Variations in disease severity outcomes for Influenza live viral challenges in man: Meta-analysis and potential role of pre-existing heterosubtypic cellular immunity

Running Title: Cellular immunity and outcomes of live influenza challenges

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ABSTRACT

Objectives: Live Influenza challenges in man are valuable models for testing the efficacy of vaccines and antiviral agents. Volunteers are treated with an investigational agent and their clinical outcomes post-challenge are compared to those of Placebo treated volunteers. Despite using a common protocol, recruitment criteria and a similar dose of the same challenge strain, we noticed differences in disease severity outcomes between Placebo groups from different studies. We investigated whether these differences were significant and, if so, whether any pattern was identifiable and its possible causes.

Methods: We compared the clinical outcomes post-challenge in Placebo groups from five clinical studies carried out between 2008 and 2013. Correlations between pre-challenge heterosubtypic cellular response (IFN-γ) and post-challenge clinical outcomes were also investigated in one study.

Results: Placebo groups from studies carried out between 2009 and 2010 attained significantly reduced (p<0.05) symptom scores post-challenge than Placebo groups from studies carried out in either 2008 or 2013. Also, in a 2010 study, the frequency of high influenza heterosubtypic cellular responders pre-vaccination was significantly lower in the test group (FLU-v) than in the Placebo group (p=0.04). Moreover, the Placebo group’s increased pre-existing heterosubtypic cellular response correlated with reductions in symptom score and viral shedding post-challenge (p≤0.023). Only post-vaccination did the Test group display an equivalent correlation.

Conclusions: The last influenza pandemic coincides with a significant reduction in disease severity outcomes. This reduction also appears to correlate with increased pre-existing influenza heterosubtypic cellular responses.
INTRODUCTION

Live influenza challenges in man are valuable models for testing the efficacy of vaccines and antiviral agents. Their basis is simple: a group of volunteers is treated with an investigational agent and their clinical outcomes post-challenge are compared to those of a group of Placebo treated volunteers. Their logistics, in contrast, are complex.

Influenza infection elicits a range of immune responses. One such response is the production of strain-specific neutralising antibodies that confer immunity against infection by the same strain (1). As a result, a key volunteer exclusion criterion in challenge studies is the detection of pre-existing neutralising antibodies (HAI>10) to the challenge strain. Another such response is the generation of antiviral cellular immune responses. Despite existing evidence as to their protective role during infection (2-4), pre-existing cellular immune responses to the challenge strain are not normally assessed during volunteer recruitment.

We have developed a novel vaccine (FLU-v) that elicits broad influenza heterosubtypic cellular responses without inducing any significant antibody response (5,6,7). In humans, FLU-v was found to be safe, well tolerated and, in a live challenge study, to induce a vaccine specific cellular response whose magnitude correlated with reductions in symptom score and viral shedding (7). No such correlations were seen in the Placebo group, but we did notice that both viral shedding and symptom score post-challenge were much lower (50%) in our Placebo group than in the Placebo group from a previous study. To establish the significance of these differences, we compared the Placebo group outcomes of several other live influenza challenge studies. All these studies, although involving different placebo agents, were carried out by the same clinical group by the same clinical group (Retroscreen Ltd), using the same recruitment
criteria, the same viral strain and dose and the same method for determining post-challenge clinical and virological outcomes. This meta-analysis revealed an experiment of nature that we believe provides interesting insights in the potential of the cellular immune system in controlling Influenza infection.
MATERIALS AND METHODS

Clinical trial data used for meta-analysis

The reported clinical outcomes post-challenge for the Placebo group of four reported independent clinical trials (3, 7, 8, 9) and one previously unreported study (Retroscreen Ltd, personal communication) were used for the meta-analysis. The Placebo agent used in each study was different, but all the studies were carried out by the same clinical group (Retroscreen Ltd) and followed a common challenge protocol (Fig. 1) that used the same well defined recruitment criteria, viral challenge strain (A/Wisconsin/67/2005, H3N2) and procedures for the assessment of disease severity and viral shedding. Exact details for each study are provided in the referenced manuscripts, but they are also briefly summarised below.

Recruitment criteria and study procedures: Healthy male subjects aged 18 to ~45 years with no clinically significant abnormal findings (i.e. physical examination, medical history or laboratory results) and no medical history of Influenza-like illness in the prior 12 months were assessed for enrolment. Only those with HAI $\leq 10$ for the Influenza challenge strain were enrolled.

