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Safety and Immunogenicity of Two Influenza Virus Subunit Vaccines, with or without MF59 Adjuvant, Administered to Human Immunodeficiency Virus Type 1-Seropositive and -Seronegative Adults▽

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The objective of this study was to evaluate and compare both the safety and tolerability and the humoral and cell-mediated immune responses for two influenza virus subunit vaccines, one with MF59 adjuvant (Fluad) and one without an adjuvant (Agrippal), in healthy and in human immunodeficiency virus type 1 (HIV-1)-infected adult individuals. To achieve this aim, an open, randomized, comparative clinical trial was performed during the 2005-2006 season. A total of 256 subjects were enrolled to receive one dose of vaccine intramuscularly. Blood samples were taken at the time of vaccination and at 1 and 3 months postvaccination. A good humoral antibody response was detected for both vaccines, meeting all the criteria of the Committee for Medical Products for Human Use. After Beyer's correction for prevaccination status, Fluad exhibited better immunogenicity than Agrippal, as shown from the analysis of the geometric mean titers, with significant differences for some virus strains; however, no definitive conclusions on the clinical significance of such results can be drawn, because the method used to estimate antibody response is currently nonstandard for influenza virus vaccines. Significant induction of an antigen-specific $CD4^+$ T-lymphocyte proliferative response was detected at all time points after immunization, for both the vaccines, among HIV-1-seronegative subjects. This was different from what was observed for HIV-1-infected individuals. In this group, significance was not reached at 30 days postvaccination (T30) for those immunized with Agrippal. Also when data were compared between treatment groups, a clear difference in the response at T30 was observed in favor of Fluad ($P = 0.0002$). The safety profiles of both vaccines were excellent. For HIV-1-infected individuals, no significant changes either in viremia or in the $CD4^+$ cell count were observed at any time point. The results showed good safety and immunogenicity for both vaccines under study for both uninfected and HIV-1-infected adults, confirming current recommendations for immunization of this high-risk category.

Influenza vaccination is recommended in many countries to at-risk individuals, such as elderly people, patients with reduced immune responses, and those with chronic diseases (6, 21, 42, 47). The current immunization strategy, which is focused mainly on the control of the disease, has benefits at both the individual and the community level (18, 32, 34).

In human immunodeficiency virus (HIV)-positive subjects, influenza virus may continue to replicate for weeks or months, prolonging shedding and increasing the risk of complications, hospitalizations, and death (15, 27, 33, 39).

Although immunization of HIV type 1 (HIV-1)-infected patients against influenza has been studied thoroughly in recent years, vaccination coverage remains low for this group of subjects, as is also the case for other high-risk groups (1, 12, 17, 24–26, 41, 43, 46, 52, 53). This may be due to both the hypothesized negative effects of vaccination on viremia levels and $CD4^+$ lymphocyte counts (10, 23, 35, 40, 45) and the inadequate immune responses of severely immunodepressed individuals. Several factors can influence the efficacy of vaccination, e.g., age, the immunocompetence of the recipient, the closeness of the match between the vaccine and circulating strains, the clinical outcome measured, and annual influenza attack rates (2, 47).

The availability of influenza virus vaccines with adjuvants, such as those containing the oil-in-water emulsion MF59, has recently opened new, interesting perspectives for the prevention of the disease, not only in elderly subjects but also in adults belonging to well-recognized risk categories (3, 4, 17, 24). Several studies of the elderly have demonstrated the higher immunogenicity of influenza virus vaccines with MF59 adjuvant than of split or subunit vaccines with no adjuvant (13, 19, 31, 38, 44). However, few studies have been carried out both on healthy adults (16) and on patients with underlying clinical conditions, such those infected with HIV-1 (17). In addition, relatively little is known about the mechanisms triggered in humans by MF59, which lead to the activation of T

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cells specific to the major surface antigens of influenza virus, such as hemagglutinin (HA), and their role in helping B cells produce specific antibodies (36). It is well known that $CD4^+$ T helper cells play a pivotal role in the mechanisms of immune control during several virus infections, also facilitating cytotoxic T-cell expansion and activities (48). Understanding these mechanisms could be of benefit to immunocompromised patients, particularly HIV-1-infected individuals, especially considering that $CD4^+$ memory T cells, which are the main targets of HIV infection, are involved in the response to influenza vaccination (51). Therefore, evaluation of the functional T-cell response, in addition to measurement of the serologic response, could provide additional information that would help us better estimate vaccine protection against influenza (29).

