RESEARCH ARTICLE

Production of TNF-alpha ex vivo is predictive of an immune response to flu vaccination in a frail elderly population*

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ABSTRACT. ***Objective:*** **To investigate the relationship between the response to influenza vaccination and the ability to produce proinflamatory cytokines in elderly subjects.** ***Methods:*** **Whole blood samples from 25 elderly subjects collected before influenza vaccination were stimulated with the influenza vaccine in order to evaluate the secretion of five specific cytokines: TNFα, IFNα, IFNγ, IL2 and IL10. The results were correlated with the increased HAI antibody titres two weeks after vaccination.** ***Results:*** **Only 30% of elderly individuals seroconverted after vaccination. Although 50 to 70% of the cohort did not produce TNFα, IFNα, IFNγ, IL2 or IL10, all of the individuals who seroconverted were able to produce TNFα. Furthermore production of IFNγ, with or without production of IFNα/β, was not associated with a better response to the vaccine.** ***Conclusion:*** **Production of TNFα appears to be primordial for an efficient vaccine response, and may provide a predictive marker for the humoral response to vaccination. It may also provide the basis for evaluating agents designed to rescue TNFα-producing cells. This study emphasises a need to rescue TNF-producing cell function.**

Key words: vaccination, cytokines, elderly subjects, interferon, TNFα

Influenza vaccination is not protective for all recipients in particular, in the elderly of whom only 17 to 53 % develop an antibody response that attains a protective level [1, 2]. Indeed, the development of an immune response involves the intervention of numerous cellular components and secreted cytokines, which become deficient in the elderly [2, 3]. Although several studies have been devoted to investigation of the synthesis of different cytokines throughout the immune response, little is known about predictive markers of the response to influenza vaccination [4-6]. We report herein the results of a study designed to determine the relationship between the capacity to produce specific cytokines in response to influenza antigens *ex vivo*, prior to influenza vaccination, and the efficiency of the response to vaccination in elderly individuals. The results of this study show clearly that ability to produce TNFα is essential in order to obtain an efficient serological response to influenza vaccination, and that other cytokines are not indispensable.

METHODS AND MATERIALS

Study design

This investigation was an ancillary of a study conducted in 2005 aiming to evaluate the effect of interferon alpha2 administration *per os* on the influenza vaccine response in elderly, institutionalised individuals [1].
The *in vitro* production of cytokines by whole blood incubated in the presence of the vaccine preparation was determined and the results were correlated with the increase in haemagglutination inhibitory (HAI) antibody titres.

Study population

Immune tests were performed on whole blood samples from 25 of 48 individuals aged 75 or more, who had been

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included in the study completed in 2005, and who were recruited in the long-term units of our University Hospital in Paris, France. The population studied had not previously been exposed to the New York strain of the influenza virus. Individuals with neoplasia, autoimmune diseases or type 1 diabetes, or concomitant treatment with glucocorticoid or immunosuppressive drugs, or who had undergone a splenectomy or tonsillectomy were excluded from the study. At least one prior influenza vaccination in the previous five years was the principal inclusion criterion. Subjects were vaccinated with a single intramuscular injection of influenza vaccine (Solvay Pharma France) composed of the New Caledonia 20/99 strain (H1N1) of influenza A virus, the New York strain (H3N2) influenza A virus, and the Jangsu strain of influenza B virus.

The study protocol was approved by the Cochin Hospital institutional ethics board, and all subjects included in the study gave written voluntary informed consent.

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Methods

Venous blood samples were collected on the day of vaccination, 14 and 180 days thereafter. One ml of whole blood collected in citrate tubes was incubated for 18h with a mixture of each vaccinal strain, diluted to 1/100 from the influenza vaccine preparations. Plasmas (supernatants) were separated by gentle centrifugation and stored at -80°C. Production of cytokines TNFα, interferon gamma (IFNγ), IL-2 and IL-10 were measured using the CBA techniques BD^{TM} Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II (San Diego); levels of IFNγ, TNFα<200 pg/mL, were considered as negative, as were IL-2 and IL-10 levels of <20 pg/ml. Type 1 interferon (IFNα and IFNβ) was measured in supernatants at D0, D14, D180 using a biological assay as previously described [7, 8]. IFNα/β levels of <2 IU/mL were considered to be negative.