Following recruitment and treatment (Placebo or test agent), volunteers were challenged on Day 0 by nasal instillation with 1 ml of solution containing approximately $10^{5.25}$ 50% tissue infective dose per ml of live A/Wisconsin/67/2005 (H3N2, tissue culture grown). From days 5 to 7 volunteers received antiviral treatment (e.g. oseltamivir) before being released from quarantine on Day 7.

Physical examinations and clinical laboratory tests were performed at screening, pre- and post-treatment (both pre-challenge), and daily from Day -2 pre-challenge to Day 7 post-challenge. A final assessment was carried out around Day 28 post-challenge. Volunteer self-recorded
observations pre- and post-challenge, as well as the scripted symptom questionnaires, were assessed by clinical staff.

Symptom scoring and virology and HAI tests: Symptom score was determined using a standardised scoring system (3,10) based on subject self-assessment and clinician’s examination. A range of parameters (e.g. Runny Nose, Stuffy Nose, Sneezing, Sore Throat, Earache, Malaise, Cough, Shortness of Breath, Headache and Muscle/Joint ache) were assessed and scored from 0 to 3, corresponding to from absent to severe.

Viral shedding in the nasopharyngeal samples was determined by TCID50 assay as described in the WHO manual of Animal Influenza Diagnosis and Surveillance 2002 (11). Briefly, serial ten-fold dilutions of virus-containing daily post-challenge nasal lavage samples were inoculated into 96-well microtitre plates seeded with Madin-Darby canine kidney (MDCK) cells. Cytopathic effects in individual wells were determined after 5–6 days incubation at 37°C. Viral shedding was defined as a viral culture titre greater than 1.5 log10 TCID50/ml.

Haemagglutinin-specific antibody titres against the challenge virus in volunteer’s sera samples were determined by HAI assay using chicken erythrocytes as described in the WHO manual of Animal Influenza Diagnosis and Surveillance 2002 (11).

Regulatory Approval and Ethical Considerations:

All studies included in the meta-analysis were reported as conducted in accordance with Good Clinical Practice, the Declaration of Helsinki (1964 and 2008), and all regulatory requirements. As we are also reporting previously undisclosed experimental data, we confirm that our FLU-v study (7) was approved by the Plymouth Independent Ethics Committee under REC reference.
The trial was registered under EUDRA Identifier 2009-014716-35 and NCT01226758. Written informed consent was obtained from all participants.

**Vaccine description**

FLU-v is a sterile equimolar mixture of four polypeptides encoding immunoreactive conserved regions within Influenza (5,6, 7). These sequences, which are shown below, were synthetically manufactured (Bachem AG, Bübendorf, Switzerland) in accordance with current Good Manufacturing Practice.

- **M1** - DREALMEWLKTRPIPLSTKGLGFLVFTLTVP (32 aa)
- **NPA** - DIFLARSLRGLRDVHKSC (21 aa)
- **NPB** - PGIADIDLTLARSMVVVR (20 aa)
- **M2** - IIILHLILWILDLFKCIYRLF (24 aa)

FLU-v was administered subcutaneously in 1.0 ml volume as a single 500 μg dose in saline emulsified (1:1) with adjuvant ISA-51 (Seppic, France). The Placebo was saline emulsified with ISA-51. The adjuvant is composed of a light mineral oil and a surfactant system designed to make a water-in-oil emulsion. Functionally, ISA-51 is not known to preferentially favour the induction of Th1-like responses (12).

**Heterosubtypic Cellular immunity – Cytokine ELISA**

Blood was harvested pre-challenge on days -21 (i.e. pre-vaccination) and -2 (i.e. 19 days post-vaccination) and PBMCs isolated and frozen. Thawed PBMCs were seeded at 2x10^5 cells/well (96 well plate) in RPMI-1640 (Sigma, UK), supplemented with 25 mM HEPES, Penicillin (100 units/ml), Streptomycin (100 μg/ml) and 10% FCS, and containing one of the following test antigens: 1 μg/ml Con A (Sigma, UK), 1 μg/ml Bovine Serum Albumin (BSA; Sigma, UK) or live...
Influenza A/Swine/Iowa/15/30 (H1N1, Multiplicity of Infection 10). Virus (egg grown) was obtained from NIBSC as low-endotoxin preparations suitable for in vitro cellular analysis. Each antigen was tested in triplicate. After 24 h incubation at 37°C and 5% CO₂, IFN-γ production in the cell supernatant for each of the test antigens was determined using a validated ELISA assay (BD, UK; Human IFN-γ kit 555142). Response levels were calculated as pg/ml of IFN-γ produced against a standard provided with the assay kit. Minimum level of detection for the assay is 9 pg/ml IFN-γ.

