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H1N1 viral proteome peptide microarray predicts individuals at risk for H1N1 infection and segregates infection versus Pandemrix® vaccination

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Summary

A high content peptide microarray containing the entire influenza A virus [A/California/08/2009(H1N1)] proteome and haemagglutinin proteins from 12 other influenza A subtypes, including the haemagglutinin from the [A/South Carolina/1/1918(H1N1)] strain, was used to gauge serum IgG epitope signatures before and after Pandemrix® vaccination or H1N1 infection in a Swedish cohort during the pandemic influenza season 2009. A very narrow pattern of pandemic flu-specific IgG epitope recognition was observed in the serum from individuals who later contracted H1N1 infection. Moreover, the pandemic influenza infection generated IgG reactivity to two adjacent epitopes of the neuraminidase protein. The differential serum IgG recognition was focused on haemagglutinin 1 (H1) and restricted to classical antigenic sites (Cb) in both the vaccinated controls and individuals with flu infections. We further identified a novel epitope VEPGDKITFEATGNL on the Ca antigenic site (251–265) of the pandemic flu haemagglutinin, which was exclusively recognized in serum from individuals with previous vaccinations and never in serum from individuals with H1N1 infection (confirmed by RNA PCR analysis from nasal swabs). This epitope was mapped to the receptor-binding domain of the influenza haemagglutinin and could serve as a correlate of immune protection in the context of pandemic flu. The study shows that unbiased epitope mapping using peptide microarray technology leads to the identification of biologically and clinically relevant target structures. Most significantly an H1N1 infection induced a different footprint of IgG epitope recognition patterns compared with the pandemic H1N1 vaccine.

Keywords: epitopes; haemagglutinin; immunoglobulin G; influenza; peptide microarray; vaccination

Introduction

The influenza pandemic 2009, caused by novel triple reassorted swine origin influenza A virus H1N1, was first identified in the USA.[1–4] The European Centre for Disease Control estimated that 1975 laboratory confirmed death cases were reported all over Europe in the first year.[5] The seasonal influenza vaccines, either adjuvanted or non-adjuvanted, exhibited no protective effect as measured by a haemagglutinin (HA) inhibition assay in young adults and children, although individuals born before 1950 showed protective serum IgG titres.[3,6,7] The lack of pre-existing neutralizing antibodies against the pandemic influenza virus increased the susceptibility in the general population.[8] Only 31% of B-cell epitopes were conserved in the pandemic (swine-origin influenza virus) strain compared with the seasonal variant flu strains; moreover, of the eight conserved epitopes, only a single

Abbreviations: GAL, GenePix array list file; HA, influenza haemagglutinin protein; ILI, influenza-like illness; SEREX, Serological analysis of recombinant tumor cDNA expression libraries

epitope was from HA.[9] The HA ectodomain is a homotrimeric complex with four distinct antigenic sites with two polypeptide chains HA1 and HA2. Each homotrimer is comprised of a large globular head that binds to glycan receptors and a distal stem region.[10,11] Haemagglutinin is the target of neutralizing antibodies in the context of pandemic influenza 2009[12] and often described as the immunogenicity-defining surface protein of influenza viruses.[11,13,14] Therefore, we mapped in this study the serum IgG epitope recognition profiles after a natural pandemic flu infection and Pandemrix® vaccination using a high content influenza peptide microarray.[15,16]

Serum antibody-based protein target identification (SEREX) has been used to successfully identify a number of biologically relevant targets in cancer[17–20] and to show differences in the epitope recognition pattern in the course of HIV infection versus (gp120) HIV vaccination.[21] We choose a similar approach to define the epitope recognition pattern in serum using synthetic linear peptide stretches to define (i) the immune recognition pattern to segregate individuals at high risk of infection with influenza virus, (ii) differences in IgG recognition patterns induced by vaccination versus infection, and (iii) shared epitope recognition patterns comparing different HA proteins from influenza A strains, including the HA from the pandemic strain influenza A virus [A/South Carolina/1/1918(H1N1)]. Up to now, immunological differences between time-points (e.g. before/after flu vaccination) or between different patient groups were assayed by ELISA.[22] Using this method, only increase in titres or reactivity to different targets could be measured. However, recognition of recombinant proteins in ELISA usually represents recognition of 20–40 different epitopes (linear and conformational). Peptide array screening is a feasible way to map differences in humoral immune responses, though an important limitation may be the exclusion of conformational epitopes, as described in recent reports.[23,24] The epitope-mapping analysis was possible in the current study using material from a prospective study,[25–27] in which 2000 individuals were followed before and after vaccination, or H1N1 infection.