The main purpose of the present study was to investigate and compare the humoral and $CD4^+$ cell responses to two influenza virus vaccines, one with and one without MF59 adjuvant, in uninfected or HIV-1-infected adults. An additional aim was to evaluate the tolerability and safety of the vaccines by monitoring viremia (HIV-1 RNA) and $CD4^+$ cell counts at regular intervals after immunization.

MATERIALS AND METHODS

Study design, population, and vaccines used. The present spontaneous, multicenter, open-label, randomized, comparative study was carried out after approval by the local ethics committees of all the participating centers. During the period 2005 to 2006, 256 subjects between the ages of 18 and 65 years were enrolled at the Rehabilitation Centre of San Patrignano, Province of Rimini, Italy. Following written informed consent, subjects entering the study were interviewed. Detailed clinical and vaccination histories were collected, and physical examinations were carried out. Between November and December 2005, all subjects, depending on their HIV serostatus, were randomly assigned to vaccine groups at a ratio of 1:1. HIV-seronegative groups A and B and HIV-seropositive groups C and D received intramuscularly a single dose of an influenza virus vaccine with MF59 adjuvant (Fluad) or a subunit vaccine (Agrippal S1), both commercially available (Chiron, Siena, Italy).

The antigenic composition of the two trivalent inactivated influenza virus vaccines was that recommended by the World Health Organization for the northern hemisphere and accepted, in Italy, by the Ministry of Health for the influenza season during which the study was carried out. The vaccines contained 15 μg of HA for each of the following strains of influenza virus: A/New Caledonia/20/99 (H1N1), A/California/7/2004 (H3N2), and B/Shanghai/361/2002.

Blood samples were collected at the time of the vaccination visit (T0) and then at 30 and 90 days postvaccination (T30 and T90, respectively) for assessment of antibody responses in all subjects and for determination of the $CD4^+$ T-lymphocyte counts and HIV-1 RNA levels in HIV-1-infected individuals. Cell-mediated immunity was evaluated for a subset of subjects.

Safety and tolerability evaluation. Postvaccination adverse events (AEs) were monitored by the investigators for 1 month. Solicited local and systemic side effects were obtained directly from each enrolled subject using a clinical diary, which was completed during the 4 days after vaccination. All reactions were classified by severity as mild, moderate, or severe.

Plasma viremia. Plasma samples from the subset were tested by a sandwich nucleic acid hybridization test for the direct quantitation of HIV-1 RNA, using 50 copies of RNA/ml of plasma as the cutoff for detection (Versant HIV RNA assay, version 3.0 ultra [bDNA]; Bayer). Undetectable levels were arbitrarily assigned a value of 25 copies/ml.

$CD4^+$ T-lymphocyte count. Whole blood was collected in Vacutainers containing tetrasodium EDTA. The absolute number of $CD4^+$ T lymphocytes was determined on a FACSCalibur cytometer using a commercially available kit (Multiset; Becton Dickinson).

Antibody assays. Antibodies to each of the three components of the vaccines were tested by use of a hemagglutination inhibition test (14).

The antibody response was evaluated according to the parameters set out by the Committee for Medicinal Products for Human Use (CHMP) for subjects between the ages of 18 and 60 years: a T30/T0 geometric mean titer (GMT) ratio of ≥2.5 and seroconversion and postvaccine seroprotection rates of ≥40% and ≥70%, respectively (50). Seroconversion refers to an increase of at least fourfold in the antibody titer between T0 and T30. Seroprotection was defined as an antibody titer of ≥1:40.

