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Research paper

Pitfalls in anti-influenza T cell detection by Elispot using thimerosal containing pandemic H1N1 vaccine as antigen

A. Chauvat [a,b,c,d,1], N. Benhamouda [a,b,c,d,1], E. Loison [e], M.L. Gougeon [e], A. Gey [a], E. Levionnois [a], P. Ravel [f], V. Abitbol [g], S. Roncelin [a], E. Marcheteau [a,b], F. Quintin-Colonna [d], W.H. Fridman [a,c], O. Launay [b,c,h], E. Tartour [a,b,c,d,*]

[a] Assistance Publique-Hôpitaux de Paris (AP-HP), Hôpital Européen Georges Pompidou (HEGP), Service d'Immunologie Biologique, Paris France
[b] INSERM, CIC-BT-505, Paris, France
[c] Universite Paris Descartes, Faculté de Médecine, Paris, France
[d] INSERM U970 PARCC, Paris, France
[e] Institut Pasteur, Antiviral Immunity, Biotherapy and Vaccine Unit, Paris, France
[f] CNRS UMR 5048 Centre de Biochimie structurale; INSERM U554; Universite de Montpellier 1 et 2, France
[g] AP-HP, Groupe Hospitalier Cochin Broca Hôtel-Dieu Service de gastro-entérologie, Paris, France
[h] AP-HP, Groupe Hospitalier Cochin Broca Hôtel-Dieu, Centre d'investigation clinique de vaccinologie Cochin Pasteur (CIC-BT 505), Paris, France

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ABSTRACT

Monitoring T cells in combination with humoral response may be of value to predict clinical protection and cross-protective immunity after influenza vaccination. Elispot technique which measures cytokine produced after antigen-specific T cell stimulation is used routinely to detect and characterize anti-viral T cells. We found that the preservative thimerosal present in most H1N1 pandemic vaccines, induced *in vitro* abortive activation of T cells followed by cell death leading to false-positive results with the Elispot technique. The size of the spots, usually not measured in routine analysis, appears to be a discriminative criterion to detect this bias. Multi-dose vials of vaccine containing thimerosal remain important for vaccine delivery and our results alert about false-positive results of Elispot to monitor the clinical efficacy of these vaccines. We showed that this finding extends for other T cell monitoring techniques based on cytokine production such as ELISA. Although measuring *in vitro* immune response using the whole vaccine used for human immunization directly reflects *in vivo* global host response to the vaccine, the present study strongly supports the use of individual vaccine components for immune monitoring due to the presence of contaminants, such as thimerosal, leading to a bias in interpretation of the results.

1. Introduction

Neutralizing antibody responses are the reference criteria for evaluating the efficacy of influenza vaccination. However, T cell mediated immunity also represent an important biomarker of clinical protection after influenza infection especially in older subjects (McElhaney et al., 2006; McKinstry et al., 2011). T cells play a major role in the cross-protective immunity against various types of influenza (Hillaire et al., 2011) (Scheible et al., 2011). In addition, a correlation between baseline or early induced influenza-specific $CD4^+$T cell and humoral response has also been reported (He et al., 2008; Galli et al., 2009). These studies highlight the increasing value of monitoring T cells in combination with humoral response after influenza vaccination. The Elispot technique which measures cytokine produced after antigen-

* Corresponding author at: Hopital Européen Georges Pompidou, Service d'immunologie biologique, France. Tel.: +33 156093942; fax: +33 156092080.
E-mail address: eric.tartour@egp.aphp.fr (E. Tartour).
[1] Contributed equally.

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specific T cell stimulation is a routine method to detect and characterize T cells (Godard et al., 2004). Due to its easiness, the recent possibility to detect multiple cytokines at the single cell level and the development of automated reader for spot counting, Elispot technique has been extensively used for the monitoring of vaccines (Pittet et al., 2001; Gazagne et al., 2003; Bercovici et al., 2008; Britten et al., 2008; Vogt et al., 2008; Winstone et al., 2009). We found that most pandemic influenza 2009 vaccines induced false-positive results with this technique due to the presence of the preservative thimerosal in these vaccines.

2. Methods

2.1. Vaccines and antigens

All the following vaccines except Pandemrix were obtained from Sanofi-Pasteur (Lyon, France). Mutagrip® (0.5 ml/dose) contains Hemagglutinin (HA) and Neuraminidase (N) proteins from the following three influenza strains (A/Brisbane/59/2007 [H1N1]-like, A/Brisbane/10/2007 [H3N2], B/Brisbane/60/2008-like). Each dose includes 15 μg of the various HA proteins but no thimerosal. Panenza®, in its multidose format (10 doses), contains for each dose 15 μg of HA derived from the A/California/7/2009 [H1N1]-like strain and 45 μg of thimerosal.