Materials and methods

Influenza peptide microarrays

The peptide arrays were custom manufactured by JPT (Berlin, Germany) and the manufacturing process based on SPOT synthesis has been described elsewhere.[28] The Influenza A virus [A/California/08/2009(H1N1)] proteome and HA proteins from 12 other influenza A subtypes (Table 1) were used as a template and overlapping peptides were generated, i.e. as 15-mer peptides overlapping by five amino acid residues with a total of 256 positive controls in each subarray resulting in 2304 unique features in triplicate subarrays.[29]

Table 1. List of influenza targets used in the microarray including the whole proteome of the Influenza A virus [A/California/08/2009(H1N1)] in addition to previous vaccine and pandemic H1 proteins

	Influenza protein antigen	GenBank ID
1	Matrix protein 1 [Influenza A virus (A/California/08/2009(H1N1))]	ACP44177.1
2	Matrix protein 2 [Influenza A virus (A/California/08/2009(H1N1))]	ACP44178.1
3	Haemagglutinin [Influenza A virus (A/California/08/2009(H1N1))]	ACP52565.1
4	Neuraminidase [Influenza A virus (A/California/08/2009(H1N1))]	ACT36689.1
5	Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	ACQ63248.1
6	Polymerase PB1 [Influenza A virus (A/California/08/2009(H1N1))]	ACQ63247.1
7	Nucleocapsid protein [Influenza A virus (A/California/08/2009(H1N1))]	ACQ63246.1
8	Polymerase PB2 [Influenza A virus (A/California/08/2009(H1N1))]	ACQ63245.1
9	Nuclear export protein [Influenza A virus (A/California/08/2009(H1N1))]	ACP44180.1
10	Non-structural protein 1 [Influenza A virus (A/California/08/2009(H1N1))]	ACP44161.1
11	Haemagglutinin [Influenza A virus (A/Uruguay/716/2007 X-175(H3N2))]	ACD47234.1
12	Haemagglutinin [Influenza A virus (A/Solomon Islands/3/2006 (Egg passage)(H1N1))]	ABU99069.1
13	Haemagglutinin [Influenza A virus (A/Viet Nam/1203/2004(H5N1))]	ABW90135.1
14	Haemagglutinin [Influenza A virus (A/South Carolina/1/1918(H1N1))]	AAD17229.1
15	Haemagglutinin precursor [Influenza A virus (A/swine/Iowa/15/1930(H1N1))]	AAB52905.1
16	Haemagglutinin [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2))]	AAL87870.1
17	Haemagglutinin [Influenza A virus (strain A/swine/England/195852/92)] H1N1	AAC57167.1
18	Neuraminidase [Influenza A virus (A/Weiss/1943(H1N1))]	AAF77045.1
19	Haemagglutinin [Influenza A virus (A/Weiss/1943(H1N1))]	ABD79101.1
20	Haemagglutinin [Influenza A virus (A/Fort Monmouth/1/1947-mouse adapted(H1N1))]	AAC53844.1
21	Haemagglutinin [Influenza A virus (A/USSR/92/1977(H1N1))]	ABD60933.1
22	Haemagglutinin [Influenza A virus (A/New Caledonia/20/1999(H1N1))]	CAC86622.1

Study subjects

The pilot study consisted of 2000 individuals recruited in a prospective study to gauge immunological differences in pandemic flu-infected individuals versus individuals who received the Pandemrix® vaccine (split virion, inactivated, AS03 adjuvanted).[25–27] Individuals of the LG ILI (LifeGene Influenza-Like-Illness) study who experienced flu-like symptoms mailed a viral swab, followed by PCR-based detection of 22 viral pathogens. Most individuals with ILI symptoms presented during the 2009/2010 season with either rhinovirus, coronavirus or influenza virus infections (described in detail in ref. 27). We identified 19 individuals with a positive pandemic H1N1 RNA swab with pre-infection and post-infection serum samples. These were used to assay the detailed flu epitope recognition pattern. Serum samples from 19 (age- and sex-matched) individuals vaccinated with the adjuvanted vaccine against pandemic H1N1 influenza (Pandemrix®) were used as controls (see Fig. 1). The regional ethics committee in Stockholm (2009/1183-31) approved the study.

Peptide array–serum incubation

The serum was diluted 1 : 100 in 300 µl buffer (PBS 3% fetal calf serum 0·5% Tween 80) (Sigma Aldrich, St Louis, MO) and incubated on microarrays for 16 hr in a humid chamber at + 4°C, the slides were then washed with buffer twice and sterile distilled water thrice, followed by secondary incubation with 300 µl diluted (1 : 500) monoclonal Cy5®-labelled mouse anti-human IgG antibody [Cat no: 9042-15 lot: C4505-X595J (0·1 mg/ml) (Southern Biotech, Birmingham, AL)] for an hour at room temperature and then washed as before. The slides were spun dry in a slide centrifuge (DJB Labcare, Newport Pagnell, UK) and scanned at 635 nm using an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA). Image analysis was performed using the circular feature alignment of the GENEPIX Pro 7.0 software (Molecular Devices) and Genepix Array List (GAL) files (supplied by JPT, Berlin, Germany).