Purified antigens were kindly supplied by Novartis Vaccines S.r.l., Siena, Italy.

Cell-mediated immunity. Carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) at 5 mM in dimethyl sulfoxide was stored at −20°C until use.

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll gradient (Biochrom) centrifugation. For CFSE staining, cells were prepared in phosphate-buffered saline (PBS; Invitrogen Life Technologies) without supplemental protein at 10×10^6/ml. Stock CFSE was thawed and diluted to 5 μM in PBS, and 1 ml of this solution was added to a pellet containing 10×10^6 PBMC. After incubation for 5 min at room temperature, labeled cells were washed twice in PBS–1% fetal calf serum at 4°C, recounted, and diluted to 2×10^6/ml in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% autologous plasma. For the proliferation assay, duplicate cultures of 4×10^5 cells in 200 μl per well were established in round-bottom microtiter plates (Nalge Nunc International). PBMC were activated by a mix of 2 μg/ml of HA from each of the virus strains contained in the influenza virus vaccine used in the study, kindly supplied by Novartis Vaccines S.r.l., Siena, Italy.

Data were acquired on a FACSCanto cytometer using FACS DIVA software. Gates were set on live lymphocytes according to forward scatter and side scatter characteristics, and 50,000 $CD3^+$ T cells were collected for each sample. To estimate the frequency of proliferating cells (precursor frequency) in response to antigens, flow cytometric data files were analyzed with the "Proliferation Wizard" module using ModFit software. To analyze the $CD4^+$ antigen-specific proliferating T lymphocytes, the $CD3^+$ $CD4^+$ cells were set, and the results were expressed as $CD3^+$ $CD4^+$ T-cell proliferation per 8×10^5 cells tested (20).

Statistical analyses. The Student *t* test and chi-square test were used both in the comparison of the tolerability parameters among groups and in the HIV-1 RNA and $CD4^+$ T-lymphocyte count determinations, pre- and postvaccination, expressed as means or percentages.

Beyer's method was used for the analysis and comparison of the immunogenicity profiles (7). The comparison between groups was performed using the analysis-of-variance method.

The Mann-Whitney test was used for the evaluation of lymphocyte proliferation at the monitored time points.

SPSS was used in the analysis of data.

RESULTS

Vaccinees. A total of 256 subjects, 216 of whom were males (84.3%), with a mean age of 35 years (standard deviation [SD], 8.05), recruited during the study period, were enrolled and assigned to four groups (groups A, B, C, and D) according to HIV-1 serostatus and the vaccine administered. Table 1 gives a brief summary of the characteristics of the study population. Twenty-three and 14 subjects who were evaluated in the tolerability study did not complete the immunogenicity analysis due to the fact that blood samples were unavailable at T30 and T90, respectively. These dropouts were not dependent on any clinical factor related to the study.

Twenty, 20, 29, and 30 subjects from groups A, B, C, and D, respectively, were assigned to representative subsets of these groups for the evaluation of safety, tolerability, and humoral immunogenicity, as well as cell-mediated immunity, $CD4^+$ cell counts, and HIV-1 RNA levels.

The four groups did not differ in terms of general demographic and baseline clinical features.

Safety and tolerability. During the entire clinical follow-up, no serious AEs or significant AEs were reported, except for a case of transitory ischemic attack in a man on the same day as immunization with Fluad, which was considered unlikely to be associated with vaccination.

The tolerability profiles of both vaccines are reported in Table 2.

