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Standardization of a neutralizing anti-vaccinia antibodies titration method: an essential step for titration of vaccinia immunoglobulins and smallpox vaccines evaluation

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Abstract

The possibility of mass population vaccination with smallpox vaccine implies the development of anti-vaccinia immunoglobulins for the treatment of severe side effects following vaccination. We have chosen to develop and validate the "gold standard method" (plaque reduction neutralization assay) to titrate neutralizing anti-vaccinia antibodies in two different French laboratories belonging to the Department of Defense (CRSSA) and to the French Health Products Safety Agency (Afssaps). The results of precision, linearity and accuracy of the method led to consider the method as validated. In parallel, we have prepared and lyophilized a pool of anti-vaccinia plasma samples issued from a unique donor and qualified this preparation versus the first British standard to use it as an in-house standard with a titer of 25 international units (IU). This work will allow to titrate, in IU, sera from vaccinated persons in order (i) to titrate purified anti-vaccinia immunoglobulin preparations for vaccine severe side effect treatments; (ii) to investigate the level of neutralizing antibodies in the general population; and (iii) to investigate clinical trials of new generation smallpox vaccines. In the future, this will allow comparability of studies on either smallpox vaccines or on the serological status of the population.

Keywords: Vaccinia virus; Anti-vaccinia neutralizing antibodies; Anti-vaccinia immunoglobulins (VIGs); Smallpox vaccine; Statistics

1. Introduction

Smallpox vaccines were stockpiled in France in the 80s. The potency of these vaccines was regularly controlled by the French Health Products Safety Agency (Afssaps) (Leparc-Goffart et al., 2003). As variola virus could be possibly used as a biological weapon, a national vaccination plan was implemented in France, associating the Ministry of Health and the Ministry of Defense (Lévy-Bruhl and Guérin, 2001). Consequently, in the case of a massive vaccination of the population with animal- or cell-derived smallpox vaccines, it has been estimated that a number of serious side effects would occur (Cono et al., 2003). These complications are essentially due to the live, replicating vaccinia virus strain (NCBY or Lister strain) contained in the vaccine rather than the production process of the vaccine itself (cells compared to live animals). Therefore, the French health authorities have considered as a need to develop anti-vaccinia immunoglobulins (VIGs) with an assigned titer in order to treat the severe side effects following smallpox vaccination and particularly

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eczema vaccinatum, generalized vaccinia and progressive vaccinia (Goldstein et al., 1975; Sharp and Fletcher, 1973).

Furthermore, a number of new smallpox vaccines are under development and it will be essential during the clinical trial phases to be able to compare the results of humoral immunity. Indeed, recent publications suggested that humoral immunity is a useful biomarker of protective immunity and is essential to protect against poxvirus infection (Belyakov et al., 2003; Edghill-Smith et al., 2003; Hammarlund et al., 2003; Weltzin et al., 2003).

Therefore, there is a necessity to develop, validate and standardize an antibody titration method. The plaque-reduction serum-neutralization method, described by Katz in 1987 (Katz, 1987), is still being used as the gold standard method to titrate neutralizing anti-vaccinia antibodies (Frey et al., 2002; Hammarlund et al., 2003). We have chosen to validate this method and to standardize it by using the first British standard provided by the National Institute for Biological Standards and Control (NIBSC, UK). This standard, kept aside only for research purposes corresponds to the former World Health Organization (WHO) International Standard (anti-smallpox sera) discontinued because of the presence of HBsAg. This is why, in parallel to our method validation approach, we have decided to prepare a standard usable for laboratory routine testing. We have developed and qualified an in-house standard (coded "19584") corresponding to a pool of plasma samples from a unique donor with a relatively high amount of neutralizing anti-vaccinia antibodies.

The method was validated in two different laboratories: the Military Health Service Research Laboratory (CRSSA) belonging to the Ministry of Defense and the French Health Products Safety Agency (Afssaps). The precision (repeatability, intermediate precision and reproducibility), linearity and accuracy of the titration method were evaluated and the in-house standard was calibrated in international units.

2. Materials and methods

2.1. Vaccinia virus

Vaccinia virus stock LISv270303 (titer of $10^{5.1}$ plaque forming units (PFU)/ml) was produced on Vero cells from a cell adapted vaccinia virus originating from the American Type Culture Collection (Lister strain cell adapted, ATCC VR-862).

2.2. Cells

Vero cells were obtained from the European Directorate for the Quality of Medicines (EDQM, Council of Europe). Vero cells were grown at 37 °C, in 5% CO_2, in 199 medium (M199) supplemented with 5% heat-inactivated foetal calf serum (FCS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin. For vaccinia virus infected cells, only 0.4% FCS was used.

2.3. Standards and serum samples

The first British Standard (anti-smallpox serum 63/24), gratefully provided by the NIBSC, has an assigned potency of 1000 IU per vial. This standard, issued from sera of smallpox convalescent patients, is identical to the WHO International Standard (WHO IS) discontinued as an international reference preparation and consequently destroyed because of the presence of HBsAg.

The in-house standard (19584) was obtained by pooling and lyophilizing (performed by the European Directorate for the Quality of Medicines; Council of Europe) four plasma samples of one donor, multi-vaccinated with the smallpox vaccine (Lister strain) and containing anti-vaccinia neutralizing antibodies.

These two lyophilized standards were reconstituted with 1 ml of sterile water before use.

A negative serum coded S041 was obtained by pooling serum samples of naïve donors never vaccinated with smallpox vaccine.

All serum samples and standards were inactivated at 56 °C for 30 min before the assay.

2.4. Neutralization assay for titration of anti-vaccinia neutralizing antibodies

Serial two-fold dilutions of serum samples were incubated for 1 h at 37 °C with an equal volume of vaccinia virus suspension containing 15–55 PFU/0.1 ml. After washing and addition of 0.6 ml of M199 medium supplemented with 0.4% FCS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin confluent Vero cells were infected with 0.2 ml of each diluted serum/virus mixture (15–55 PFU per well of 24-well culture plates, 4 wells per dilution). After two days of incubation at 37 °C in a 5% CO_2 atmosphere, cells were fixed by adding in each well 0.4 ml of fixing and staining solution (0.2% crystal violet, 4.5% formaldehyde and 7.5% ethanol in PBS) to allow plaques counts. Each experiment included the neutralizing anti-vaccinia antibodies titration of the first British Standard, the in-house standard (19584) and the negative serum sample. As controls, the titration of the LISv270303 vaccinia virus was performed in each experiment and cytotoxicity of each serum sample was tested by inoculation on Vero cells.

The ND_{50} is the serum dilution that causes 50% vaccinia plaques reduction compared to the number of vaccinia plaques obtained with the negative sera.

2.5. Precision of the anti-vaccinia neutralizing antibodies titration method

For both laboratories, the precision of the anti-vaccinia immunoglobulin titration method was assessed with the in-house standard (19584) titrated versus the first British Standard (anti-smallpox serum 63/24). The statistical model used to estimate the titers was a parallel lines model (European