Vaccine (2008) **26**, 3153–3161

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EV01: A phase I trial in healthy HIV negative volunteers to evaluate a clade C HIV vaccine, NYVAC-C undertaken by the EuroVacc Consortium

Pierre-Alexandre Bart[a], Ruth Goodall[b], Tristan Barber[c], Alexandre Harari[a], Ana Guimaraes-Walker[c], Mona Khonkarly[a], Neil C. Sheppard[d], Yolanda Bangala[a], Marie-Joelle Frachette[e], Ralf Wagner[f], Peter Liljeström[g], Jean-Pierre Kraehenbuhl[h], Marc Girard[i], Jaap Goudsmit[j], Mariano Esteban[k], Jonathan Heeney[l,m], Quentin Sattentau[d], Sheena McCormack[b,*], Abdel Babiker[b], Giuseppe Pantaleo[a], Jonathan Weber[c]

[a] *Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*
[b] *MRC Clinical Trials Unit, 222 Euston Road, London NW1 2DA, UK*
[c] *St. Mary's Hospital, London, UK*
[d] *University of Oxford, Oxford, UK*
[e] *Sanofi Pasteur, Lyon, France*
[f] *University of Regensburg, Regensburg, Germany*
[g] *Karolinska Institutet, Stockholm, Sweden*
[h] *Eurovacc Foundation, Lausanne, Switzerland*
[i] *Centre National de la Recherche Scientifique, Lyon, France*
[j] *Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands*
[k] *Centro Nacional de Biotecnologia, Madrid, Spain*
[l] *Biomedical Primate Research Centre, Rijswijk, The Netherlands*
[m] *University of Cambridge, Cambridge, UK*

Received 14 March 2008; accepted 19 March 2008

KEYWORDS
HIV preventative vaccine;
NYVAC-C;
Phase I

Abstract NYVAC-C (vP2010), a recombinant vector expressing HIV subtype C *gag*, *pol*, *env* and *nef* antigens, was tested in a phase I study in healthy, HIV negative volunteers in London and Lausanne. Twenty-four participants were randomised to receive NYVAC-C (20) or matching placebo (4) at weeks 0 and 4, and assessed for safety and immunogenicity over 48 weeks. There were no serious adverse events, and no clinical or laboratory abnormalities or other events that led to withdrawal, interruption or dose reduction of the NYVAC-C/placebo. Half of the 10

* Corresponding author. Tel.: +44 20 7670 4708; fax: +44 20 7670 4685.
E-mail address: s.mccormack@ctu.mrc.ac.uk (S. McCormack).

0264-410X/$ – see front matter
doi:10.1016/j.vaccine.2008.03.083

assessed responded in the ELISpot assay under stringent criteria, which informed the sample size for a DNA—NYVAC-C comparison to NYVAC-C alone.

1. Introduction

The HIV pandemic continues, with an estimated 4.1 million new infections in 2006, the vast majority of which is acquired through heterosexual intercourse [1]. Even in populations where treatment is widely available, such as homosexual men in developed countries, the epidemic persists with estimated incidence as high as 3% per year [2]. A safe, effective HIV vaccine must remain the ultimate goal.

This paper describes the first trial in the EuroVac [3] scientific programme, an initiative funded by the European Commission through a series of three awards, bringing together 21 academic and pharmaceutical partners in an ambitious effort to bring new products into Phase I clinical trials. The first candidate that emerged from GMP production was one of the pox vectors.

The NYVAC (vP866) strain derived from a plaque-purified isolate (VC-2) of the Copenhagen strain after deletion of 18 genes encoding proteins involved in host range and virulence, has been demonstrated to be highly attenuated, and well tolerated in clinical trials [4,5]. The HIV genes expressed in the recombinant vector within this study are derived from the Chinese R5 CRF_07 strain (97CN54) [6] which has been shown to be representative of the most prevalent virus strains circulating in China and India. The EV01 trial described here was the first time this novel vaccinia construct expressing HIV subtype C *gag*, *pol*, *env* and *nef* antigens was studied in humans.

2. Materials and methods

2.1. Vaccine products

The vaccine, NYVAC-C (vP2010), was manufactured by Sanofi Pasteur (formerly Aventis Pasteur, Lyon, France) according to Good Manufacturing Practice and formulated as $>10^6$ $CCID_{50}$/mL NYVAC-C (vP2010) in 0.25 ml × buffer (10 mM Tris—HCl buffer; pH 7.5), 0.25 ml × virus stabiliser (lactoglutamate) and 0.5 ml freeze-drying medium (according to formula 65-1-3) (batch number used: NYVAC-C S3914, placebo S3915).