The most common side effects in both treatment groups

TABLE 1. Baseline characteristics of study subjects according to treatment group

Characteristic[a]	HIV-1-seronegative subjects		HIV-1-seropositive subjects	
	Group A (Fluad) (n = 81)	Group B (Agrippal) (n = 80)	Group C (Fluad) (n = 46)	Group D (Agrippal) (n = 49)
Mean yrs of age (SD)	31.4 (7.5)	32.5 (7.1)	41.0 (5.7)	40.1 (6.4)
No. (%) of subjects				
Male	66 (81.5)	71 (88.7)	40 (86.9)	39 (79.6)
Previously vaccinated for influenza	20 (24.7)	22 (27.5)	22 (47.8)	19 (38.7)
On HAART			29 (63.0)	39 (79.6)
Baseline no. (SD) of:				
HIV-1 RNA copies/ml			2,809 (9,736)	19,608 (56,471)
$CD4^+$ cells/mm^3			398 (216)	413 (197)
No. (%) at CDC stage:				
A			6 (22.2)	8 (26.6)
B			17 (62.9)	20 (66.6)
C			4 (14.8)	2 (6.6)

[a] HAART, highly active antiretroviral therapy. HIV-1 RNA level, $CD4^+$ cell count, and CDC disease stage refer to HIV-1-infected individuals enrolled in the subsets (27 individuals in group C and 30 individuals in group D).

were pain, induration, chills, general malaise, headache, and fever. Among HIV-1-seronegative subjects, these side effects were more frequent for those vaccinated with Fluad. Among HIV-1-seropositive individuals, shivers ($P \leq 0.05$) and fever ($P \leq 0.01$) were more frequently reported by those receiving Fluad than by those receiving Agrippal. Most of the reported signs and symptoms were classified as mild and disappeared within 48 to 72 h.

Plasma viremia and $CD4^+$ T-lymphocyte counts. Among the 59 HIV-1-seropositive subjects enrolled, 2 patients decided not to undergo laboratory analysis and were considered dropouts.

For the 57 samples tested, no significant changes in $CD4^+$ cell counts and HIV-1 RNA levels, analyzed by vaccine group, were observed after immunization when data obtained at the three time points (T0, T30, and T90) were compared (Table 3).

Antibody response. Overall, prevaccination antibody titers were high, with GMTs ranging from 49.3 to 98.1 and seroprotection rates between 61% and 82%.

Antibody responses to both vaccines are presented in Tables 4 and 5 for HIV-1-seronegative and -seropositive subjects, respectively.

CHMP criteria (i.e., seroconversion and seroprotection rates and ratio of postimmunization to preimmunization GMT) were met in all the considered groups of analysis.

Seroprotection rates remained high at 3 months after immunization. Nevertheless, as expected, a general decrease in the circulating antibody titers was detected at T90 compared with those at T30, as shown by GMT ratios (T90/T0) ranging from 4.74 to 6.51 and from 1.84 to 3.05 for HIV-1-seronegative and -seropositive subjects, respectively.

A further analysis of immunogenicity was performed using the method recently proposed by Beyer et al., thus correcting for the prevaccination titers (Tables 4 and 5) (7).

Using this analysis, a better immunogenicity profile was observed for the vaccine with adjuvant, Fluad, than for the vaccine without adjuvant, Agrippal. This assessment was true for all influenza virus subtypes, although statistical significance was reached only for virus strains A/H1N1 ($P = 0.005$) and B

TABLE 2. Tolerability profiles: local and systemic reactions according to vaccine group

Reaction	No. (%) of HIV-1-seronegative subjects		P[a] (A vs B)	No. (%) of HIV-1-seropositive subjects		P (C vs D)
	Group A (Fluad) (n = 81)	Group B (Agrippal) (n = 80)		Group C (Fluad) (n = 46)	Group D (Agrippal) (n = 49)	
Local						
Redness	6 (7.4)	2 (2.5)	NS	3 (6.5)	3 (6.1)	NS
Pain	44 (54.3)	19 (23.7)	<0.01	16 (34.8)	9 (18.4)	NS
Induration	18 (22)	5 (6.2)	<0.01	8 (17.4)	5 (10.2)	NS
Ecchymosis	2 (2.5)	0 (0)	NS	1 (2.1)	1 (2)	NS
Systemic						
Shivering	16 (19.7)	7 (8.7)	<0.05	13 (28.3)	5 (10.2)	<0.05
General malaise	27 (33.3)	13 (16.2)	<0.05	14 (30.4)	9 (18.4)	NS
Headache	21 (25.9)	8 (10)	<0.01	14 (30.4)	11 (22.4)	NS
Sweating	9 (11.1)	4 (5)	NS	6 (13)	4 (8.1)	NS
Myalgia	12 (14.8)	5 (6.2)	NS	6 (13)	2 (4.1)	NS
Asthenia	14 (17.3)	5 (6.2)	<0.05	5 (10.9)	3 (6.1)	NS
Arthralgia	10 (12.3)	6 (7.5)	NS	6 (13)	3 (6.1)	NS
Fever	23 (28.4)	4 (5)	<0.01	11 (23.9)	2 (4.1)	<0.01