The HAI antibody titre was measured using a standardized micro-method with four antigen Ha units of each of the three antigens as previously described [1, 9]. The seroconversion rate was defined as more than a two-fold increase in HAI antibody titre between baseline at days 0 and 14.

Statistical analysis

Descriptive statistics were expressed as mean and standard deviation (sd) or median (interquartile range) for continuous variables, or as frequency counts and percentages for qualitative variables. Characteristics of the study population were compared using Fisher's test for distributions, and the Chi-square test for means. For correlations between quantitative variables, the Spearman correlation test was used. Significance was assumed at $p<0.05$. The StatView software suite version 5.0.1 (SAS Inc., Cary, NC, USA) was used for all analyses.

RESULTS

Population at inclusion

Between November 2 and December 1, 2005, 48 individuals were randomized to receive either placebo (n = 22), or IFNα (n = 26). From this group, 25 patients had venous blood samples collected at J0, J14 and J180 days, allowing them to participate to our ancillary study. In this population, patients' ages ranged from 78 to 97 years (87.3 ± 5.3), 86% were women and their mean weight was 64.4 ± 15.2 kg. Most of them were disabled, were highly dependent, and a high prevalence of dementia, comorbidity and incontinence. Nine were assigned to the control group and 16 to the study group. These 25 subjects were not significantly different from the entire population included in the primary study [1], and were representative of institutionalised, long-term care patients.

Table 1
Number of non-producers of cytokine subjects (np) before (Day 0) and after (Day 180) the injection of the flu vaccine, and at Day 0 and Day 180.

Type of cytokine	Day 0 np (%) n = 25	Day180 np (%) n = 21	Day 0 and Day 180 np (%) n = 21
TNFα	15 (60)	15 (71)	11 (52)
IFNα/β	11 (44)	5 (24)	3 (14)
IFNγ	16 (64)	19 (90)	9 (43)
IL10	19 (76)	15 (71)	11 (52)
IL2	12 (48)	10 (47)	5 (24)

Analysis of the capacity for cytokines production

In the elderly population studied, numerous individuals did not produce detectable levels of the cytokines studied following *ex vivo* stimulation on Day 0 prior to vaccination: 60% were deficient for TNFα, 44% for IFN alpha/beta, 64 % for IFNγ, 76% for IL-10, and 48% for IL-2. Similar deficiencies in the cytokine response were also observed six months later at D180 in 52% od individuals, for TNFα and IL-10, 43% for IFNγ, 24% IL-2, and 14% for IFNα (*table 1*) were also observed six months later at D180 in 52% of individuals.

Two subjects were deficient for the production of four cytokines (TNFα, IL-2, IFNγ, IL-10) and three did not produce three of the cytokines (TNFα, IFNγ, IL-2) or (IL-10, IFN α/β, IL-10). None of the subjects developed any chronic infectious diseases during this time. In contrast, two individuals were able to produce three of the cytokines, one of them produced (IFNγ, IL-2, IL-10) and the other (IFNα/β, IL-10, TNF α), at both Day 0 and Day 180.

Analysis of the population developing a serological response against the NY strain

We saw that 8/25 (32%) subjects seroconverted (increase > 2 of HAI titre antibodies against the NY strain), and 17 had no significant increase (2 or <2) between Day 0 and Day 14. The two groups did not differ in either age or weight (*table 2*).

Analysis of the relationship between cytokine production and HAI antibodies titres

Of the five cytokines produced *ex vivo*, prior to vaccination, only production of TNFα was detected in all the subjects exhibiting an increase in antibody titre of >2 ($p<0.0001$); production of IL-10 was seen in 5/8 individuals ($p<0.01$) (*table 3*).

Table 2
Percentages of the population with and without seroconversion.