2.2. Study design

EV01 was a randomised, blinded Phase I trial conducted in two clinical centres: Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne and St. Mary's Hospital, Imperial College of Science, Technology and Medicine, London. Participants were randomised to receive two vaccinations of NYVAC-C ($n = 20$) or vehicle placebo ($n = 4$) on day 0 and day 28 by intramuscular injection into the non-dominant deltoid. Participants, clinical investigators and laboratory personnel were all blind to the allocation. The main purpose of the four placebo controls was to avoid bias in the laboratory staff conducting the immunological assays.

The primary safety endpoints were grade 3 or above events reported as

- Local (pain, cutaneous reactions including induration) or systemic (fever, chills, headache, nausea, vomiting, malaise, and myalgia) reactogenicity events solicited within 7 days of vaccination.
- Other unsolicited or laboratory adverse events reported at or before the 48-week visit (confirmed at examination or on repeat testing, respectively).
- Any event attributable to vaccine leading to discontinuation of the immunisation regimen reported at or before the 48-week visit.

Secondary safety endpoints were defined as grade 1 and 2 solicited adverse events within 7 days of a vaccination, and all grade 1 and 2 adverse events within 28 days of a vaccination.

The primary immunogenicity endpoint was $CD8/CD4^+$ T cell responses at week 6/8, and at any point following immunisation assessed using the ELISpot technique defined as positive according to internationally agreed criteria [7]. The secondary immunogenicity endpoint was antibody responses defined by the binding titration to the gp140 of CN54 strain.

2.3. Study population

Healthy volunteers were recruited through advertising in the hospital and university in Lausanne and hospitals, colleges, newspapers, magazines and on the radio in London, and given a telephone number to contact. They were provided with further information about the study, and asked to complete a short interview (by telephone or in person) to assess their suitability. They were given or sent an information sheet in an appropriate language about the trial.

Volunteers had to be 18—55 years old, available for 52 weeks from screening; at low risk of HIV and willing to remain so for the duration; willing to undergo an HIV test, genital infection screen, and pregnancy test (if appropriate); and using an effective method of contraception with their partner if heterosexually active.

Volunteers were not allowed to participate if they were pregnant or lactating; had a clinically relevant abnormality on history or examination including history of grand-mal epilepsy, severe eczema, egg allergy, immunodeficiency or use of immunosuppressives in preceding 3 months; had a history of severe local or general reaction to vaccination; were HIV-1/2 positive or indeterminate on screening or positive for hepatitis B surface antigen, hepatitis C antibody or serology indicating active syphilis requiring treatment; had grade 1 or above routine laboratory parameters; or were deemed unlikely to comply with the protocol. The use of blood products or immunoglobulins within 4 months, live attenuated vaccine within 60 days or other vaccine within 14 days were not permitted. Participants were also ineligible if they had participated in another trial of a

medicinal product completed less than 30 days prior to enrolment.

2.4. Safety evaluations

Local and systemic reactogenicity data were collected systematically with specific questions prior to immunisation, 10 min (local only) and 1 h after immunisation, at least once within the first 3 days following immunisation and at 1 week after immunisation. Vital signs (temperature, blood pressure, pulse and respiratory rate) were measured pre-immunisation, and at 10 and 60 min post-immunisation.

Participants were given a diary card, with instructions and a full verbal explanation, describing the same solicited events, which were to be filled in on the evening following immunisation and daily for 7 days, or longer if events were ongoing. This was to assist in recall, and the nurses/physicians went through this with them at the next scheduled visit. Participants were advised to return to clinic if vesicles appeared at any site at any time following immunisation.

Data on other clinical events and laboratory events were collected with an open question at each visit and through haematology and chemical pathology performed at screening, 3 days and 4 weeks after each vaccination and at weeks 24 and 48.

2.5. Immunogenicity evaluations

2.5.1. Cellular assays

The immunogenicity of NYVAC-C was assessed by the quantification of T-cell responses evaluated using the IFN-γ ELISpot assay with a panel of eight pools of 49—61 peptides (15-mers overlapping by 11 amino acids), namely $ENV_{1/2}$, $GAG_{1/2}$, GAG/POL, NEF and GPN_{1-2}. The peptides encompassed the *gag-pol-nef* and *env* proteins of HIV-1 based on the sequence of the immunogens expressed by the NYVAC-C that were derived from the CN54 clade C isolate. The assays were performed on cryo-preserved peripheral blood mononuclear cells (PBMC) at weeks 0, 4, 6, 8, 24 and 48. The following criteria were used to define the technical validity of the assay and positive responses:

(a) The assay background (unstimulated PBMC) had to be below 50 spots/10^6 PBMC and the positive control responses to staphylococcal enterotoxin B above 500 spots/10^6 PBMC.
(b) Positive responses had to be at least fourfold above background and equal to or higher than 55 spots/10^6 PBMC.

