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Humoral Response to Influenza Hemagglutinin: Oligoclonal Spectrotype and Failure of Thymopentin as Immunoadjuvant

Abstract

Influenza remains a serious cause of illness and death among certain populations. Influenza vaccines in use at present are of limited effectiveness due to the high variability of the virus, and trials all over the world are in progress to enhance their immunogenicity. Conflicting results, in fact, have been reported about the immune response to influenza vaccination in diverse populations. In this paper we analyzed the antibody response to the hemagglutinin (HA) of the H3N2 A/Shanghai 16/89 strain, which was included into the trivalent 1991-92 influenza vaccine, in four groups of subjects: 8 healthy young, 13 human immunodeficiency virus (HIV)-infected and 37 elderly healthy people, 9 of whom were treated with thymopentin (TP-5).

Our results show levels of anti-HA IgG before vaccination in HIV-infected and elderly people significantly lower than those of normal young subjects. After vaccination, HIV-infected and elderly healthy people showed a significant increase of specific antibodies, whereas a failure in the specific response in normal young subjects was observed, thus differences among the groups were no longer present. Moreover, the spectrotypic analysis of antibody response, by isoelectric focusing and reverse blotting, showed oligoclonal but polymorphic pattern in the majority of subjects, irrespective of the group, and more frequently lack of expansion of the spectrotype after vaccination, thus demonstrating the lack of the recruitment of antigen-specific B cells. Finally, the treatment with TP-5 did not influence the outcome of the vaccination in the group of elderly people. These results further emphasize the limited immunogenicity of influenza vaccination and the inefficacy of TP-5 as immunoadjuvant, in this model of vaccination.

Introduction

Despite large immunization programs, viral influenza remains a serious source of morbidity and mortality throughout the world

and a significant cause of illness and death among subjects with T-cell dysfunction, such as elderly people [1]. The crucial role of T-cell immunity in the anti-influenza protection is linked to the thymus dependence of the two

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main antigens able to induce neutralizing antibodies, hemagglutinin (HA) and neuraminidase (NA). Among the three types of human influenza viruses, namely A, B and C, A is the most variable and includes three HA subtypes (H1, H2, H3) and two NA subtypes (N1, N2) that originate from antigenic shift [2]. Point mutations of epitopes within a subtype (Antigenic drift) are also observed, as a consequence of selective antibody pressure [2]. The immunity against one subtype is not protective against a different subtype as well as a distantly related strain of the same subtype [3].

Furthermore, several papers reported conflicting results about the immune response to influenza vaccine in the elderly. Some of them, in fact, show a good antibody response, some instead report a poor response [4]. This discrepancy probably depends on many causes, including the high recirculation of influenza virus, able to continuously stimulate a specific antibody response, the relative ineffectiveness of the vaccine due to the variability of the main antigenic epitopes under immune system selective pressure, the reduced performance of immune system in the elderly, mainly in its cellular branch, and finally possible technical problems in the monitoring of antibody response [4].

The antigenicity of the currently licensed vaccines is known to be so weak, that many efforts have been provided to improve their immunogenicity through the use of immunoadjuvants [5]. Several adjuvants have been used, among which even the Freund's incomplete adjuvant, that has been administered to the Armed Forces in the USA for many years [6, 7]. More recently, α_1 -thymosin [1, 8], immunostimulating complexes (ISCOMs) [9], RU 41740, a glycoprotein from *Klebsiella pneumoniae* with a broad spectrum of immunomodulating activity [10] and muramyl tripeptide conjugated to phosphatidylethanolamine [11] have been used to improve the

immunogenicity of influenza vaccine, but conclusive results are still lacking.

The aim of this paper was to study the response to influenza vaccination with a method, such as the isoelectric focusing and reverse blotting (IEFRB) that, giving indirect information about the number of B-cell clones enrolled in the specific immune response, may be useful in the definition of the mechanisms underlying the inefficacy of the influenza vaccination. Furthermore, in a preliminary trial and in a minority of elderly subjects, we used as immunoadjuvant thymopentin (TP-5) (a pentapeptide corresponding to amino acids 32–36 from thymopoietin [12], a purified thymic hormone with a molecular weight of 5,562 daltons), which has already shown immunopotentiating properties in aged people [13].