	Antibody variation titre >2-fold	**Antibody variation titre 2- or <2-fold**	***p***
n	8	17	
Age (mean ± SD)	86.0 ± 4.84	87.1 ± 3,9	ns
Weight (mean ± SD)	58.6 ± 21.3	67.2 ± 11,7	ns

Table 3
Number of cytokine producers and relation to serological response

	Influenza antibody titre >2-fold	**Influenza antibody titre 2 or ≤ 2-fold**	***p***
n	**N = 8**	**N = 17**	
Titre >0 TNFα n (%)			
D0	8 (100)	2 (11.8)	p<0.0001
D14	6 (75.0)	6 (35.3)	ns
Mean ± SD	1882.1 ± 2036.2	137.3 ± 408.9	p<0.0001
Titre>0 INFγ n (%)			
D0	2 (25.0)	7 (41.1)	ns
D14	6 (75.0)	6 (35.3)	ns
Titre>0 INFα n (%)			
D0	2(25.0)	12 (70.6)	p<0.05
D14	3 (37.5)	7 (43.7)	ns
Titre>0 IL2 n (%)			
D0	6 (75.0)	8 (47.0)	ns
D14	7 (87.5)	9 (52.9)	ns
Titre>0 IL10 n (%)			
J0	5 (62.5)	1 (5.9)	p<0.01
D14	4 (50.0)	4 (23.5)	ns

Production of detectable levels of IFNγ, IL-2, IFNα/β at Day 0 did not appear to be linked to the humoral response to vaccination. Furthermore, the absence of production of IFNα/β or IFNγ did not affect the synthesis of antibodies against influenza.

No significant relationship was observed between the production of the other cytokines studied and the response to influenza A antigens (*table 3* and *figure 1*).

DISCUSSION

Relatively little is known about the relationship between the innate immune response, in particular cytokine production, as a predictive indicator of the efficacy of the immune response following vaccination. We have investigated the *ex vivo* capacity of whole blood to secrete five critical cytokines after stimulation with antigens of the influenza vaccine prior to vaccination in an institutionalised population aged 78 years and older. The use of whole blood contrasts with the use of isolated PBMCs since antibodies from prior influenza epidemics cross-react with the new viral components and lead to viral immune complexes that can induce cytokines [10]. The absence of detectable levels of several cytokines was observed in several individuals. IFNα/β production was undetectable in 44 % of the cohort at Day 0, and in 24% of individuals six months later, and undetectable in14 % of individuals at both time points. A partial deficiency in IFNα production in response to live or inactivated influenza virus has been reported previously in a geriatric population of subjects over 65 years [11]. The inability to produce detectable levels of IFNαβ in response to influenza antigens did not appear to have an impact however, on the development of specific antibodies to influenza virus following vaccination. Indeed, six individuals (*table 3*) who did not produce detectable levels of IFNα/β *in vitro* in the presence of the vaccine preparation prior to vaccination developed a significant serological response to the vaccination, while in other individuals the ability to produce IFNα/β was insufficient to confer