2.5.2. Flow cytometry analysis

The distribution of T-cell responses in CD4 and CD8 T-cell populations was assessed using polychromatic flow cytometry in responders with more than 100 SFU/10^6 blood mononuclear cells measured in the IFN-γ ELISpot assay. Cryo-preserved blood mononuclear cells ($1-2 \times 10^6$) were stimulated overnight in 1 ml of complete media containing golgiplug (1 μl/ml, BD Biosciences (BD)) and αCD28 antibodies (Ab) (0.5 μg/ml, BD) as described [8]. For stimulation of blood mononuclear cells, peptide pools were used at 1 μg/ml for each peptide. SEB stimulation (200 ng/ml) served as positive control. The following Abs were used in various combinations: CD4-FITC, CD8-PerCP-Cy5.5, IFN-γ-APC and IL-2-PE. All Abs were from BD. At the end of the stimulation period, cells were washed, permeabilized (FACS Perm 2 solution; BD) and stained as previously described [8]. Data were acquired on a FACScalibur and analysed using CellQuest™. The number of lymphocyte-gated events ranged between 10^5 and 10^6 in the flow cytometry experiments shown. With regard to the criteria of positivity of ICS, the background in the unstimulated controls never exceeded 0.01—0.02%. An ICS to be considered positive had to have a background less than 20% of the total percentage of cytokine-positive cells in the stimulated samples.

Specimens were collected according to the schedule and the assessments were conducted in a single laboratory (CHUV) by five personnel using standardised equipment and operating procedures. Samples from all volunteers were tested at all time points using a pool of peptides derived from cytomegalovirus, Epstein Barr virus and influenza (CEF).

2.5.3. Humoral assays

Antibody responses to NYVAC-C were analysed using ELISA, measuring titres of IgG to gp140 CN54 at weeks 0, 4, 8 and 24. All participant sera were screened at a 1/100 dilution, and subsequently at 1/20. Participants with any positive result in this screen were further investigated by performing full binding titration: At each time point, four separate ELISA experiments (repetitions) on the same participant sample were performed. For each repetition, serum was titrated from 1/20 to 1/240 (seven dilutions). All these analyses were performed at the Sir William Dunn School of Pathology, Oxford University, by a single person using standardised equipment and operating procedures. Normal human serum and serum from HIV-1 seropositive patients were used as controls in all screening ELISAs.

2.6. Statistical methods

All clinical event and routine laboratory data were included in the safety analysis.

The following assumptions were made: when the same event was reported on the clinic form and diary card and the grades reported were different, the maximum grade was reported; any event existing prior to immunisation that persisted after the vaccination was only considered to be an adverse event if the severity/grade of the event increased post-immunisation; within the 4 weeks following each vaccination, a recurrence of any event was defined as an adverse event that occurred after the first event, with a period of no solicited events of the same type in between. The duration of an event was defined as the number of days from the start of the event to the first event-free day.

The T-cell responses were analysed as present or absent and reported as the number (%) of participants responding to each peptide pool. For the IgG binding titration to CN54-gp140 the analysis was performed as follows: at each dilution and background (no serum) the optical density was assessed three times and then averaged. For each repetition

and dilution, baseline optical density was subtracted from the optical density at weeks 4, 8, and 24. For each week and repetition, a regression line was then fitted, from which the end-point titre was derived (the lowest concentration where there is an optical density above the mean background plus two standard deviations). If the estimated end-point titre was within the dilution range, the repetition was classified as positive. For each participant and week, results were classified as 'negative' if there were zero or one positive repetitions, as 'borderline positive' if there were two positive repetitions, and as 'positive' if three or four positive repetitions.

2.7. Trial management

The study documents were reviewed and approved by St. Mary's LREC (Local Research Ethics Committee) and GTAC (Gene Therapy Advisory Committee) for the UK, and Commission d'éthique de la recherche clinique (Université de Lausanne) for Switzerland. Regulatory approval was also obtained from the competent authorities in both countries, MHRA (Medicines and Healthcare Products Regulatory Agency) for the UK and Swissmedic for Switzerland.

The trial was overseen by a Trial Coordinating Committee (TCC) which included the Principal Investigators from each centre, a representative from Sanofi Pasteur, and two independent members including the Chair. A Data and Safety Monitoring Committee (DSMC) was also appointed to review the design and protocol, but was only needed to meet during the trial if three or more participants experienced an unexplained, unexpected grade 3 or 4 clinical or laboratory event.