Four groups of subjects were vaccinated according to standard schedule: 37 elderly healthy people, 9 of whom treated with TP-5, 8 normal young subjects and 13 human immunodeficiency virus (HIV)-infected individuals.

Our results showed limited differences among the four groups of subjects both in ELISA and in the spectrotypic pattern of antibody response, as analyzed by IEFRB. The large majority of the subjects showed a restricted number of specific B-cell clones involved in the response to influenza antigen. Finally, we observed the lack of an enhancing effect of the immunoadjuvant TP-5 in this model of influenza vaccination.

Materials and Methods

Patients

After informed consent, 37 healthy elderly individuals, 8 healthy young adults and 13 HIV-infected patients were included in the study. They were divided into four groups: (1) Group A, including 8 young adults (2 males, 6 females), mean age 26 years, range 22–28,

all in good health. (2) Group B, including 28 healthy elderly people (7 males, 21 females), mean age 87 years, range 76–95, all institutionalized. (3) Group C, including 13 HIV-infected patients (10 males, 3 females), mean age 32 years, range 22–45, of whom 2 in CDC stage II, 7 in CDC stage III and 4 in CDC stage IV. (4) Group D, including 8 healthy elderly people (all females and institutionalized) treated with an immunoadjuvant (TP-5, Timunox; Cilag, Cologno Monzese, Italy), mean age 82 years, range 70–90.

Subjects from groups B and D were selected according to the Senieur Eurage Protocol.

Vaccine and Immunization

All participants had already been vaccinated before against influenza, thus they were only treated with a single dose of 0.5 ml of the 1991–92 preparation of trivalent, inactivated whole virus, influenza vaccine containing 10 µg each of A/Taiwan 1/86 (H1N1), A/Shanghai 16/89 (H3N2) and B/Yamagata 16/88 (Agrippal, Sclavo, Italy). The vaccine was administered intramuscularly in the deltoid region. Group D subjects were also treated with nine intramuscular injections of TP-5 (50 µg/ml) every other day, starting 1 week before vaccination and going on to 2 weeks after vaccination. Venous blood samples were collected prevaccination and at 30 and 60 days postvaccination. Only responses to the HA of A/Shanghai 16/89 (H3N2) vaccine component are reported in this study.

ELISA for Anti-H3N2 HA Antibodies

Quantitative analysis of anti-H3N2 HA antibodies was performed by ELISA, as previously reported [14, 15]. Briefly, flat-bottomed 96-well microtiter polystyrene plates (Pro-bind; Falcon) were coated (3 h, 37 °C) with 100 µl of H3N2 HA (a kind gift of Dr. A. Doublas, Division of Virology and WHO Influenza Center, National Institute for Medical Research, London, UK) diluted to 50 µg/ml in carbonate/bicarbonate buffer, 0.05 M, pH 9.6. The coated plates were washed with PBS containing 0.035% Tween 20 (PBS-T). After a postcoat step with PBS containing gelatin 1% w/v (1 h, at room temperature), the plates were incubated for 2 h at RT with 100 µl of serum samples diluted 1/50, 1/100, 1/200 and 1/400 in PBS-T. After four washings with PBS-T, 100 µl of a 1/1,000 dilution in PBS-T of an alkaline phosphatase (AP)-conjugated goat F(ab')₂ anti-human IgG, IgA or IgM, Fc fragment specific (Tago, Burlingame, Calif., USA), were added for 2 h at RT. The amount of enzyme bound to each well was determined by the addition of 100 µl of substrate (pnpp, Sigma p104-105) diluted at 1 mg/ml in TRIS-HCl, 0.1 M, pH 8.6. The reaction was stopped after

20 min with the addition of NaOH 3 M and the optical density (OD) of each well read with an automated spectrophotometer.

When sera were tested in uncoated wells, background OD units were read. Specificity was demonstrated by inhibition assay with soluble HA.