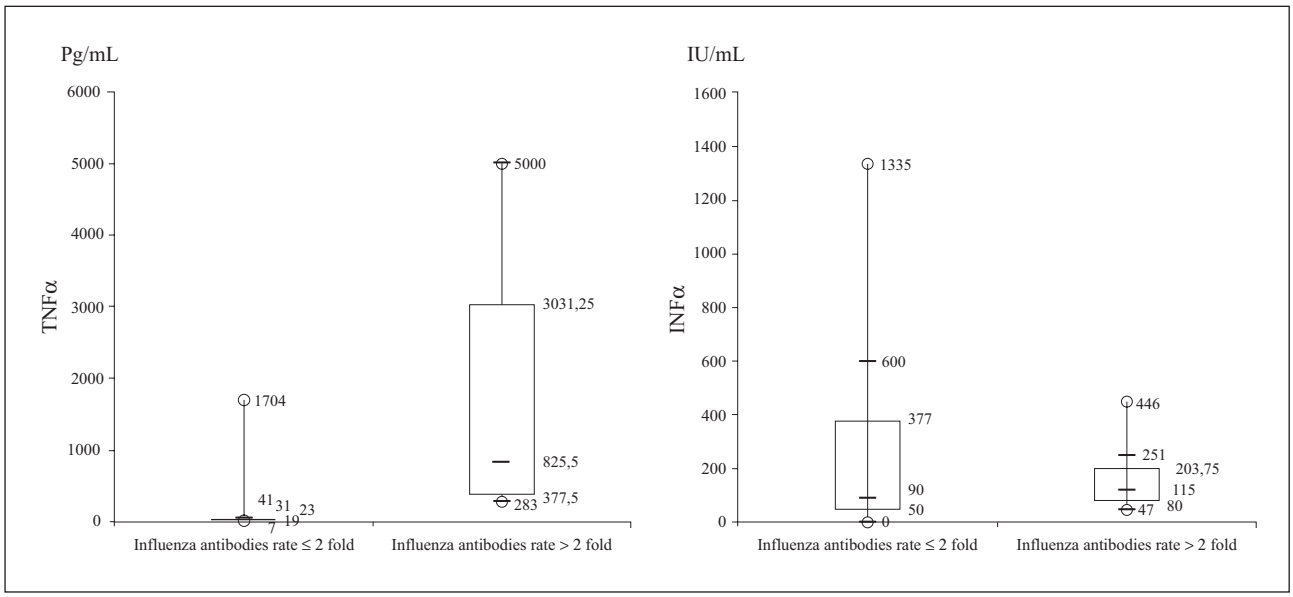

Figure 1
Whisker-plots of TNFα and IFN α on Day 0 in the groups with an influenza antibody rate >2-fold and ≤ 2-fold.

seroconversion. The production of other cytokines such as IL-2 and IFNγwas also deficient in this population. Although 64% and 48% of patients did not produce IFNγ and IL-2 respectively, this did not impair their immune response to vaccination. Furthermore, production of IFNγ (with or without IFNα/β) was insufficient to achieve seroconversion.

TNFα production *in vitro* in response to influenza antigens was also impaired in the elderly population under study. Thus, 60% of the subjects did not produce detectable levels of TNFα at day 0, and 52% did not produce TNFα at either Day 0 and Day 180. In contrast, all of the subjects who responded to vaccination produced detectable levels of TNFα *in vitro* ($p<0001$). The large proportion of individuals in the elderly population under study (70%) who did not develop a serological response following influenza vaccination may be related to a blockade of a specific function or deficiency in the cell population (mononuclear cells, including monocytes and macrophages) responsible for the production of TNFα, which may lead to a loss of the capture and/or presentation of antigens. Production of TNFα is also a determining factor in the dendritic cell (DC)-mediated CD8+ T cell response against the influenza virus [12]. Moreover, some reports have shown that anti-TNFα therapy can decrease the response to the influenza vaccine [13-17]. Decreased production of TNFα after exposure to LPS in an elderly population has also been reported and may reflect an impaired host defence [18].

Production of IL-10 in response to influenza antigens is also associated with a positive serological response, but to a lesser degree than the production of TNFα.

Determination of the capacity of an individual to secrete TNFα in response to exposure to influenza antigens may predict the success of subsequent vaccination, although the cost of the test needs to be low relative to the cost of vaccination. The results of the present study show that the capacity to produce TNFα is absent or low in a majority of individuals in an elderly population. Stimulation or a rescue of TNFα-producing cells may provide a means of increasing the rate of success of vaccination in the elderly [19-21]. The addition of TNFα [19] or adjuvant [20] administered some days prior the administration of the vaccine may improve the percentage of elderly individuals who develop a protective antibody response against a new virus variant. In future studies it would also be of interest to evaluate the effect of administration of TNFα-producing cells derived from stem cell cultures before or together with injection of the vaccine.

We acknowledge that conclusions of our study are limited because of the sample size, and we cannot be sure that our statistically significant differences are not merely due to multiple testing. Including patients in this study had been difficult, even though our criteria were not very selective because patients were frail and severely demented. A comparison and extrapolation of these results seems, however, difficult, but this study gives weight to the setting up of a larger study to confirm these observations. It would be also of interest to compare our results with those obtained from younger patients.

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