The data management, site monitoring, analysis and report writing were coordinated by the UK Medical Research Council Clinical Trials Unit (MRC CTU) in collaboration with the Clinical Centres, Sanofi Pasteur and the EuroVac scientific programme coordination.

3. Results

3.1. Study population

Of 36 volunteers screened 12 were not enrolled for reasons described in the study flow diagram (Fig. 1). Twenty-four volunteers were enrolled into the study between 4th August and 5th November 2003.

Four participants, median age 38 years old, received a placebo vaccination. Two (50%) were female and three (75%) were of white ethnicity. The baseline characteristics of the participants receiving NYVAC-C are described in Table 1, and were similar to those receiving placebo.

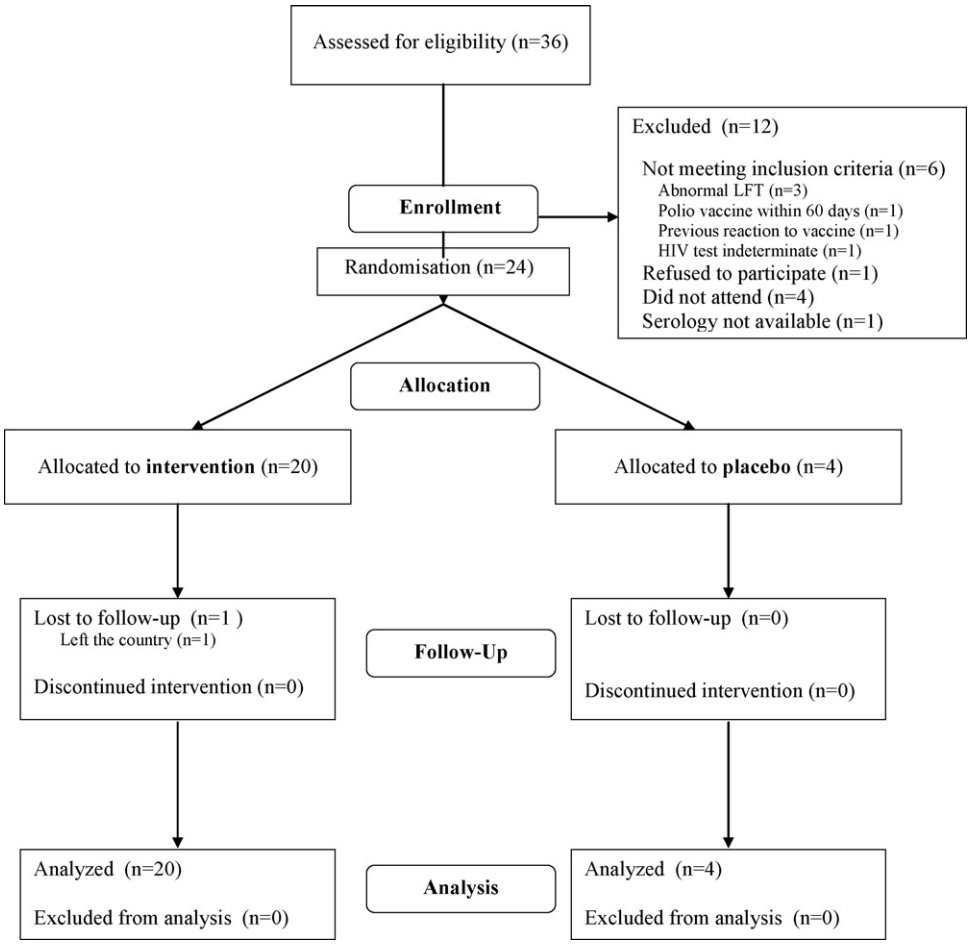

Fig. 1 Flow chart of study recruitment and follow-up.