Analysis

Each slide was analysed by using GENEPIX 7.0 image analysis software (Molecular Devices) and the GAL file that contained the peptide information for each spot as supplied by the manufacturer (JPT, Berlin, Germany). The detectable spots that are not internally uniform are flagged as 'bad' (i.e. unreliable). This is efficiently estimated by the following criterion (described in detail elsewhere[16]).

$$([\text{F635 Mean}] > (3 \cdot 5 * [\text{F635 Median}])) \text{ and } ([\text{F635 Median}] > 40)$$

which identifies the spots with a mean foreground value different from the spots exhibiting median fluorescence intensity values. The spots were visually inspected after flagging and the results were saved separately for each sub-array. The positive control spots in each sub-array were selected after careful screening, as described previously.[29] Two paired strategies were adopted in this context: differential recognition of epitopes, i.e. peptides exhibiting statistically different recognition from immunoglobulin present in the test group compared with the control group, (strong recognition in one group versus weak or no recognition in the other group), as well as 'exclusive recognition' of peptide targets that are recognized above a defined threshold for detection in serum from a test group but never in a control group (or vice versa).[15,16,29,30] The epitope mapping on the crystal structure of the 2009 H1N1 influenza virus HA (PDB ID-3LZG)[31] and HA receptor-binding domain (PDB ID -3MLH)[32] were performed using the protein databank Jmol, an open-source Java viewer for chemical structures using a three-dimensional application (http://www.jmol.org).[33]

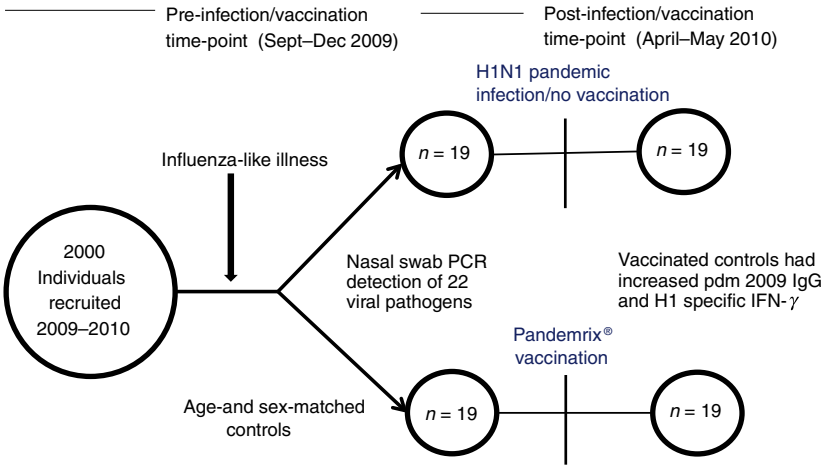

Figure 1. Overview of the study cohort. Subject recruitment from a larger cohort of 2000 individuals, 19 individuals showed a positive nasal swab PCR for pandemic flu, these were matched for age and sex in the control group who were Pandemrix® vaccinated.

Monospecific antibody production and H1N1 neutralization assay

A polyclonal monospecific rabbit antibody specific to VEPGDKITFEATGNL was prepared by Genscript (Piscataway, NJ) and the specificity was verified by an indirect ELISA (data not shown). The peptide-specific polyclonal reagents were prepared in three different rabbits and affinity-purified using the immobilized VEPGDKITFEATGNL peptide attached to a linker. The affinity-purified reagent did not show cross-reactivity to other, non-relevant peptide targets. The three antibody reagents were individually used in neutralization experiments.

The molecular epidemiology laboratory, at the University of Siena, performed the virus neutralization assay using the cytopathic effect as the readout against the influenza virus H1N1 A/California/7/2009 as previously described.[34] The affinity-purified monospecific antibody (heat-inactivated for 30 min at 56°C) was serial twofold diluted starting from 10^{-1} up to 10^{-10} dilutions and incubated at 37°C for 1 hr with 100 $TCID_{50}$/50 μl of influenza live virus (in equal volume/well) H1N1 A/California/7/2009. The incubation of monospecific antibody and virus allows the neutralization of virus by specific antibodies. The mixture was then added to 90% confluent monolayered MDCK (Madin Darbin Canine Kidney) susceptible cells in 96-well flat-bottomed microtitre plates. These plates were incubated at 37°C in 0·5% CO_2 for 5 days, to allow infection of the cell substrate in case live virus was not neutralized by serum antibodies. The cytopathic effect was monitored every day and at the end of the incubation period, each well of the 96-well microtitre plates was checked under an optical microscope for the presence of cytopathic effect in the cell lawn and compared with the negative and positive controls. The virus neutralization titre was defined as the antibody dilution by means of which 50% of the wells were protected against a virus-induced cytopathic effect. The Spearman–Kärber formula was used to calculate the virus neutralization titre of each sample.

Statistical analysis

The data were pre-processed (quality control, background subtraction, outliers and false positives detection and removal) and normalized as previously described.[28,29] Differential recognition was estimated by using Significance Analysis for Microarrays,[35] a non-parametric penalized *t*-test for microarrays. Exclusive recognition was performed by fixing a threshold for detection (corresponding to the average response in the negative controls + 2 × standard deviation) and by counting the number of detection hits among cases for the peptides that have a level of recognition always below this value in all the controls.[16] The number of hits is then reported together with the average recognition. All the procedures were performed by using in-house-made R scripts, and packages from the project BIOCONDUCTOR(www.bioconductor.org).[36]

Results

The differential epitope–serum IgG recognition patterns segregate into clusters based on pandemic influenza infection and Pandemrix® vaccination

Serum from each individual was tested for IgG recognition using the influenza peptide microarray platform. Serum IgG epitope reactivity from 19 individuals before and after H1N1 infection was compared with serum reactivity (sex- and age-matched) in individuals before and after Pandemrix® vaccination from the same prospective study cohort. Peptides that were recognized differentially ($P < 0{\cdot}001$) in both groups, flu infection versus vaccination (significance analysis of microarrays method[35]), were identified and represented as fold change (Table 2 and Fig. 2). Data are presented as the number of times a particular epitope in the group that experienced H1N1 infection is recognized compared with the vaccination group (below onefold change indicates a higher response in the vaccination group).