Strong heterosubtypic cellular responses were defined as those in which an individual's IFN-γ response to the Influenza A/Swine/Iowa/15/30 (H1N1) virus were ≥4-fold higher than the individual's IFN-γ response to the negative control (i.e. BSA + medium).

**Statistical analysis**

Inter-group differences in total mean symptom score and viral shedding were determined by single-factor ANOVA analysis. Pairwise differences between the studies were determined by T-test (2-way), with adjustment of significance for multiple pairwise comparisons by the Tukey-Kramer HSD (Honest Significant Difference) method. Heterosubtypic responder frequencies were analysed by the Friedman Exact test, whilst correlations between clinical outcomes and heterosubtypic cellular response levels were determined using the Spearman Rank correlation test.
RESULTS

Mean total symptom score post-challenge: inter-study variability

We have previously reported (7) how in an Influenza live challenge study carried out in 2010, vaccination with FLU-v induced an IFN–γ response to the vaccine whose magnitude correlated with reductions in both viral titre (p=0.01) and total symptom score (p=0.02). No such correlation was seen in the Placebo group. Although we saw no significant differences in mean total symptom score post-challenge between the FLU-v and Placebo group, we did noticed a significant reduction in mean total symptom score post-challenge in our 2010 Placebo group compared to the Placebo group of a previous unreported study carried out by Retroscreen in 2008 (mean total symptom score: 11.4±13.0 vs 37.1±27.5, AVR±SD, our Placebo vs 2008 Placebo, p=0.006).

This significant difference in outcomes was surprising to us because, although the nature of the Placebo agent was different in the two trials, the historical 2008 Placebo dataset (n=12) had been obtained by the same clinical group (Retroscreen Ltd), using the same recruitment criteria, the same viral strain and dose and the same method for determining symptom score. More importantly, the outcomes of this 2008 Placebo constituted the baseline data used to calculate the sample size required to meet the endpoints of our trial.

This difference in outcomes also raised the question of whether our observation was unique, or significant differences in outcome were a common observation in live challenge studies. To address this question we compared the outcome of both our 2010 Placebo and the Retroscreen 2008 Placebo against those reported for Placebo groups in other published studies carried out
in 2008 (3), 2009 (9) and 2013 (8). These studies followed the same common standard protocol (Fig. 1), recruitment criteria and procedures used in the 2010 and 2008 Placebos.

Statistical analysis (ANOVA) of these five studies (Table 1) revealed a significant difference (p=0.004) in the mean total symptom score of the Placebo groups. Subsequent pairwise comparisons (T-test with Tukey-Kramer’s HSD adjustment for significance) revealed that the mean total symptom score for the Placebo Group in the 2008 Wilkinson study (3) was significantly higher than that seen in our 2010 Placebo (60.8±10.7 vs 11.4±13.0, p=0.000), but not different to that in the Retroscreen’s 2008 Placebo (60.8±10.7 vs 37.1±27.5, p>0.050). In contrast, the mean total symptom score for the Placebo Group in the 2009 Lillie study (9) was significantly lower than that seen in both the Retroscreen’s 2008 Placebo (15.3±15.1 vs 37.1±27.5, p=0.030) and the 2008 Wilkinson Placebo (15.3±15.1 vs 60.8±10.7, p=0.000), but not different to that in our 2010 Placebo (15.3±15.1 vs 11.4±13.0, p>0.050). A final comparison of these four different Placebo groups to that of the Placebo group in the 2013 Ramos’ study (8) (55.5±54.8) reveals that mean total symptom score in this study is not different to that seen in either the Wilkinson’s 2008 Placebo or the Retroscreen’s 2008 Placebo (p>0.050 for both), but it is significantly higher than that seen in both Lillie’s 2009 Placebo (p=0.022) and our 2010 Placebo (p=0.007). These results indicate that, following influenza live challenge, mean total symptom scores in Placebo group volunteers were significantly lower in 2009-2010 than they were in either 2008 or 2013.