Table 1 Baseline characteristics in NYVAC-C recipients

Characteristic		Centre		Total
		CHUV ($n = 10$)	St. Mary's ($n = 10$)	
Demographic and clinical data				
Sex	Female	5 (50%)	4 (40%)	9 (45%)
Age (years)[a]		28 (20—51)	36 (22—49)	28 (20—51)
Ethnicity	White	10 (100%)	8 (80%)	18 (80%)
Arm circumference (cm)[a]	Male	31 (29—33)	33 (27—37)	31 (27—37)
	Female	30 (28—30)	29 (27—31)	30 (27—31)
	Total	30 (28—33)	31 (27—37)	30 (27—37)
Height (cm)[a]	Male	178 (169—185)	179 (170—189)	178 (169—189)
	Female	168 (164—171)	171 (162—177)	168 (162—177)
	Total	171 (164—185)	176 (162—189)	173 (162—189)
Weight (kg)[a]	Male	68 (63—70)	80 (67—94)	70 (63—94)
	Female	64 (55—67)	65 (57—70)	64 (55—70)
	Total	66 (55—70)	73 (57—94)	68 (55—94)
Diastolic blood pressure (mmHg)[a]		73 (65—84)	73 (60—90)	73 (60—90)
Systolic blood pressure (mmHg)[a]		114 (100—135)	110 (106—130)	111 (100—135)
Routine laboratory parameters[b]				
Haemoglobin[a] (g/dl)		13.9 (11.0—15.3)	14.9 (12.6—16.5)	14.0 (11.0—16.5)
White cell count[a] (10^9 cells/l)		5.8 (3.5—10.2)	7.1 (6.4—10.5)	6.6 (3.5—10.5)
Neutrophils[a] (10^9 cells/l)		3.4 (1.6—6.1)	4.2 (3.6—6.7)	4.0 (1.6—6.7)
Lymphocytes[a] (10^9 cells/l)		1.7 (1.0—3.5)	2.1 (1.8—2.9)	2.0 (1.0—3.5)
Platelets[a] (10^9 cells/l)		291 (181—344)	244 (152—362)	282 (152—362)
ALT[a] (U/l)		18 (7—44)	21 (13—41)	19 (7—44)
Alkaline phosphatase[a] (U/l)		54 (31—69)	66 (52—104)	60 (31—104)
Bilirubin[a] (μmol/l)		10 (10—14)	10 (7—19)	10 (7—19)
Creatinine[a] (μmol/l)		98 (81—104)	93 (70—141)	97 (70—141)
Glucose[a] (μmol/l)		5.0 (3.9—6.4)	4.4 (3.7—6.0)	4.8 (3.7—6.4)

[a] Median (range).
[b] Blood taken at screening visit a median of 7 days (IQR 4—31 days) before trial entry.

The immunisation schedule was completed as planned by all participants. A total of 96 clinic visits were expected. One participant (NYVAC-C) missed visit 6, 3 days after the second immunisation but attended for the following visit 1-week after the second immunisation and a diary card was completed for the whole week, and one participant (NYVAC-C) failed to attend the week 48 appointment, and the data from this final visit are missing from the safety analyses.

3.2. Safety results

There were no serious or severe/extreme (grade 3/4) events, although two volunteers (both NYVAC-C) were admitted for elective surgery for pre-existing conditions during follow-up, one for lipo-suction and one to repair an inguinal hernia. Neither event met the protocol definition of a serious adverse event (based on ICH GCP criteria) [9].

Vital signs before and after immunisations were similar. There were no moderate (grade 2) local reactions following the first immunisation and only one after the second (induration recorded by a participant that received NYVAC-C on a diary card) (Fig. 2). The most common solicited local reaction was pain at the injection site. The proportion of participants receiving NYVAC-C with erythema increased significantly after the second immunisation ($p < 0.002$). Four volunteers (all NYVAC-C) reported eight moderate systemic events (one chills/rigors, two malaise, one myalgia, three headache, and one nausea), three following the first immunisation and one following the second. The most common solicited systemic reaction was malaise. The median duration of solicited events was 2 days (range 1—9 days). Five events lasted for 5 days or more and these were all in participants receiving NYVAC-C.

There were no grade 2 laboratory abnormalities. Eleven participants (eight NYVAC-C, three placebo) developed grade 1 abnormalities that were not present at screening in the following routine laboratory parameters: platelets (one NYVAC-C), ALT (three NYVAC-C), AST (one placebo), bilirubin (one NYVAC-C), creatinine (four NYVAC-C, one placebo) and glucose (one NYVAC-C, one placebo). None of these was considered clinically significant.

Five NYVAC-C recipients reported a non-solicited adverse event within 28 days of an immunisation, which was considered related to study product. All of these were mild (grade 1): oral ulceration on day 2 following immunisation lasting 7 days; pain at injection site on day 8 lasting 3 days; blood streaks when blowing nose on day 0 lasting 2 days, haematoma at injection site on day 2 lasting 11 days, and itchiness at injection site on day 7 lasting 5 days.