The epitope–serum IgG recognition patterns segregate into clusters based on the group, i.e. individuals who later contracted a flu infection versus the group who received vaccination (see Fig. 2). The prospective study design allowed the characterization of serum reactivity before vaccination as well as before pandemic H1N1 infection. We identified a cluster of 262 epitopes that were differentially recognized pre-vaccination/infection and a cluster of 250 epitopes differentially recognized post-vaccination/infection (represented in heat maps Fig. 2).

Pre-existing pandemic influenza-specific IgG before the flu season in individuals with previous vaccinations

Before the flu season onset, 13 peptide epitopes were differentially recognized in the group who later experienced the H1N1 infection based on their strong serum recognition pattern (as defined by fluorescence intensity). Only two of these 13 epitopes from the 2009 pandemic flu strain were recognized, i.e. polymerase PA (GRDRIMAWTVVNSIC) and neuraminidase (NFSIKQDIVGINEWS). This was in contrast to the serum reactivity pattern from individuals who chose to be vaccinated, in whom a total of 17 epitopes were differentially recognized, 10 of the 17 epitopes from the pandemic strain (six from polymerase PA, two from matrix1,

Table 2. Top differentially recognized epitopes by the serum IgG of individuals (n = 19) who later experienced pandemic flu infection compared to epitopes recognized by the serum IgG of individuals (n = 19) before Pandemrix® vaccination

Influenza protein	Position	Epitope	Fold change
Haemagglutinin [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2))]	221–235	SRYSKKFKPEIAARP	2·5086
Haemagglutinin [Influenza A virus (strain A/swine/England/195852/92)]	196–210	VHHPPTNNDQQSLYQ	2·1607
Haemagglutinin [Influenza A virus (A/Viet Nam/1203/2004(H5N1))]	371–385	EQGSGYAADKESTQK	2·0748
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	81–95	GRDRIMAWTVVNSIC	1·8249
Haemagglutinin [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2))]	136–150	KTSSWPNHDTNRGVT	1·8051
Haemagglutinin [Influenza A virus (A/Solomon Islands/3/2006 (Egg passage)(H1N1))]	91–105	ESWSYIVEKPNPENG	1·7574
Haemagglutinin [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2))]	276–290	RNSGSGIIISDTSVH	1·7171
Neuraminidase [Influenza A virus (A/California/08/2009(H1N1))]	386–400	NFSIKQDIVGINEWS	1·6996
Haemagglutinin [Influenza A virus (A/USSR/92/1977(H1N1))]	91–105	KSWSYIAETPNSENG	1·6751
Haemagglutinin [Influenza A virus (A/Solomon Islands/3/2006 (Egg passage)(H1N1))]	81–95	NPECELLISRESWSY	1·6252
Haemagglutinin [Influenza A virus (A/Uruguay/716/2007 X-175(H3N2))]	191–205	DKLYIWGVHHPGTDN	1·5817
Haemagglutinin [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2))]	91–105	SSWSYIVETSSSDNG	1·5811
Haemagglutinin [Influenza A virus (strain A/swine/England/195852/92)]	136–150	KATSWPNHETTKGAT	1·5536
Polymerase PB1 [Influenza A virus (A/California/08/2009(H1N1))]	21–35	TFPYTGDPPYSHGTG	0·6926
Matrix protein 1 [Influenza A virus (A/California/08/2009(H1N1))]	61–75	GFVFTLTVPSERGLQ	0·6106
Matrix protein 1 [Influenza A virus (A/California/08/2009(H1N1))]	106–120	EITFHGAKEVSLSYS	0·5994
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	601–615	SVKEKDMTKEFFENK	0·5862
Haemagglutinin [Influenza A virus (A/Weiss/1943(H1N1))]	001–15	MKARLLVLLCALAAT	0·5804
Haemagglutinin [Influenza A virus (A/South Carolina/1/1918(H1N1))]	76–90	GWLLGNPECDLLLTA	0·5623
Haemagglutinin precursor [Influenza A virus (A/swine/Iowa/15/1930(H1N1))]	51–65	QLGKCNIAGWILGNP	0·5549
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	631–645	GSIGKVCRTLLAKSV	0·4808
Haemagglutinin [Influenza A virus (A/California/08/2009(H1N1))]	251–265	VEPGDKITFEATGNL	0·4787
Haemagglutinin precursor [Influenza A virus (A/swine/Iowa/15/1930(H1N1))]	006–20	GYHANNSTDTVDTVL	0·4658
Haemagglutinin [Influenza A virus (A/Fort Monmouth/1/1947-mouse adapted(H1N1))]	76–90	GWILGNPECESLLSK	0·4449
Haemagglutinin [Influenza A virus (A/Viet Nam/1203/2004(H5N1))]	506–520	EEARLKREEISGVKL	0·4331
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	676–690	LEPGTFDLGGLYEAI	0·4202
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	611–625	FFENKSETWPIGESP	0·4165
Haemagglutinin [Influenza A virus (A/Viet Nam/1203/2004(H5N1))]	76–90	WLLGNPMCDEFINVP	0·3758
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	651–665	ASPQLEGFSAESRKL	0·3728
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	671–685	ALRDNLEPGTFDLGG	0·3703

