the greater severity of histologic findings in smoke-exposed mice suggests that smoking may induce an aggravated inflammatory response prior to the development of morphologic changes of the respiratory tract. The molecular and cellular changes that occur before the emergence of clinically apparent symptoms remain to be elucidated [15].

Despite the association between cigarette smoking and inflammatory changes of the respiratory tract, smokers have a

lower incidence of certain diseases with an inflammatory component, including ulcerative colitis, sarcoidosis, and Parkinson's disease [16]. This may be due to the anti-inflammatory effects of nicotine, a major component of cigarette smoke. In a study evaluating the effects of nicotine on the inflammatory response to influenza infection in mice, Razani-Boroujerdi et al. found that nicotine suppressed the migration of leukocytes to the region of inflammation, but increased the influenza viral burden in the lung [17]. However, cigarette smoke contains thousands of constituents in addition to nicotine, including strong inflammatory inducers such as tar. Our study found that both inflammatory changes and the influenza virus titer in the lung were increased in mice exposed to cigarette smoke. Similarly, Gualano et al. hypothesized that short-term smoke exposure would protect against subsequent influenza infection via activation of pro-inflammatory mediators, but instead found that even short-term exposure (4 days) exacerbated the host response to influenza [18]. The effect of smoke exposure on the immune response to influenza may also depend on the infectious dose. Robbins et al. demonstrated that smoke exposure attenuated the airway inflammatory response to low-dose influenza virus inoculation, but increased lung inflammation after infection with high-dose influenza [19]. Further experiments using varying durations of cigarette smoke exposure and influenza inoculum doses are needed to clarify the relationship between smoke exposure and inflammation.

Annual influenza vaccination is generally recommended for individuals with chronic respiratory diseases or structural lung diseases that result from chronic cigarette smoke exposure (e.g. chronic bronchitis and emphysema), but not for cigarette smokers per se. Cigarette smoking could influence the immune response to influenza vaccination in various ways. Smoking affects both the cellular and humoral immune response, resulting in decreased production of immunoglobulins, an impaired antibody response to antigens, a decrease in CD4+ lymphocyte cells, depressed natural killer cell activity, and other alterations of the immune system [20-24]. However, an antibody response to influenza vaccination was reported to be induced in both smokers and non-smokers, although smokers showed a greater subsequent decline in the antibody titers [6]. Cruijff et al. likewise reported that smoking had no effect on the efficacy of vaccination for protection against clinical influenza illness in the elderly [7]. There are no established criteria for assessing the immunogenicity of influenza vaccination in mice. The Committee for Proprietary Medicinal Products (CPMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) criteria for assessing vaccines include an HI antibody titer of $\geq$1:40 and a seroconversion factor exceeding 2.5 [25]. In our study, immune responses above 5.0 were achieved following influenza vaccination regardless of smoke exposure, although the observed trend towards lower antibody titers in smoke-exposed mice suggests that exposure to cigarette smoke may have reduced the immunogenicity of the vaccine.

In this study, CE mice infected with the influenza virus showed signs of aggravated influenza illness (as demonstrated by increased body weight loss, inflammatory histologic changes, and virus proliferation compared with NCE mice), but smoke exposure had no significant effect on the immune response to influenza vaccination. Further epidemiologic and immunologic studies in human populations are needed to evaluate the effectiveness of influenza vaccination for preventing influenza-associated morbidity in smokers.