The range of interassay variability was below 15%. This was checked by using the same serum positive for IgG, A and M in each plate, at six dilutions ranging from 1:50 to 1:1,600.

IEFRB

Spectrotypic qualitative analysis of anti-H3N2 HA antibodies was performed by IEFRB in agarose gel (0.5 mm, 1% agarose, 10% sorbitol and 3% ampholines, pH 3.5–9.5; Pharmacia-LKB Biotechnologies AB, Bromma, Sweden), as previously reported [16, 17]. 17 µl of serum, 1:4 in NaCl 0.9%, were applied near the anode on paper sample strips on a Multiphor (Pharmacia-LKB) apparatus. Proteins were allowed to migrate for 3 h (the voltage was progressively increased from 300 to 1,200 V). Sample paper strips were removed after 1 h. The pH measurements were performed at +10 °C with the agarose plate still lying on the cooling plate of the Multiphor, using a flat-bottomed electrode (Orion Instruments). Then, the IEF-separated proteins were blotted for 90 min onto a nitrocellulose sheet prewetted with distilled water, laid on the agarose gel and covered with Whatman 3MM dry paper and stratified paper towel. All this material was pressed under a 1-kg weight. Nonspecific binding was blocked by immersing the nitrocellulose sheet in 5% dried nonfat milk 0.1% Tween-20 in PBS for 1 h at RT. The membrane was then incubated with 50–100 µCi of H3N2 HA labelled with ¹²⁵I by chloramine T method according to McConahey and Dixon [18]. The nitrocellulose sheet was then washed 10 times in 0.1% Tween-20 in PBS at RT, air-dried and finally exposed to Kodak X-Omat AR film (Eastman-Kodak Co., Rochester, N.Y., USA) at –70 °C for autoradiography.

Statistical Analysis

Statistical analysis of the differences among the levels of specific antibodies in each group, at day 0 and 30 and 60 days after vaccination, and among the different groups at the above reported times, was performed by analysis of variance (ANOVA) on natural logarithm (ln)-transformed figures followed by Bonferroni's *t* test correction. The analysis of the difference of clinical data among the groups was carried out by χ^2 .

Table 1. Geometric mean titers (arbitrary OD units) of anti-influenza IgG in normal young subjects (group A), normal elderly subjects (group B), HIV-infected patients (group C) and normal elderly TP-5-treated subjects (group D) at day 0 (t0), 30 days (t1) and 60 days (t2) after vaccination

Group	Subjects	t0 ¹	t1	t2
A	8	400.27	311.16	366.72
B	28	224.85	323.62 ²	297.66 ²
C	13	201.35	291.34 ³	278.25 ³
D	9	258.53	251.78	255.56

¹ t0: group A vs. group C (t = 3.170), p < 0.05; group A vs. group B (t = 3.090), p < 0.05.

² Group B: t1 vs. t0 (t = 3.586), p < 0.05; t2 vs. t0 (t = 2.475), p < 0.05.

³ Group C: t1 vs. t0 (t = 4.322), p < 0.05; t2 vs. t0 (t = 3.770), p < 0.05.

Table 2. Geometric mean titers (arbitrary OD units) of anti-influenza IgA in normal young subjects (group A), normal elderly subjects (group B), HIV-infected patients (group C) and normal elderly TP-5-treated subjects (group D) at day 0 (t0), 30 days (t1) and 60 days (t2) after vaccination

Group	Subjects	t0	t1	t2
A	8	136.21	176.91	168.62
B	28	207.66	304.69 ¹	302.59 ¹
C	13	116.12	208.84 ²	182.39 ²
D	9	203.86	224.28	240.99

¹ Group B: t1 vs. t0 (t = 4.499), p < 0.05; t2 vs. t0 (t = 4.663), p < 0.05.

² Group C: t1 vs. t0 (t = 3.715), p < 0.05; t2 vs. t0 (t = 2.868), p < 0.05.