[a] NS, not significant.

TABLE 3. Plasma HIV-1 RNA levels and $CD4^+$ T-lymphocyte counts, monitored before and after influenza virus vaccination, for 57 HIV-1-seropositive patients

Vaccine group and time point	HIV-1 RNA copies/ml (mean ± SD)	No. of $CD4^+$ T cells/mm^3 (mean ± SD)
Subjects vaccinated with Fluad (n = 27)		
T0	2,809 ± 9,736	398 ± 216
T30	1,964 ± 6,241	438 ± 260
T90	939 ± 2,590	455 ± 227
Subjects vaccinated with Agrippal (n = 30)		
T0	19,608 ± 56,471	413 ± 197
T30	19,017 ± 39,548	458 ± 377
T90	12,716 ± 33,465	486 ± 403

(P = 0.023) in HIV-1-seronegative subjects and for type A/H3N2 (P = 0.003) in HIV-1-seropositive subjects. A value near significance was also observed for HIV-1-seropositive subjects for the A/H1N1 virus strain (P = 0.097).

Finally, an analysis was also performed comparing the different groups on the basis of seropositivity for HIV-1. As expected, HIV-negative subjects mounted a stronger antibody response than HIV-positive subjects (P, 0.002 to 0.0001, depending on the influenza virus strain).

Cell-mediated immunity. Results related to cell-mediated immunity are summarized in Fig. 1. Both vaccines induced statistically significant increases in memory T cells, both at T30 and at T90, compared to baseline levels at T0 in HIV-1-seronegative individuals. No significant differences between vaccine groups were observed at the time points monitored (Fig. 1A).

Among HIV-1-infected patients, the frequency of proliferating $CD3^+$ $CD4^+$ T cells increased significantly, both at T30 and at T90, compared to T0 for the Fluad recipients. This result was obtained for the Agrippal group only at T90 (Fig. 1B). Furthermore, in the comparison between vaccine groups, a clear difference in favor of Fluad with respect to Agrippal was observed at T30 (P = 0.0002).

DISCUSSION

There is significant evidence that adjuvants enhance the immunogenicity of influenza virus vaccines, with a favorable impact on the age-related decline of the immune response in elderly people (5, 11, 13, 22, 31, 38).

Few studies, to our knowledge, have been reported on the effects of vaccines with MF59 adjuvant administered to adults and to HIV-1-seropositive patients (9), for whom there is a great need to improve the immune response to vaccination (17, 28, 37, 52).

Similarly, no large data sets exist on the immunological outcome of vaccination, other than the titration of specific antibodies by hemagglutination inhibition, the standard method used for evaluating immunogenicity.

In this study, we investigated two influenza virus vaccines available on the market, one with and one without the adjuvant MF59, administered to uninfected and HIV-1-infected adults.

No AEs of concern were observed during the entire safety follow-up, confirming the excellent safety profiles of both vaccines in both healthy adults and HIV-1-seropositive individuals. In addition, the results clearly show that vaccination affects neither HIV viremia nor the $CD4^+$ T-cell count, as previously reported (24).