Mean total viral shedding post-challenge: inter-study variability

We then proceeded to test whether the observed differences in mean total symptom score across the studies were also reflected in the mean total viral shedding measurements. Total
viral shedding data was not reported in the Wilkinson study (3) and hence we could not include this study in the analysis. Nonetheless, a comparison of the remaining four studies revealed a significant difference (p=0.040) in mean total viral shedding. Subsequent pairwise analysis revealed that, as shown in Table 1, mean total viral shedding in the Retroscreen’s 2008 Placebo (10.1±2.9, AVR±SD) was significantly higher than in Lillie’s 2009 Placebo (3.3±4.3, p=0.012), our 2010 Placebo (4.0±4.4, p=0.022) and Ramos’ 2013 Placebo (3.2±4.5, p=0.006).

**Heterosubtypic immunity**

In an attempt to determine the possible reasons for the differences amongst the groups, we first analysed the infection rate for each of the studies. Infection rate was defined as the percentage of volunteers with at least one positive result by TCID50 between days 1 and 5 after live influenza challenge. As shown in Table 1, and despite the wide range of values, no statistical differences (p>0.05) were found in the infection rates across the different studies: Wilkinson’s 2008 Placebo (100%), Retroscreen’s 2008 Placebo (66.6%), Lillie’s 2009 Placebo (45.5%), our 2010 Placebo (61.5%) and Ramos’ 2013 Placebo (48.4%). The similarity in infection rate across the studies suggests that the mechanism responsible for the differences in outcomes is most likely a post-infection mechanism. If correct, this would exclude neutralising antibody responses, but not cellular immune responses. Unfortunately, cellular responses to the challenge virus were not assessed in any of these studies, either pre- or post-challenge. Moreover, if any cellular responses were measured, the antigen (e.g. virus or vaccine) and the method of analysis (e.g. ELISA or ELISPOT) used were all different, thus rendering any direct comparison impossible.
As stated earlier, we have previously established (7) that cellular responses to a vaccine correlated with reductions in both viral load and symptom score. Since the period of reduced total mean symptom scores and viral shedding (2009-2010) identified from our earlier meta-analysis coincided with the dates of the last influenza pandemic, we decided to test if in our study strong pre-existing heterosubtypic cellular responses to a H1N1 swine influenza strain were common and, if so, whether their intensity negatively correlated with symptom score and viral shedding. Ideally, we would have preferred to use the pandemic Influenza A/California/7/2009 (H1N1) strain, but WHO recommends the use of biosafety level 2 plus [BSL-2 plus] facilities with biosafety level 3 [BSL-3] practices with this strain (13). As these facilities are not available to us, we settled for a BSL-2 swine strain: A/Swine/Iowa/15/30 (H1N1).

In our 2010 study, we had dosed and challenged a total of twenty-eight volunteers. However, for this post-hoc analysis, frozen PMBC samples were available from only fifteen volunteers (seven from the Placebo group and eight from the FLU-v group). We found (Table 2) strong pre-vaccination IFN−γ responses to the recall A/Swine/Iowa/15/30 (H1N1) virus (i.e. ≥4-fold IFN−γ response to negative control) in all but one of the Placebo volunteers (median 7.0 fold-increase). In contrast, in the vaccinated (FLU-v) group, strong pre-vaccination IFN−γ response to the recall A/Swine/Iowa/15/30 (H1N1) virus (median 3.4 fold-increase) were found in only one volunteer. Post-vaccination, the frequency of strong IFN−γ responders became similar in both groups (5 vs 4, Placebo vs FLU-v), but the overall level of IFN−γ response to the recall swine virus still remained higher in the Placebo group (median 10.3 vs 5.2, Placebo vs FLU-v).
Correlation analysis revealed a significant negative correlation in the Placebo group between the intensity of the heterosubtypic IFN–γ response to the A/Swine/Iowa/15/30 (H1N1) virus and both the mean total symptom score ($r=-0.771$, $p=0.036$; Fig 2a) and the mean total viral shedding ($r=-0.768$, $p=0.022$; Fig 2b). In the FLU-v group, no significant correlations were seen pre-vaccination ($p>0.05$), but a significant negative correlation was established post-vaccination between the intensity of the heterosubtypic IFN–γ response to the A/Swine/Iowa/15/30 (H1N1) virus and the mean total symptom score ($r=-0.667$, $p=0.035$; Fig 2c).
DISCUSSION