Pandemrix® from GlaxoSmithKline (Marly-le-Roi. France) contains for each dose 3.5 μg of HA derived from the A/California/7/2009 [H1N1]-like strain, the AS03 adjuvant and 5 μg of thimerosal.

Vaxigrip® (0.5 ml/Dose) contains HA and N proteins from the following three influenza strains (A/California/7/2009 [H1N1]-like, A/Perth/16/2009 [H3N2]-like and B/Brisbane/60/2008). Each dose includes 15 μg of the various HA proteins. This vaccine does not contain thimerosal.

Hemagglutinin (HA) peptide pools were kindly provided by Prof. Autran (CERVI at Pitié-Salpetrière hospital, Paris). The HA peptide pool contained 63 long peptides (12 and 18mers) from A/California/7/2009 [H1N1]-like HA protein. Peptides were designed with a 15 mer overlap between each peptide.

Thimerosal was purchased from Sigma-Aldrich (St Quentin-Fallavier. France) and diluted in sterile water to obtain a 0.5 g/ml stock solution.

PMA and ionomycin were obtained from Sigma-Aldrich.

2.2. PBMC separation

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coat (BC) of healthy donors (HD) provided by the French blood institute (EFS, La Plaine St. Denis) or from inflammatory bowel disease patients vaccinated with Mutagrip® or Pandemrix®. Written informed consent was obtained from each patient. The protocol was approved by an ethics committee.

2.3. Elispot experiment

All assays were performed using Diaclone (Besançon, France) IFNγ Elispot kits as already described (Pere et al., 2011). Briefly, multiscreen™ 96-well membrane plates (Millipore, Billerica) were first sensitized with 25 μl of 70% ethanol for 30 s at room temperature. After washing, anti-IFNγ capture antibody (mAb) at 10 μg/ml in PBS was added to each well for coating and the plates were incubated at 4 °C overnight followed by a saturation step with 100 μl of PBS 2% skimmed milk for 2 h at room temperature. After washing with PBS, 50 μl of antigen and 50 μl of cell suspension were added to each well. Vaccines were added to final concentrations previously defined to be optimal for T-cell stimulation in Elispot assays. HA peptide pools were used at 2 μg/ml for each peptide. PMA and Ionomycin were used together as a positive control, at 100 ng/ml and 10 μM, respectively. All antigens and cells were diluted in serum-free AIM-V medium. Cells were incubated with antigens for 20 h in a cell incubator. Cell suspensions were removed by tapping and 100 μl of PBS 0.1% Tween 20 were added to each well for 10 min at 4 °C to lyze adherent cells. After washing, 100 μl of 1 μg/ml detection biotinylated anti-IFNγ mAb was added for 90 min at room temperature. After 3 washes, 100 μl of a ready-to-use solution of streptavidin conjugated with alkaline-phosphatase (Strepta-AP) was added to each well and incubated at room temperature for 1 h. Plates were washed 3 times before adding 100 μl of a ready-to-use 0.2 μM filtered NBT/BCIP chromogenic substrate to each well. Spots developed for 5 min before reaction was stopped by rinsing the plates with sterile water 3 times on each side. Plates were air-dried before image analysis.

2.4. Elispot analysis

Elispot wells images were captured with an Immunospot® Series 5 UV Analyzer (Cellular Technology Limited, Cleveland) and analyzed with ImmunoSpot®5.0 professional analysis software (Cellular Technology Limited). Two sets of parameters were used for spot counting to discriminate small and standard size spots.

"Standard spot program" parameters were defined automatically by the software and generally used to count spots of cognate secretions. The sensitivity was set at $145 \pm 5\%$ (arbitrary unit) and spot size was gated between 6.10^3 and 82.10^3 μm². As sensitivity is based on density; high sensitivity should be set to detect faint spots.

"Small spot program" parameters were used to detect small spots obtained with Panenza Sensitivity was increased to$160 \pm 5\%$ and spot size was gated between 10^3 and 82.10^3 μm².

The number of cells specifically responsive to the vaccines and expressed as spot-forming cells was calculated after subtracting negative controls (PBMC incubated with medium).

2.5. Purification of cell populations

For $CD4^+$ and $CD8^+$T cell purification, cells were sorted on a BD FACS ARIA cell sorter (BD Biosciences, Le-Pont-de-Claix, France). Cell purity greater than 99% was obtained.

2.6. Flow cytometry cell staining

Cells were stained with CD4 and CD8 mAb (BD Biosciences), washed with PBS-0.5%BSA and a 7AAD (amino-actinomycin D) viability staining solution (BD Biosciences,