In agreement with previous studies on elderly people, which showed a higher frequency of local side effects for vaccines with adjuvant than for those without adjuvant (13, 19, 31, 38), the local and systemic adverse reactions reported in our study were classified as mild and resolved within 48 to 72 h after

TABLE 4. Immunogenicities of Fluad and Agrippal[a] in HIV-1-seronegative subjects

Parameter	Virus and vaccine					
	A/H3/California/07/04		A/H1/New Caledonia/20/99		B/Shangai/361/02	
	Fluad	Agrippal	Fluad	Agrippal	Fluad	Agrippal
No. (%) of subjects who underwent seroconversion	53 (66)	49 (69)	49 (61)	42 (59)	51 (64)	45 (63)
No. (%) of subjects with seroprotection						
T0	49 (61)	49 (69)	55 (69)	47 (66)	63 (79)	51 (72)
T30	77 (96)	69 (97)	75 (94)	64 (90)	77 (96)	66 (93)
T90	70 (95)	65 (98)	69 (93)	60 (91)	70 (95)	62 (94)
GMT						
T0	76.9	99.6	109.4	82.8	137.2	100.6
T30	787.9	740.9	1,030.7	630.7	929.2	685.2
T90	429.7	554.1	712.3	392.7	650.3	517
GMT ratio						
T30/T0	10.24	7.43	9.42	7.61	6.77	6.81
T90/T0	5.59	5.56	6.51	4.74	4.74	5.14
Adjusted mean titer at T30 according to Beyer method	5.59 ± 1.93	5.28 ± 1.77	6.08 ± 2.23	5.07 ± 2.06	6.25 ± 1.82	5.4 ± 2.64
Corresponding mean antibody titer at T30	240.4	194.28	337.4	167.52*	380.36	212.22**

[a] Samples were tested for 80 and 71 subjects, respectively, at T30 and for 74 and 66 subjects, respectively at T90. *, P = 0.005; **, P = 0.023.

TABLE 5. Immunogenicities of Fluad and Agrippal[a] for HIV-1-seropositive subjects

Parameter	Virus and vaccine group					
	A/H3/California/07/04		A/H1/New Caledonia/20/99		B/Shangai/361/02	
	Fluad	Agrippal	Fluad	Agrippal	Fluad	Agrippal
No. (%) of subjects who underwent seroconversion	18 (54)	22 (45)	15 (45)	20 (41)	16 (48)	23 (47)
No. (%) of subjects with seroprotection						
T0	23 (70)	37 (75)	26 (79)	39 (79)	28 (85)	39 (79)
T30	31 (94)	42 (86)	31 (94)	44 (90)	31 (94)	44 (90)
T90	28 (90)	40 (83)	28 (90)	39 (81)	28 (90)	43 (90)
GMT						
T0	73.6	89.6	97.6	94.1	102.9	123.9
T30	398.9	407	535.4	468.8	481.9	478.8
T90	198.8	176.6	297.9	173.1	210.9	265.9
GMT ratio						
T30/T0	5.42	4.54	5.48	4.97	4.68	3.86
T90/T0	2.7	1.97	3.05	1.84	2.05	2.15
Adjusted mean titer at T30 according to Beyer method	4.32 ± 1.77	3.02 ± 1.96	4.53 ± 2.08	3.69 ± 2.38	3.8 ± 1.73	3.14 ± 1.97
Corresponding mean antibody titer at T30	95.88	40.58*	115.6	64.7	69.5	44.2

[a] Samples were tested for 33 and 49 subjects, respectively, at T30 and for 31 and 48 subjects, respectively, at T90. *, $P = 0.003$.

vaccination. Interestingly, Fluad was better tolerated by HIV-1-seropositive subjects than by seronegative subjects. There were no significant differences in the incidences of either local or systemic adverse reactions between the Fluad and Agrippal groups, except for fever ($P \leq 0.01$) and shivering ($P \leq 0.05$), which were more frequent after Fluad vaccination (Table 2). This could reflect a difference in the activation threshold of the immune response to immunization between HIV-1-infected and uninfected subjects, depending on the deteriorated status induced by the primary infection. Lower secretion of proinflammatory cytokines could be responsible for this phenomenon. With this in mind, we are investigating the functional pattern of the T-cell response after influenza vaccination, both in healthy and in immunocompromised patients.