Results

Clinical Data

There were no reports of any adverse side effects after vaccine administration. No influenza-like episodes during the year after

Table 3. Geometric mean titers (arbitrary OD units) of anti-influenza IgM in normal young subjects (group A), normal elderly subjects (group B), HIV-infected patients (group C) and normal elderly TP-5-treated subjects (group D) at day 0 (t0), 30 days (t1) and 60 days (t2) after vaccination

Group	Subjects	t0 ¹	t1	t2
A	8	6.48	28.59	23.85
B	28	35.04	116.85	111.11 ²
C	13	1.89	17.71 ³	20.11 ³
D	9	31.93	41.75	85.74

¹ t0: group C vs. group B (t = 4.349), p < 0.05.

² Group B: t2 vs. t0 (t = 2.472), p < 0.05.

³ Group C: t1 vs. t0 (t = 5.425), p < 0.05; t2 vs. t0 (t = 6.243), p < 0.05.

⁴ Group D: t2 vs. t0 (t = 3.685), p < 0.05.

vaccination in subjects belonging to groups A, C and D were observed. On the contrary, 4 out of 28 (14%) subjects of group B had influenza-like episodes during the year after vaccination. This difference was not significant.

Quantitative Analysis of Specific Antibodies by ELISA

The study of specific anti-H3N2 HA IgG before vaccination showed that healthy young people (group A) had a baseline level of IgG significantly higher than the IgG levels of the elderly (groups B and D) and HIV+ subjects (group C) (table 1). The difference among groups was no longer present at day 30 (t1) and day 60 (t2) after vaccination. This fact is due to an increase of specific IgG after vaccination in the elderly and HIV+ individuals, but not in young healthy subjects. At t2, however, the mean IgG levels of the elderly and HIV+ subjects did not show any further increase. The treatment with TP-5 did not modify the IgG-specific response.

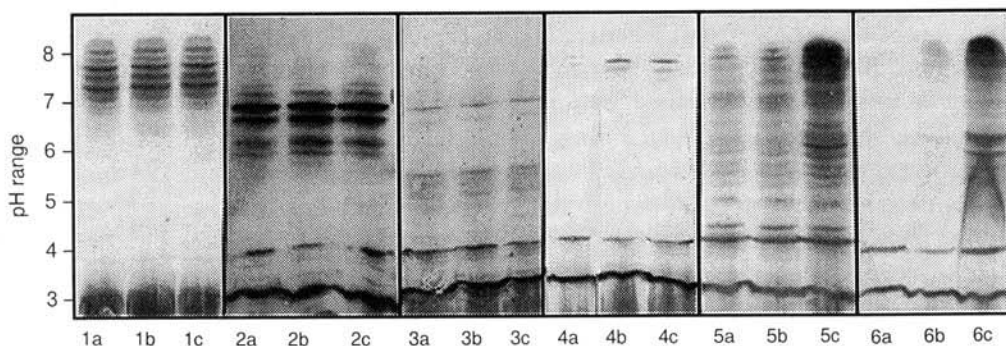


Fig. 1. Spectrotype of anti-H3N2 HA antibodies from subjects undergoing vaccination with a single dose of 0.5 ml of the 1991–92 preparation of trivalent influenza vaccine containing A/Taiwan 1/86 (H1N1), A/Shanghai 16/89 (H3N2) and B/Yamagata 16/88. The number of the subject (1–6) and the time of the follow-up evaluation (a = time 0, b = 30 and c = 60 days after vaccination) are reported on the X-axis, while isoelectric point of bands (pH range) is reported on the y-axis. The reported spectrotypic patterns are representative of all the subjects, since no difference was observed among the four studied groups. Most

subjects showed an oligoclonal spectrotypic pattern (a few clusters of bands) with a constant pattern during the follow-up evaluation (subjects 1–3). Dominant clones were observed in alkaline (lanes 1a–1c), neutral (lanes 2a–2c) or acidic areas (lanes 3a–3c). Subject 4 displayed a monoclonal pattern (only one cluster of bands), with an increase in band intensity after vaccination (lanes 4b, 4c vs. 4a). Finally, subjects 5 and 6 showed an oligoclonal pattern with an increase in band intensity (lanes 5b, 5c vs. 5a) and in the number of bands (lanes 6c vs. 6a, 6b) after vaccination, respectively.