The values are presented as fold change. Higher fold change (> 1·5) indicates stronger recognition in individuals who got the pandemic flu and lower fold change (< 0·6) indicates stronger recognition in individuals who later got the flu vaccination. (adj. P value < 0·001 for all the epitopes).

one from polymerase PB1 and one epitope from HA VEPGDKITFEATGNL) (exhaustive list in Table 2).

Pandemic influenza infection generates new neuraminidase-specific IgG reactivity

After the flu season, 14 epitopes segregated best the vaccinated versus flu-infected individuals (see exhaustive list in Table 3): four peptide epitopes were derived from the pandemic flu strain and only a single epitope GRDRIMAWTVVNSIC from polymerase PA [Influenza A virus (A/California/08/2009(H1N1))], this epitope was recognized equally before and after infection. Novel flu antigen epitope reactivity was generated after the flu season leading to the recognition of the epitopes QGALLNDKHSNGTIK, NDKHSNGTIKDRSPY from neuraminidase [Influenza A virus (A/California/08/2009 (H1N1))], and LAKGEKANVLIGQGD from polymerase PB2 [Influenza A virus (A/California/08/2009(H1N1))]. In the vaccinated group, 16 epitopes were strongly recognized, eight were associated with the pandemic flu 2009 strain (one from matrix 1, six from polymerase PA and only one from HA, i.e. VEPGDKITFEATGNL). New reactivity was generated in the vaccinated group to LQSLQQIESMIEAES from polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]. Serum in the vaccinated individuals showed also strong recognition of the pandemic flu internal proteins matrix 1 (M1) and polymerase PA.

Differential serum IgG recognition is focused on HA (H1) and is restricted to classical antigenic sites

At both pre- and post-pandemic infection/vaccination, the IgG epitope focus targeted HA and defined H1 antigenic sites (reviewed in detail in refs 10,11). The peptide epitope SRYSKKFKPEIAARP from the HA [Influenza A

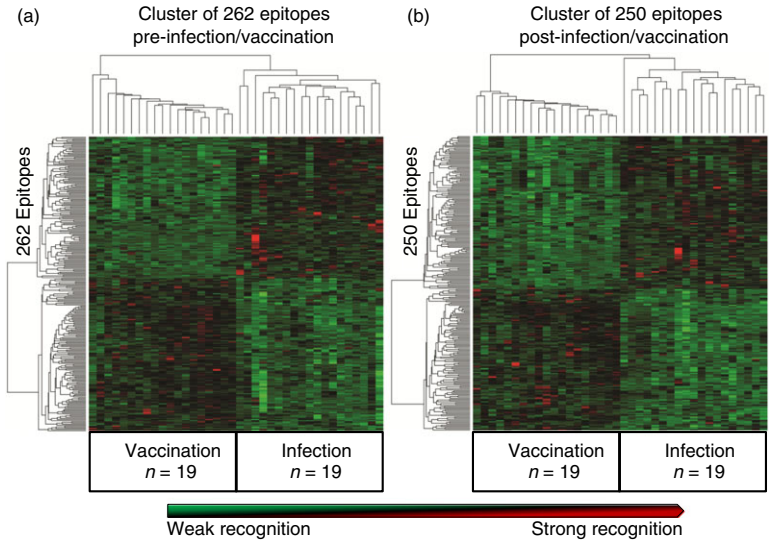

Figure 2. Peptide array recognition patterns. The differential epitope–serum IgG recognition patterns segregate into clusters based on the clinical history, i.e. H1N1 infection and the vaccination group. Peptide epitopes show a statistically different IgG serum recognition in the vaccinated (control) group compared with group with H1N1 infection. Strong recognition (in red) in one group versus weak (in green) or absent recognition in the other group). (a) Clusters of 262 epitopes are recognized differentially before flu infection (n = 19) and vaccination (n = 19). (b) Clusters of 250 epitopes are recognized differentially after pandemic flu infection or vaccination. The arrow indicates the strength of recognition in red, strong recognition; and green, weak recognition.

virus (A/Swine/Indiana/P12439/00 (H1N2))] was strongly recognized in serum from individuals both before and after flu infection (2·50-fold and 2·60-fold change, respectively); this epitope belongs to the Ca antigenic site on the H1 and is highly homologous to the SRYSKKFKPEIAIRP epitope from HA [Influenza A virus (A/California/08/2009(H1N1))] on the HA receptor binding domain. Sera from individuals who experienced pandemic flu infection showed IgG reactivity to the H1 antigenic site Cb (91–105 amino acids) from different H1 strains, excluding the pandemic strain (see Table S1 and Table S2). This contrasted with serum from vaccinated individuals, which exhibited serum IgG also to Cb but to the epitope 76–90 amino acids from the H1 HA including GWLLGNPECDLLLTA from Influenza A virus [A/South Carolina/1/1918(H1N1)] (Spanish flu) (list in the Supporting information, Tables S1 and S2).