In a previous study, Frey and colleagues studied the safety, tolerability, and immunogenicity of the influenza virus vaccine with MF59 adjuvant, comparing it with a vaccine without adjuvant in nonelderly subjects. The safety and tolerability profiles were generally favorable for both vaccines, and in terms of immunogenicity, Fluad showed only a modest benefit compared to that for elderly people (16).

In our experience, both vaccines showed very good immunogenicity profiles, and the CHMP criteria for the licensure of influenza virus vaccines were fully met for both HIV-1-seronegative and -seropositive subjects (Tables 4 and 5). With regard to these serological requirements, no significant differences between vaccine groups were observed, either among infected or among uninfected individuals. The immunogenicity results are only partially in agreement with those of a previous study conducted with HIV-1-seropositive subjects receiving highly active antiretroviral therapy (24), which showed better immune stimulation with Fluad than with Agrippal vaccination.

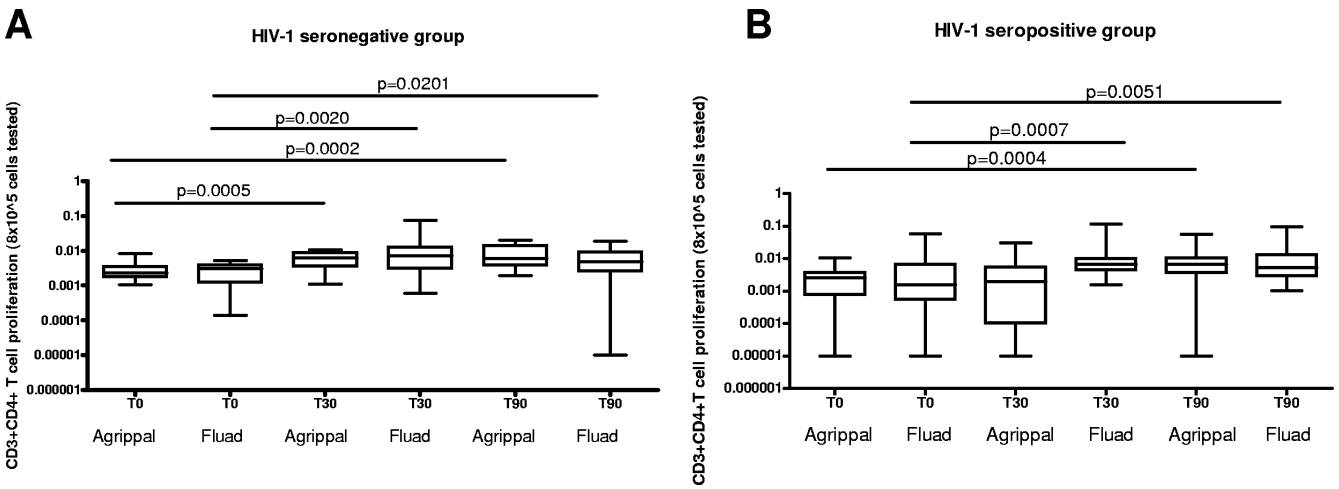

FIG. 1. Precursor frequencies (determined by back calculation with Modfit software) of antigen-specific $CD4^+$ T cells in subjects immunized with the vaccine without an adjuvant versus the vaccine with MF59 adjuvant, by HIV-1 serostatus. These values represent the frequency of antigen-stimulated T cells minus the frequency of spontaneously proliferating T cells.