The analysis of specific anti-H3N2 HA IgA showed no significant difference among groups at any time. The longitudinal evaluation in each group showed an increase only in groups B and C (table 2), as already observed for IgG. The increase was significant ($p < 0.05$) only between the IgA levels at t0 and t1 and t0 and t2, but not between t1 and t2.

Lastly, the specific anti-H3N2 HA IgM levels before vaccination were significantly different in groups B and D (table 3); the specific IgM levels in HIV+ and young healthy individuals were in fact extremely low. No difference was observed after vaccination among groups. The longitudinal evaluation of specific IgM showed that, 2 months after the vaccination, the IgM levels were maintained

significantly higher than before the vaccination in all groups, apart from group A.

Qualitative Analysis of Specific Antibodies by IEFBR

Spectrotypic analysis of specific anti-H3N2 HA antibodies showed a monomorphic pattern of specific antibody response. A large majority of subjects showed a restricted heterogeneity of antibodies specific for the HA, that resulted in a few bands with the IEFBR method (fig. 1). Dominant bands, when present, were observed in alkaline (fig. 1, lanes 1a–c), neutral (fig. 1, lanes 2a–c) or acidic areas (fig. 1, lanes 3a–c). The restricted number of bands is indicative for a restricted number of antibodies reacting with

HA and, as a consequence, for a limited diversity of B cells: the IEFBR pattern characterized by a few bands is hence representative of an oligoclonal B-cell recruitment. Two subjects even displayed a monoclonal pattern (one of these is shown in figure 1, lanes 4a-c). Only a few cases in each group showed a negative pattern, i.e. it was not possible to discriminate any band, whereas the majority of subjects displayed a positive pattern, sometimes with an increase in intensity and/or in the number of bands after vaccination (fig. 1, lanes 5a-c and 6a-c). These data indicate that, independently on the health status of the subject, the response to HA is limited to a restricted number of antibody-secreting cells and that, after the vaccination, new B-cell clones are only occasionally recruited.

Discussion

This study confirms the poor immunogenicity of the influenza vaccination and shows the failure of TP-5 as immunoadjuvant of this vaccination in the antibody response to H3N2 HA.

To evaluate the immunogenicity of this vaccination, we studied the anti-H3N2 HA-specific antibody response before and after the vaccination in four groups of subjects: normal young volunteers, elderly healthy people, elderly healthy people treated with TP-5 as immunoadjuvant and HIV-infected individuals. Although quantitatively unbalanced, the four groups were representative of diverse, but characterized physiopathological conditions.

The specific anti-HA antibody response was firstly studied by ELISA. This method allows us to identify the antibody response at single Ig isotype level and, therefore, gives more information than the HA inhibition assay, which, up until now, has been used in the majority of studies.

Young healthy subjects showed a high level of specific anti-HA IgG previous to the vaccination. Specific IgG and IgA levels, however, remained unchanged after vaccination, whereas it was possible to observe an increase, although not significant, of IgM. These results could be compatible with the outcome, in immunologically virgin subjects, of a single dose vaccination, that, evoking a primary immune response, would result in an increase of IgM only. However, these subjects were vaccinated yearly, thus, the lack of an increase of specific antibodies is likely to be regarded as a consequence of the inability of the vaccine itself in eliciting an efficient anti-HA antibody response and the high IgG level previous to the vaccination as a memory more of naturally occurring immunizations, than of previous vaccinations.

In the institutionalized elderly people that accepted to participate in this study, the IgG levels were significantly lower than those of young subjects and the IgG increase observed 30 days after the vaccination was not longlasting. These data and concomitant observation of the high IgM levels before vaccination are indicative for the inefficacy of the vaccine to expand memory cells and induce a stable IgG synthesis. Elderly subjects are generally reported as having higher levels of anti-influenza antibodies before vaccination than younger subjects [2], when measured by HA inhibition assay. In our study this was true only for IgA and IgM but not for IgG, which, on the contrary, were significantly lower in elderly subjects. One possible explanation is that specific antibodies mainly participating in the inhibition of hemagglutination are IgA and IgM but not IgG.