A novel epitope on the antigenic site of the pandemic flu HA is exclusively recognized in serum from vaccinated individuals

Exclusive recognition analysis was performed, i.e. whether epitopes are recognized strongly and exclusively in one group (flu infection) and never or always below a set cut-off in another group (flu vaccination) or vice versa (Table 4). We identified an epitope VEPGDKITFEATGNL from the pandemic flu HA that was exclusively recognized in serum from vaccinated individuals before the flu season (16/19 individuals). This was also found to be true for the post-flu season period (serum from 17/19 individuals). Consequently, we further mapped the epitope VEPGDKITFEATGNL (251–265 amino acids) from HA [Influenza A virus (A/California/08/2009(H1N1))] using the PDB entry 3LZG and 3MLH of the crystal structure of the 2009 H1N1 influenza virus HA receptor-binding domain[32] (Fig. 3).

Antibodies directed against VEPGDKITFEATGNL epitope do not neutralize the pandemic influenza *in-vitro*

As the VEPGDKITFEATGNL epitope from the pandemic H1 (251–265 amino acids) was exclusively recognized in serum from control individuals, both before and after vaccination, we tested whether a polyclonal (rabbit), peptide-affinity purified mono-specific antibody could neutralize the virus *in vitro*. Each of the mono-specific affinity-purified antibody preparations (n = 3) showed an H1 neutralizing titre of < 10, except for the hyper-immune sheep serum tested in the assay as a positive control (data not shown), demonstrating that the epitope-specific antibody recognizes, but does not neutralize, A/California/7/2009 flu live virus, with the tests applied in the current report.

Discussion

The aim of this study was to characterize the serum–IgG epitope recognition profiles in the course of a natural

Table 3. Top differentially recognized epitopes by the serum IgG of individuals (n = 19) after pandemic flu infection compared with epitopes recognized by the serum IgG of individuals (n = 19) after Pandemrix® vaccination (≥ 6 months)

Influenza protein	Position	Epitope	Fold change
Haemagglutinin [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2))]	221–235	SRYSKKFKPEIAARP	2·6082
Haemagglutinin [Influenza A virus (A/Uruguay/716/2007 X-175(H3N2))]	496–510	SIRNGTYDHDVYRDE	2·484
Neuraminidase [Influenza A virus (A/California/08/2009(H1N1))]	136–150	QGALLNDKHSNGTIK	1·9467
Haemagglutinin [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2))]	276–290	RNSGSGIIISDTSVH	1·8472
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	81–95	GRDRIMAWTVVNSIC	1·774
Neuraminidase [Influenza A virus (A/California/08/2009(H1N1))]	141–155	NDKHSNGTIKDRSPY	1·7618
Haemagglutinin [Influenza A virus (A/USSR/92/1977(H1N1))]	91–105	KSWSYIAETPNSENG	1·7297
Polymerase PB2 [Influenza A virus (A/California/08/2009(H1N1))]	716–730	LAKGEKANVLIGQGD	1·7199
Haemagglutinin [Influenza A virus (A/Solomon Islands/3/2006 (Egg passage)(H1N1))]	146–160	TGVSASCSHNGESSF	1·7175
Haemagglutinin [Influenza A virus (A/Solomon Islands/3/2006 (Egg passage)(H1N1))]	91–105	ESWSYIVEKPNPENG	1·682
Haemagglutinin [Influenza A virus (A/Solomon Islands/3/2006 (Egg passage)(H1N1))]	81–95	NPECELLISRESWSY	1·6314
Haemagglutinin [Influenza A virus (strain A/swine/England/195852/92)]	136–150	KATSWPNHETTKGAT	1·6203
Haemagglutinin [Influenza A virus (A/Uruguay/716/2007 X-175(H3N2))]	96–110	QNKKWDLFVERSKAY	1·6177
Haemagglutinin [Influenza A virus (A/Uruguay/716/2007 X-175(H3N2))]	41–55	IVKTITNDQIEVTNA	1·5078
Haemagglutinin [Influenza A virus (A/Viet Nam/1203/2004(H5N1))]	541–555	LAIMVAGLSLWMCSN	0·6801
Matrix protein 1 [Influenza A virus (A/California/08/2009(H1N1))]	61–75	GFVFTLTVPSERGLQ	0·6667
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	586–600	LQSLQQIESMIEAES	0·5818
Haemagglutinin [Influenza A virus (A/California/08/2009(H1N1))]	251–265	VEPGDKITFEATGNL	0·5575
Haemagglutinin [Influenza A virus (A/South Carolina/1/1918(H1N1))]	76–90	GWLLGNPECDLLLTA	0·5523
Haemagglutinin [Influenza A virus (A/Viet Nam/1203/2004(H5N1))]	71–85	CSVAGWLLGNPMCDE	0·4881
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	631–645	GSIGKVCRTLLAKSV	0·4658
Haemagglutinin [Influenza A virus (A/Viet Nam/1203/2004(H5N1))]	506–520	EEARLKREEISGVKL	0·4647
Neuraminidase [Influenza A virus (A/Weiss/1943(H1N1))]	451–465	DTVDWSWPDGAELPF	0·4502
Haemagglutinin [Influenza A virus (strain A/swine/England/195852/92)]	421–435	IENLNKKVDDGFLDV	0·3979
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	651–665	ASPQLEGFSAESRKL	0·3918
Haemagglutinin [Influenza A virus (A/Fort Monmouth/1/1947-mouse adapted(H1N1))]	76–90	GWILGNPECESLLSK	0·3912
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	676–690	LEPGTFDLGGLYEAI	0·3911
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	671–685	ALRDNLEPGTFDLGG	0·3711
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	611–625	FFENKSETWPIGESP	0·3679
Haemagglutinin [Influenza A virus (A/Viet Nam/1203/2004(H5N1))]	76–90	WLLGNPMCDEFINVP	0·3172