In order to take into account the generally high baseline seroprotection rates among the study population, comparative immunogenicity analysis was also conducted using Beyer's method, correcting for the presence of preexisting antibodies. This could represent a more reliable indicator, even if theoretical, of the immunological stimulation by the influenza virus antigens present in the two vaccines. By using this new approach, Fluad demonstrated better immunogenicity than Agrippal in subjects not infected with HIV-1 for all influenza virus strains, especially for the H1N1 and B strains (Table 4). A better immunogenicity profile of the vaccine with the adjuvant was also confirmed with HIV-1-infected individuals, mainly for the A/H3N2 influenza virus strain (Table 5). No definitive conclusions on the clinical significance of such results can be drawn, however, due to the fact that Beyer's method, which was used to estimate antibody response, though interesting, is currently nonstandard for influenza virus vaccines. Furthermore, due to the fact that significant differences between vaccine groups were observed only after correction for prevaccination status, it is difficult to provide any solid biological explanation of why the vaccine with MF59 adjuvant had a greater effect than Agrippal for some influenza virus strains and not for others. In fact, any attempt to explain the phenomenon by relating the results either to personal vaccination history or to virological data on influenza virus strain circulation during previous years is simply not viable.

In consideration of the matters discussed above, we believe further investigation into the immunogenicity of vaccines with MF59 adjuvant is highly desirable, especially for groups at high risk of influenza virus infection, in order to better focus the benefit of the adjuvant in eliciting immune responsiveness, particularly for potential nonresponders or low responders.

In a recent study monitoring a cohort of intravenous drug users living in a rehabilitation community, we described the impact of an influenza epidemic sustained by a drifted A/H3N2 virus (8). Data from virological surveillance in the last few years has shown this strain to be the predominant one (30, 51). Influenza attack rates were higher for HIV-1-infected individuals than for other residents (relative risk, 1.77; 95% confidence interval, 1.32 to 2.37), and those infected with HIV were also more likely to develop complications than non-HIV-infected individuals (relative risk, 5.13; 95% confidence interval, 2.52 to 10.20). This study underlines the need for vaccines with wider protective efficacy for this high-risk group, and particularly for patients with severe immunodeficiency (15, 17, 24, 27, 33, 39).

Recent studies have recognized intrinsic limitations of the serological methods currently used as a sole measure to evaluate influenza virus vaccine efficacy, and it has been suggested that evaluation of the cellular immune response could provide additional information to enable better estimation of protection against the disease (29, 49). We monitored the precursor frequency of the proliferating $CD4^+$ T cells in response to specific antigen stimulations by using the flow dye dilution assay. This test, compared to traditional methods for assaying antigen-specific proliferation, offers the additional ability to evaluate the phenotype of the responding cells and is able to determine their rate of proliferation.

Among immunocompetent HIV-1-seronegative subjects, the frequency of proliferating antigen-specific $CD4^+$ T cells was essentially the same for those immunized with either the vaccine without adjuvant or the vaccine with MF59 adjuvant. In contrast, the presence of the MF59 adjuvant induced an enhanced response in HIV-1-infected patients. In fact, among these subjects, a statistically significant increase in the frequency of proliferating T cells was already detected at day 30 postvaccination for the Fluad recipients, in contrast to what was observed for those immunized with Agrippal. A clear benefit of the formulation with the adjuvant was also observed from the comparison between vaccine groups evaluated 1 month after immunization. It is plausible that the robust expansion of the $CD4^+$ memory T cells in infected subjects treated with the vaccine with MF59 adjuvant may translate into a better ability of this vaccine than of Agrippal to confer protection against influenza. This is of particular importance for individuals with impaired immune competence, as stressed above.

In conclusion, the results of this study show that currently available influenza virus subunit vaccines, both with and without MF59 adjuvant, are safe and immunogenic for healthy adults, whether infected with HIV-1 or not.

Vaccines with MF59 adjuvant could represent a new tool for the prevention of influenza in subjects with reduced immune responsiveness, such as HIV-infected individuals (17, 24), elderly people (13, 19, 31, 38, 44), and those with underlying chronic debilitating diseases (3, 4), as recently demonstrated by other authors. Additional studies with this vaccine are required in order to better evaluate the magnitude and duration of both the humoral and cell-mediated responses driven by MF59 adjuvant in various population groups. These data may then contribute to a better understanding of the immune parameters of protection, which may ultimately lead to a change in the current recommendations for the use of vaccines with MF59 adjuvant for adults at risk, such as those infected with HIV-1.

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