The young HIV+ individuals in this study showed low levels of prevaccination specific Ig, but a significant increase of all the specific Ig isotypes after vaccination was observed. Such an increase was also maintained 2

months after the vaccination. The main feature of HIV+ individuals is a progressive failure of the immune response. However, there are reports of a normal [19], as well as of an impaired antibody response after influenza vaccination [20]. The response to the vaccination of the HIV+ patients in this study, however, is the most unexpected and it is troublesome to explain why a vaccination poorly immunogenic in healthy young individuals gives its best results in a model of immunodeficiency. It could be hypothesized that the increase of antibody levels in the HIV patients is the consequence of the polyclonal B-cell activation demonstrated in these patients, but certainly further studies in larger populations are necessary to confirm and understand this phenomenon.

As a further progress in understanding the limited immunogenicity of influenza vaccination, we monitored the development of the specific immune response after vaccination with IEFBR. To our knowledge, this is the first time that the qualitative analysis of antibody response to one influenza antigen, as obtained by IEFBR, has been performed. Spectrotypic analysis allows us to gain insights on the approximate amount of B-cell clones actively engaged in the specific antibody secretion. This is indirectly evaluated by the number of bands or band clusters that are obtained as a result of specific binding onto nitrocellulose paper (spectrotype). Numerous bands or band clusters are originated by a large number of diverse antibodies, recognizing different epitopes of the same antigen, that are produced by a large number of B-cell clones (polyclonal spectrotype), while a few discrete bands indicate the involvement of a limited number of B cells (oligoclonal spectrotype) and 3–5 adjacent bands a single B-cell clone (monoclonal spectrotype).

The spectrotype was interestingly very similar in all the groups studied: in all the sub-

jects it was oligoclonal and in 2 subjects it was even monoclonal. However, some individual spectrotypic characteristics should be underlined. Irrespective of the group of subjects, it was observed that the immunodominant clones were found, in the diverse individuals, in alkaline, neutral or acidic areas. This difference is indicative for the variability in the structure of the specific antibodies, and may reflect both the characteristics of the influenza virus and the individual immunological experiences. These antibodies, in fact, could have been induced by a natural infection and, therefore, by a wild-type HA partially different, due to antigenic drift, by the HA used for vaccination and for the IEFBR. As a consequence, the variability of the wild-type HA may cause the variability of the antibodies cross-reacting with the HA used as a challenge antigen in IEFBR.

It is particularly interesting that in none of the vaccinees was polyclonal spectrotype observed, which is often observed in response to proteic antigens. This phenomenon is indicative for the inability of the HA to stimulate a large number of different B cells, probably as a consequence of the paucity of epitopes available for the recognition. In particular it might be related to the glycosylation of HA that may render inaccessible most of the epitopes. Finally, in the majority of the subjects the oligoclonal pattern of specific antibodies was maintained unchanged after the vaccination. This observation might be further evidence of the poor stimulation of the immune system upon vaccination.

Lastly, we tried, in a limited number of elderly individuals, to circumvent the inefficacy of the vaccination by treatment with TP-5. Not only were we unable to point out an improvement in the production of specific antibodies of the diverse isotypes, but, more notably, we did not observe any increase in the number of B-cell clones in IEFBR, follow-

ing this immunoadjuvant treatment. In a previous work [14], instead, we observed that thymic hormones may improve the immune response by an earlier recruitment of B-cell clones.

In conclusion, this study confirms, by a more analytical technical approach, the poor immunogenicity of the currently available influenza vaccines and shows the lack of adjuvant effect of TP-5, limited to the production of the anti-H3N2 HA antibodies. These ob-

servations may indicate that the desired immunogenicity of influenza vaccines should be searched through the construction of new, more antigenic vaccines, together with the use of more effective adjuvants.

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