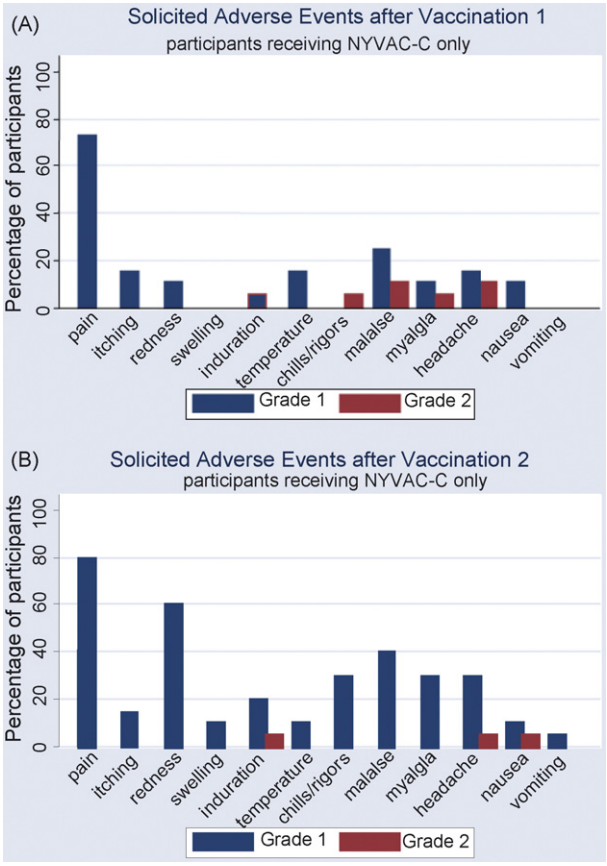

Fig. 2 Number of solicited adverse events (episode of maximum grade) within 7 days of (a) vaccination 1 and (b) vaccination 2.

All of the volunteers reported at least one non-solicited adverse event during total follow-up to week 48. Sixty-seven events in 23 volunteers were considered mild and 15 events in 12 volunteers moderate. Nine events in eight (7 NYVAC-C, 1 placebo) participants were considered possibly or probably related to study product, only one of which was moderate and this was fatigue occurring in a participant 10 weeks after the first NYVAC-C immunisation. This event was associated with mild weight loss (3 kg from a baseline of 65 kg), and both were considered possibly related to vaccine. Complete resolution occurred after 12 weeks.

3.3. Immunogenicity results

Due to a technical error with the equipment used to separate cells prior to freezing at St. Mary's Hospital Laboratory, there were no baseline and no primary endpoint samples available from participants that had been enrolled at St. Mary's. All 12 CHUV participants were included in the ELISpot analyses. One of the two participants receiving placebo had detectable ELISpot responses. These were to the GPN peptide pool and were seen at baseline and all subsequent weeks (4, 6, 8, 24 and 48). T-cell responses were detected in five out of 10 NYVAC-C recipients with available stored samples (50%, 95% CI: 19—81%) (Table 2 and Fig. 3). Responses to *env* represented the majority of the total responses (i.e. ENV1 50% and ENV2 10%) and were found in all responders in the range of 58—120 SFU/10^6 cells (Fig. 3). The *env*-specific T-cell responses were therefore further characterized, and successfully mapped in four volunteers to identify five distinct epitopes, two of which are new, and three have been previously described in HIV-infected subjects (NVWATHACVPTDPNP, KNCSFNISTSIRGKV, TDPNPQEVV).

Multicolor flow cytometry analysis was carried out on two 6-week samples with more than 100 SFU/10^6 blood mononuclear cells measured in the IFN-γ ELISpot assay. This analysis confirmed that both CD4 and CD8 T-cell responses were induced and that responses were polyfunctional, as indicated by the presence of both IFN-γ and IL-2 secreting T cells (Fig. 4).

Specimens were available from all trial participants up to and including week 24 for assessment of IgG antibody responses to CN54 gp140 antigen using ELISA. All participants were negative at 1/100 dilution. At 1/20 dilution 12 specimens (10 NYVAC-C, 2 placebo) gave signals above the background, meriting further investigation as described under Section 2. Three (15%, 95% CI: 3—38%) participants (all in the NYVAC-C group) had at least one positive antibody response at maximal end-point titre of 61, 116 and 50 at weeks 4, 8, and 24, respectively.

Only one of these three participants was assessed in the ELISpot assay, as the other two were St. Mary's participants. This participant was a responder in the ELISpot assay. Of the four remaining participants with a positive ELISpot, 1 had a borderline positive IgG response and three were negative.

4. Discussion

Previous trials of HIV immunogens using pox vectors have been disappointing in comparison to Adenovirus vector in terms of cellular responses. Although only 10 of the individuals exposed to NYVAC-HIVC had viable cells for the ELISpot assessment, 5 (50%) of these were responders under the most stringent criteria. This is encouraging and warrants further investigation, particularly in light of the favourable safety profile reported here.