The values are presented as fold change. Higher fold change (> 1·5) indicates stronger recognition in individuals who had the pandemic flu and lower fold change (< 0·6) indicates stronger recognition in flu-vaccinated individuals. (adj. P value < 0·001 for all the epitopes.

pandemic influenza infection and Pandemrix® vaccination using a high-content influenza peptide microarray. One salient finding is the pre-existing serum IgG to pandemic HA in vaccinated individuals before the onset of the flu season, probably due to past exposures and previous vaccinations (the interviews of the study participants showed that individuals who chose to be vaccinated did so previously, before 2009/2010 and vice versa.[27]) Not only pre-existing serum IgG, resulting from previous flu vaccinations, but also pre-existing protective antibodies from past exposures to H1 strains[37–40] may contribute to this humoral response. A broader pandemic flu epitope recognition pattern is reflected in 10 shared pandemic influenza epitopes in serum from individuals before the flu season onset, as well as in seven epitopes from pandemic flu internal proteins in serum from individuals who chose to be vaccinated. The biological role of these antibodies, directed to internal proteins, is not clear. Previous reports suggested that non-neutralizing antibodies may decrease morbidity and lead to increased viral clearance through binding to Fc receptors and subsequently to CD8 cells.[41–45] Conserved influenza internal proteins may therefore help to elicit memory B cells that are long-lived[46,47] and contribute to heterosubtypic immunity during antigenic drifts.

Cross-protection can be induced by antibodies against the generic N1 that are reactive against the pandemic N1, as described previously for N2 subtypes.[48,49] Moreover, in the absence of neutralizing HA antibodies, neuraminidase-specific antibodies would be instrumental to mount protective immune responses against the pandemic virus.[49] Of note, we observed in serum from the current cohort an increase in neuraminidase epitope reactivity, but no pandemic H1 epitopes were commonly recognized. This could be related to the fact that we tested reactivity only against linear epitopes, which is a caveat of the chip design used in the current study, and biologically relevant conformational epitopes may have been missed.[23,24]

Table 4. List of exclusively recognized peptide epitopes in serum from the pandemic flu infection group (n = 19) but never in the Pandemrix® vaccination control group (n = 19) (or vice versa)

Protein	Position	Epitope	Average intensity	Number of subjects (19)
H1N1 pandemic (pre-infection)				
Haemagglutinin [Influenza A virus (A/Solomon Islands/3/2006 (Egg passage)(H1N1))]	81–95	NPECELLISRESWSY	0·39	16/19
Haemagglutinin precursor [Influenza A virus (A/swine/Iowa/15/1930(H1N1))]	291–305	PFQNIHPVTIGECPK	0·65	9/19
Pandemrix® Vaccination (pre-vaccination)				
Haemagglutinin [Influenza A virus (A/California/08/2009(H1N1))]	251–265	VEPGDKITFEATGNL	0·57	16/19
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	651–665	ASPQLEGFSAESRKL	0·70	15/19
H1N1 pandemic (post-infection)				
Haemagglutinin [Influenza A virus (A/Uruguay/716/2007 X-175(H3N2))]	496–510	SIRNGTYDHDVYRDE	0·67	12/19
Nuclear export protein [Influenza A virus (A/California/08/2009(H1N1))]	61–75	RNEKWREQLGQKFEE	0·36	10/19
Pandemrix® vaccination (post-vaccination)				
Haemagglutinin [Influenza A virus (A/California/08/2009(H1N1))]	251–265	VEPGDKITFEATGNL	0·49	17/19
Polymerase PA [Influenza A virus (A/California/08/2009(H1N1))]	651–665	ASPQLEGFSAESRKL	0·68	17/19

The top two peptide epitopes are strongly recognized in serum of individuals (before/after Pandemrix® vaccination) or always below the cut-off in the sera of individuals (before/after pandemic flu infection). Note that the epitope VEPGDKITFEATGNL (from the pandemic flu) was exclusively recognized in serum from individuals both before and after Pandemrix® vaccination, yet never in serum from individuals who experienced later H1N1 infection.