Influenza infection elicits a range of natural antibody and cellular immune responses to the virus. Some of these responses are specific to the infecting viral strain (homosubtypic responses), whilst others are cross-reactive to other viral strains (heterosubtypic responses). Amongst the homosubtypic responses, neutralising antibodies directed to the Haemagglutinin (HA) and Neuraminidase (NA) antigens are of particular interest. Infection by one influenza strain elicits neutralising HA/NA antibody responses that confer lifelong immunity against infection by the same strain (1). For over 50 years influenza public health programs worldwide have built upon this observation by using vaccines that induce homosubtypic neutralising HA/NA antibody responses. Heterosubtypic responses, despite increasing evidence of their potential protective role during infection at both the antibody (14-16) and cellular level (2-4), have not yet been successfully exploited in the clinic.

Notwithstanding their universal use, HA/NA based vaccines suffer from major shortcomings. As new variants of the virus emerge every year, the new circulating viral strains must be first identified before new formulations of the vaccine are prepared every year, which in turn means that every year the population must be re-vaccinated (17). A clear need remains for a vaccine that can address these shortcomings.

Live challenge studies in man are valuable models for the development of effective therapies (e.g. vaccines and antivirals) against Influenza. They allow the efficacy of a candidate treatment to be assessed by comparing disease severity outcomes post-challenge between volunteer groups treated with either the candidate therapy or a Placebo.
Recognising the importance of neutralising antibody responses in influenza protection, the identification of pre-existing neutralising antibody titres (i.e. HAI>10) to the challenge strain is a key universal exclusion criterion during volunteer recruitment in live challenge studies (3, 7, 8, 9). In contrast, neither pre-existing heterosubtypic immune responses (antibody or cellular) nor pre-existing homosubtypic cellular responses to the challenge strain are regularly assessed during volunteer recruitment.

In 2010 we carried out a live challenge study in humans using a novel vaccine (FLU-v) designed to elicit cellular immune responses against influenza. Despite induction of a FLU-v specific IFN-γ response (6, 7) that correlated with reductions in viral shedding and symptom score (7), no significant differences in clinical outcome was seen between the Placebo and FLU-v groups. However, we did identify a clear and significant reduction in both viral shedding and symptom score in our 2010 Placebo group compared to a Placebo group from a study carried out by Retroscreen in 2008. This 2008 Placebo group is significant because its outcomes constitute the baseline data used to calculate the sample size needed to meet the endpoints of our 2010 trial.

As a certain degree of variability is expected in all biological systems, we decided to investigate how consistent viral shedding and symptom score outcomes were across five live challenge studies carried out between 2008 and 2013. Meta-analysis of historical data is extensively used in clinical research (18-23) and, under certain stringent rules, is even allowed by both FDA and EMEA to assess the efficacy of a treatment (24,25). These rules state that all the data analysed must come from clinical trials that used the same eligibility criteria, measured comparable variables and were carried out by the same investigators. Since all the five clinical studies considered in our meta-analysis were carried out by the same clinical group (Retroscreen Ltd),
using the same well defined recruitment criteria, following a common challenge protocol that used a similar dose of the same viral strain (A/Wisconsin/67/2005, H3N2) and assessed the same parameters (i.e. symptom score and viral shedding), we were confident of the validity of our approach. Of course, the nature of the Placebo in these five studies was different, but since we and Retroscreen Ltd agreed to use historical data from the 2008 Placebo group to determine the required sample size of our 2010 study, we believe this decision was consistent with and supports our multi-study comparative approach.

The meta-analysis revealed that of the five studies analysed, the two carried out between 2009 and 2010 (7, 9) achieved total mean symptom scores post-challenge that were significantly lower (~50%) than those seen in studies carried out in either 2008 (3) or 2013 (8). Viral shedding was also significantly higher in the 2008 studies than in the 2009 and 2010 studies, and, in contrast to symptom score, it was also higher than in the 2013 study.