The EuroVacc investigators were able to amend the protocol in time to collect ELISpot samples 2 weeks after the second NYVAC-C immunisation, after emergent data suggested the peak response may occur at this time point. This was confirmed as all five responders were detected at week 6, whereas only three would have been identified if week 8 had been the only time point. However, no ELISpot responses were durable, with only one individual eliciting a positive response at week 24, and none by week 48. As part of a non-protocol study IgM responses to Env were examined, and detected in a greater proportion of participants than IgG. Moreover, serum from the patient with the greatest Env-specific IgM titre demonstrated antibody-dependent complement-mediated inactivation of the autologous isolate in a PBMC-based assay, suggesting that the Env-specific IgM could have antiviral activity [10].

Two important lessons arose from this trial. Firstly, that it is not feasible to go from bench to completion of clinical trial within a 3-year scientific grant. The EuroVac I grant started in 2000, with the intention of delivering five immunogens using common inserts. The first immunogen, NYVAC-C, emerged in the third quarter of 2003, and the

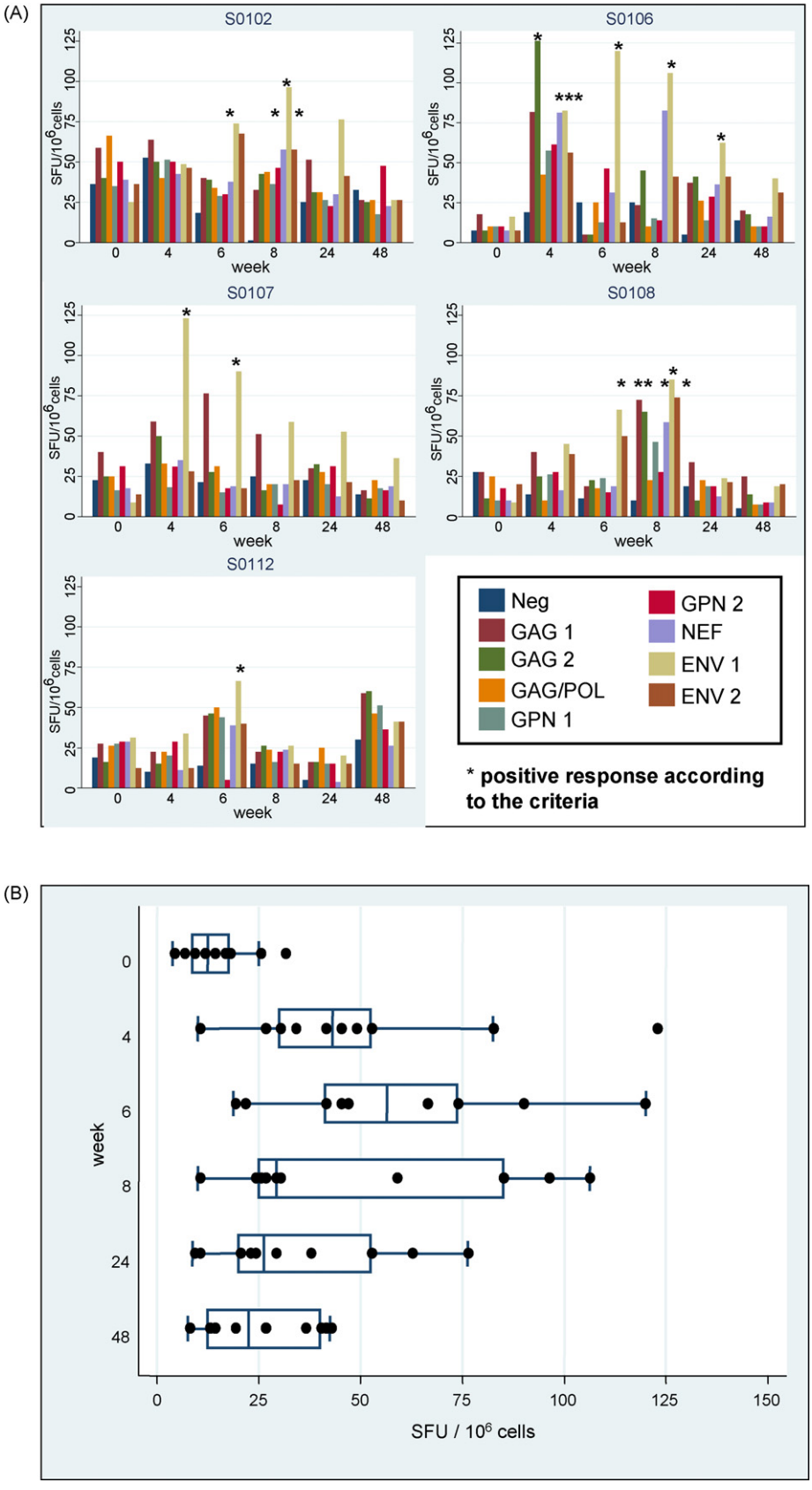

Fig. 3 Magnitude of the T-cell responses. (A) Individual patterns of the T-cell responses as measured by the frequencies of the IFN-γ secreting cells against different peptide pools encompassing the *env*, *gag*, *pol* and *nef* proteins in the ELISpot assay are shown for all responders. Each bar corresponds to the reactivity against a different peptide pool and positive responses are indicated by a star. (B) Distribution T-cell responses against ENV1 at different time points.

Table 2 ELISpot responses for participants receiving NYVAC-C, by participant and week

Participant	Week 0	Week 4	Week 6	Week 8	Week 24	Week 48
S0101	—	—	—	—	—	—
S0102	—	—	Env1	Env1 Env2 Nef	—	—
S0104	—	—	—	—	—	—
S0105	—	—	—	—	—	—
S0106	—	Env1 Gag1 Gag2 Nef	Env1	Env1	Env1	—
S0107	—	Env1	Env1	—	—	—
S0108	—	—	Env1	Env1 Env2 Gag1 Gag2 Nef	—	—
S0109	—	—	—	—	—	—
S0111	—	—	—	—	—	—
S0112	—	—	Env1	—	—	—