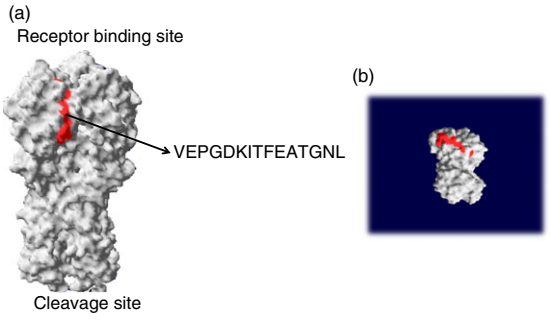

Figure 3. (a) The epitope VEPGDKITFEATGNL (highlighted in red) exclusively recognized in serum from individuals (n = 17) who were vaccinated mapped on the crystal structure of the 2009 H1N1 influenza virus haemagglutinin receptor-binding domain (PDB ID-3LZG). (b) The epitope mapped on the Crystal structure of the 2009 H1N1 influenza virus haemagglutinin receptor-binding domain (PDB ID-3MLH).

Antibodies against swine influenza virus were found to neutralize pandemic flu in experimental models.[50,51] However non-neutralizing antibodies produced against the swine origin H1 – due to past exposures – could enhance virus fusion and promote infection.[52] Most significantly, the subjects in the current study who experienced H1N1 infection (confirmed with a positive PCR), exhibited increased levels of IgG against SRYSKKFKPEIAARP from the HA [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2))], which is highly homologous to the SRYSKKFKPEIAIRP epitope from HA [Influenza A virus (A/California/08/2009(H1N1))] on the HA receptor binding domain. As a single amino acid mutation in the antigenic site is able to generate a new epidemic strain,[53] there is the possibility of 'misdirected reactivity'[54,55] leading to symptomatic flu infection.

The pandemic HA exhibits conservation of most of the epitopes from the antigenic sites Cb, Sa and Sb compared with the HA of the 1918 Spanish flu, yet significant differences have been described in the Ca antigenic site.[31] The IgG serum reactivity in vaccinated individuals to GWLLGNPECDLLLTA on the antigenic site Cb was directed against vestigial esterase domain on the HA1 [Influenza A virus (A/South Carolina/1/1918(H1N1))] (Spanish flu). This epitope GWLLGNPECDLLLTA from the HA of (Spanish flu) was recognized by serum immunoglobulin at both pre- and post-vaccination time-points (see Supporting information, Tables S1 and S2), supporting the notion that IgG to the Spanish flu may be able to induce cross-protection against the 2009 pandemic flu. In addition, serum from these individuals also exhibited IgG reactivity directed against the non-conserved Ca site to VEPGDKITFEATGNL from the 2009 pandemic H1.

Our data show that serum from the individuals that chose to be vaccinated exhibited strong pre-existing IgG reactivity towards pandemic flu epitopes, particularly to the pandemic flu HA epitope VEPGDKITFEATGNL. This reactivity was completely absent from the IgG-epitope recognition repertoire of the flu-infected individuals before and after the flu season. Most likely, this epitope

represents a dominant response associated with repetitive flu vaccination (the interviews of the study participants indicated as previous to 2009/2010 influenza vaccination). A rabbit mono-specific antibody directed against this epitope was prepared that did not neutralize the pandemic flu in vitro; functional significance of this epitope could not be established. A possible explanation is that a 15-mer linear peptide that was used to immunize the rabbits does not resemble the same folding of this epitope within the native protein. However, the antibodies detected in serum from vaccinated individuals against this epitope may activate complement and subsequently contribute to virus neutralization in a biological system.[56,57] This test was beyond the scope of the study, since epitope-specific complement-activating human reagents were not available. Future experiments will also need to address whether high-avidity antibodies from flu-protected individuals[58,59] can be identified with the peptide microarray assay and whether the target epitopes could be used for guided vaccine development. Although the current study may be limited because only IgG directed against linear epitopes could be characterized with conformational epitopes being missed, a distinct IgG recognition pattern was identified. These patterns segregated based on H1N1 infection and vaccination and could be clearly linked to a clinically relevant endpoint: flu infection leads to a different array of epitope recognition compared with the flu vaccine. Immune profiling of individuals, protected from infectious pathogens, may help to tailor new vaccine candidates and help to identify clinically relevant markers of immune protection.

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Disclosures

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Differentially recognized epitopes by serum IgG from individuals (n = 19) who later experienced the pandemic flu infection compared with epitopes recognized by the serum IgG of individuals (n = 19) before Pandemrix® vaccination.

Table S2. Differentially recognized epitopes by serum IgG from individuals (n = 19) post pandemic flu infection compared with the epitopes recognized by serum IgG of individuals (n = 19) after Pandemrix® vaccination ($\geq$ 6 months). The values are presented as fold change.