An accurate determination of the mechanism(s) responsible for these differences was not possible as the immune/pharmacological effector mechanisms assessed were different for each study. However, because (a) infection rates (determined as the percentage of challenged volunteers that develop a positive TCID50 between days 1 and 5 post-challenge) across all five studies were not statistically different, (b) neutralising antibodies act primarily at the pre-infection stage, and (c) all volunteers had HAI titres to the challenge strain ≤10, we do not believe that the observed inter-study differences were caused by differences in the volunteers’ HAI titres.

Assessment of cellular responses amongst the studies was not possible. Pre-existing cellular responses to the challenge virus are not regularly assessed in these studies and, when cellular
responses are measured, the antigen (e.g. virus or vaccine) and the method of analysis (e.g. ELISA or ELISPOT) used are all different, thus rendering any direct comparison impossible.

Nonetheless, three observations lead us to consider the possibility that differences in the pre-existing influenza heterosubtypic cellular responses may be, at least partially, responsible for the observed inter-study differences in Placebo group outcomes post-challenge. Firstly, the two studies showing significant reductions in mean total symptom scores were those carried in 2009-2010. These dates coincide with the last influenza pandemic. Secondly, we (7) and others (3, 4) have shown significant negative correlations between the intensity of the cellular response and measurements of influenza disease severity. Thirdly, the reduction in viral shedding, but not in either symptom score or rate of infection, in the 2013 study compared to the 2008 Placebo suggests that a post-infection mechanism is controlling viral replication.

Although lack of data prevented us from comparing the role of heterosubtypic cellular responses across the five studies considered, we believed that some relevant evidence could still be obtained through additional testing of PBMC samples from our 2010 study. Unfortunately, we did not have a complete sample set for this post-hoc analysis and hence we accept that the small size of the sample (15 individuals) further limits the power of this analysis.

Nonetheless, we found significant correlations between the pre-existing IFN-γ responses to influenza A/Swine/Iowa/15/30 (H1N1) and reductions in both total mean symptom score and total mean viral shedding in the Placebo group.

An additional and surprising finding of our analysis was that the frequency of pre-existing high IFN-γ responders to A/Swine/Iowa/15/30 (H1N1) was much higher in the Placebo group than in the vaccine (FLU-v) group. Moreover, although no significant correlation between the IFN-γ
response to the influenza A/Swine/Iowa/15/30 (H1N1) strain and reductions in viral shedding was seen pre-vaccination in the FLU-v group, this correlation became evident post-vaccination. Of course, we have no evidence that the pattern of heterosubtypic cellular responses (i.e. to A/Swine/Iowa/15/30, H1N1) is the same as that of the homosubtypic cellular responses (i.e. to the challenge strain A/Wisconsin/67/2005, H3N2). However, we would maintain that it is not unreasonable to expect it to be so.

We have no explanation as to how, despite the randomisation and double-blind nature of the study, our Placebo group ended up with a higher number of volunteers with strong heterosubtypic cellular responses than our FLU-v group. Recruitment criteria and randomisation in our study was not different to the other studies included in our meta-analysis. A post-hoc analysis of pre-existing HAI responses in our volunteers to the actual 2009-2010 pandemic strain (A/California/7/2009, H1N1) did not reveal any positive individual in either the Placebo or the FLU-v group (data not shown). Nonetheless, we cannot completely rule out a difference in the exposure rate to the virus between the two groups. A report by Presanis et al (26) suggests that the rate of asymptomatic infection in England during the pandemic [June 2009 to February 2010] was as high as 65%. With the benefit of hindsight, and since our study took place shortly after the end of the pandemic, it is our opinion that the list of exclusion criteria used (i.e. history of Influenza-like illness over the previous 12 months and HAI > 10) was ill suited to prevent the recruitment of asymptomatically infected individuals.

Increased levels of influenza heterosubtypic cellular responses in the population after the pandemic, could also help to explain the particular results of the Placebo group in the Ramos’ 2013 study. McMichael et al (27) showed that T cell responses to influenza are detectable years...
after initial natural exposure, although their number declines rapidly with time. As T cell responses are widely acknowledged to play a key anti-viral role, it is possible that exposure to the challenge virus may have caused the expansion of a small pool of memory influenza heretosubtypic T cell clones. The expansion of this small population may not have been sufficient to significantly reduce symptom severity (total symptom score), but it may have been able to have a negative effect on the rate of viral proliferation (total viral shedding).