Placebo recipients: S0103, no response to any pool at any time; S0110, response to GPN pool at all time points, no response to any other pools at any time points.

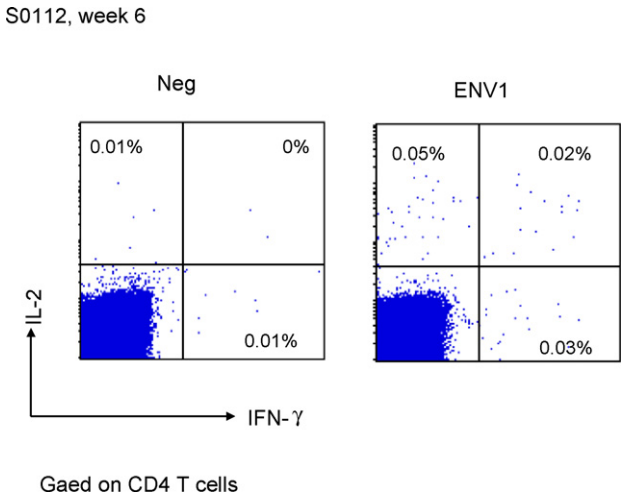

Fig. 4 Detection of IFN-γ but also IL-2 secreting ENV-specific CD4 T cells in a responder.

production was heavily underpinned by Sanofi Pasteur (then Aventis). The second lesson was that sharing standardised operating procedures is insufficient to guarantee quality control of biological assays. Although experienced laboratory technicians from the London site visited the central laboratory in Lausanne and observed the standardised operating procedures in practise, and laboratory staff from Lausanne observed procedures in London, the baseline specimens collected from London participants failed to yield any cells. This was due to a fault within the machine used to freeze cells prior to cryopreservation. By the time the error was detected all the participants had been enrolled and passed the primary endpoint (week 8). Corrective action was taken immediately and there were no further failures in this or the subsequent trial.

Acknowledgements

We are indebted to the altruistic commitment and enthusiasm of the volunteers. We also thank the Chair of the Trial Coordinating Committee (Geoffrey Schild) and the Data and Safety Monitoring Committee (Anne McLaren (Chair), Ferdinand Wit, Jürg Schifferli), and the investigators and staff at the participating institutions, including: CHUV: Romilda Gamboni, Veronique Descartes, Severine Burnet; St. Mary's: Miranda Cowen, Ken Legg, John Clarke; MRC CTU: Elizabeth Brodnicki, Mary Rauchenberger, Patrick Kelleher, Sarah Banbury.

Funding: Funds for the trial came from the European Commission through a scientific award (QLK2-CT-1999-01321) which was distributed to the partners, and coordinated initially by Prof. Mark Girard (CNRS-BioMerieux, Lyon, France) and Prof. Jaap Goudsmit (Academisch Ziekenhuis bij de Universiteit van Amsterdam, Netherlands) and later by Professor Peter Liljeström based at the Karolinska Institute, Stockholm, Sweden. In addition each institutional partner provided financial support through the time of core senior staff and access to clinical facilities and technical assistance.

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