References

1. Nicholson KG, Wood JM, Zambon M. Influenza. Lancet 2003; 362:1733-45.
2. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA 2003;289: 179-86.
3. Arcavi L, Benowitz NL. Cigarette smoking and infection. Arch Intern Med 2004;164: 2206-16.
4. Richardson MA. Upper airway complications of cigarette smoking. J Allergy Clin Immunol 1988;81:1032-5.
5. Finklea JF, Sandifer SH, Smith DD. Cigarette smoking and epidemic influenza. Am J Epidemiol 1969;90:390-9.
6. Finklea JF, Hasselblad V, Riggan WB, Nelson WC, Hammer DI, Newill VA. Cigarette smoking and hemagglutination inhibition response to influenza after natural disease and immunization. Am Rev Respir Dis 1971;104:368-76.
7. Cruijff M, Thijs C, Govaert T, Aretz K, Dinant GJ, Knottnerus A. The effect of smoking on influenza, influenza vaccination efficacy and on the antibody response to influenza vaccination. Vaccine 1999;17:426-32.
8. Langley RJ, Kalra R, Mishra NC, Hahn FF, Razani-Boroujerdi S, Singh SP, Benson JM, Peña-Philippides JC, Barr EB, Sopori ML. A biphasic response to silica: I. Immunostimulation is restricted to the early stage of silicosis in Lewis rats. Am J Respir Cell Mol Biol 2004;30:823-9.
9. Zhang WD, Evans DH. Detection and identification of human influenza viruses by the polymerase chain reaction. J Virol Methods 1991;33:165-89.
10. Admon D, Engelhard D, Strauss N, Goldman N, Zakay-Rones Z.

Antibody response to influenza immunization in patients after heart transplantation. Vaccine 1997;15:1518-22.
11. Kark JD, Lebiush M, Rannon L. Cigarette smoking as a risk factor for epidemic a(h1n1) influenza in young men. N Engl J Med 1982;307:1042-6.
12. Kark JD, Lebiush M. Smoking and epidemic influenza-like illness in female military recruits: a brief survey. Am J Public Health 1981;71:530-2.
13. Dye JA, Adler KB. Effects of cigarette smoke on epithelial cells of the respiratory tract. Thorax 1994;49:825-34.
14. Leuchtenberger C, Leuchtenberger R, Ruch F, Tanaka K, Tanaka T. Cytological and cytochemical alterations in the respiratory tract of mice after exposure to cigarette smoke, influenza virus, and both. Cancer Res 1963;23:555-65.
15. Gebel S, Gerstmayer B, Bosio A, Haussmann HJ, Van Miert E, Müller T. Gene expression profiling in respiratory tissues from rats exposed to mainstream cigarette smoke. Carcinogenesis 2004;25:169-78.
16. Sopori M. Effects of cigarette smoke on the immune system. Nat Rev Immunol 2002;2:372-7.
17. Razani-Boroujerdi S, Singh SP, Knall C, Hahn FF, Peña-Philippides JC, Kalra R, Langley RJ, Sopori ML. Chronic nicotine inhibits inflammation and promotes influenza infection. Cell Immunol 2004;230:1-9.
18. Gualano RC, Hansen MJ, Vlahos R, Jones JE, Park-Jones RA, Deliyannis G, Turner SJ, Duca KA, Anderson GP. Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. Respir Res 2008;9:53.
19. Robbins CS, Bauer CM, Vujicic N, Gaschler GJ, Lichty BD, Brown EG, Stämpfli MR. Cigarette smoke impacts immune inflammatory responses to influenza in mice. Am J Respir Crit Care Med 2006;174:1342-51.
20. Andersen P, Pedersen OF, Bach B, Bonde GJ. Serum antibodies and immunoglobulins in smokers and nonsmokers. Clin Exp Immunol 1982;47:467-73.
21. Hughes DA, Haslam PL, Townsend PJ, Turner-Warwick M. Numerical and functional alterations in circulatory lymphocytes in cigarette smokers. Clin Exp Immunol 1985;61:459-66.
22. Fisher GL, McNeill KL, Finch GL, Wilson FD, Golde DW. Functional evaluation of lung macrophages from cigarette smokers and nonsmokers. J Reticuloendothel Soc 1982;32:311-21.
23. Tollerud DJ, Clark JW, Brown LM, Neuland CY, Mann DL, Pankiw-Trost LK, Blattner WA, Hoover RN. Association of cigarette smoking with decreased numbers of circulating natural killer cells. Am Rev Respir Dis 1989;139:194-8.
24. McCrea KA, Ensor JE, Nall K, Bleecker ER, Hasday JD. Altered cytokine regulation in the lungs of cigarette smokers. Am J Respir Crit Care Med 1994;150:696-703.
25. Committee for Proprietary Medicinal Products (CPMP). Note for Guidance on Harmonisation of Requirements for Influenza Vaccines. CPMP/BWP/214/96. Available at: http://www.emea.europa.eu/pdfs/human/bwp/021496en.pdf. Accessed 25 July 2010.