In summary, we believe our results provide evidence of an unplanned “experiment of nature” that adds to the existing body of evidence on the ability of heterosubtypic cellular immunity to reduce influenza disease severity in humans (2-4). As such, it supports our efforts, and those of other groups, in developing vaccines that elicit heterosubtypic cellular immune responses against influenza. Whether it constitutes sufficient evidence to justify the consistent screening of volunteers for pre-existing cellular immunity to the challenge strain during recruitment, we leave that decision to any researcher planning to use live influenza challenge models in man in the future.
REFERENCES


with reduction in symptomatology and virus shedding in a randomised Phase Ib live viral challenge in man. CVI in press.


valganciclovir for the treatment of cytomegalovirus infection and disease in

2005. A trial of valganciclovir prophylaxis for cytomegalovirus prevention in lung


vaccination in allogeneic stem cell recipients: induction of human cytomegalovirus
(HCMV)-specific cytotoxic T lymphocyte responses even in patients receiving a

24. EMEA document CPMP/ICH/364/96, available from

25. FDA Documents 21 CFR 314.126, available from
accessed January 2012.

a Bayesian evidence synthesis. BMJ. 343:d5408.
Table 1

Summary of descriptive statistics for the post-challenge outcome of all analysed studies. Total symptom score is the sum of all measured symptoms scores for an individual from day 1 to day 7 following challenge with influenza A/Wisconsin/67/2005 (H3N2). Infection rates are the percentage of challenged volunteers with at least one daily nasal sample positive for influenza A/Wisconsin/67/2005 (H3N2) post-challenge. Total viral shedding represents the sum of all measured viral shedding for an individual from day 1 to day 5 post-challenge with influenza A/Wisconsin/67/2005 (H3N2). Viral shedding on days 6 and 7 post-challenge is not considered as under the clinical protocol used all individuals receive anti-viral treatment (e.g. oseltamivir) on those days. Abbreviations: Average (AVR), Standard Deviation (STDEV), Lowest value in range (min), Highest value in range (max). N/A indicates that no data is available.

Table 2

Heterosubtypic cellular immune responses pre- and post-vaccination in 2010 FLU-v study. Values are represented as the fold-increase in IFN-γ response to A/Swine/Iowa/15/30 (H1N1) compared to the negative control. IFN-γ (pg/ml) responses to the negative control pre- and post-vaccination for both groups are 99.2±25.7 vs 80.6±12.9 pg/ml. IFN-γ (pg/ml) response to the positive control (ConA) pre- and post-vaccination for both groups are 311±85 vs 378±35 pg/ml.
Figure 1

Consort profile. Trial profile and baseline demographic data for enrolled volunteers in all five studies analysed. The reported median age of the volunteers in the studies ranged from 24 to 30 years. Where this information is provided, studies are reported to have been carried out between August and November. The section in grey refers to data not incorporated in the meta-analysis of Placebo groups, but that was used for the comparison of cellular immunity described later on in the manuscript.

Figure 2

Correlation analysis between heterosubtypic cellular responses and measurements of disease severity post-challenge. Values are represented as the fold-increase in IFN−γ response to A/Swine/Iowa/15/30 (H1N1) compared to the negative control. Figures 2A and 2B represent the correlations between the Placebo group's pre-existing heterosubtypic cellular response and, respectively, its mean total symptom score and mean total viral shedding post-challenge. Figure 2C represents the correlation between the FLU-v group's post-vaccination heterosubtypic cellular response and its mean total symptom score post-challenge. All analyses were carried out using the Spearman rank correlation test.
RECRUITMENT CRITERIA

Individuals aged 18-45

Absence of abnormal clinical findings (e.g. physical examination, medical history, laboratory results, etc)

No history of Influenza-like illness for the 12 months prior to enrolment

No prior Influenza vaccination for at least 1 year

HI/IS10 to challenge virus

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| Withdrawn consent / Excluded | N = 1 | N = 3 | N = 0 | N = 0 | N = 1 | N = 0 |

Challenge (intranasal)

1 ml of ~10^5 TCID50 of influenza A/Wisconsin/67/2005 (H3N2)

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