Residual humoral immunity sustained over decades in a cohort of vaccinia-vaccinated individuals

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Running head: Residual Immunity from smallpox vaccine

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Abstract

In 2019, Singapore experienced a case of imported Monkeypox. As with smallpox, disease can be prevented through vaccination, which was mandatory for Singaporean infants until 1981. However, the degree of residual immunity in older vaccinated Singaporeans remains unknown. Sera from individuals born from 1946-1984 were therefore tested and those born prior to 1981 were found to have higher anti-vaccinia IgG and neutralizing activity titres. This suggests that protective humoral immunity remains which could reduce disease severity in an orthopoxvirus outbreak. Correlation between IgG and neutralizing titres was observed indicating that serology could be used as a surrogate marker for immunity.

KEYWORDS: Smallpox, Monkeypox, vaccinia,
Background

In May 2019, Singapore experienced its first case of imported Monkeypox arising from the ongoing outbreak in Nigeria. Additional imported cases, which in some instances resulted in secondary transmission, have been reported in other countries including Israel and United Kingdom[1]. In addition, while natural variola virus is not easily accessed, synthetic biological techniques capable of generating recombinant poxviruses increases the risk of deliberate release[2]. Vaccinia virus vaccines e.g. Jynneos offers protection against orthopoxvirus infections including smallpox and monkeypox and was mandatory for Singapore infants, but was halted in March 1981 after eradication of smallpox worldwide[3,4]. In the event of re-emergence of such viruses in Singapore, knowledge of the level of residual immunity would be critical in implementing the appropriate protective measures including vaccination. Various studies have shown that immunity to smallpox obtained through either infection or vaccination can last for years despite the lack of exposure[5–7]. In particular, serum neutralizing titres appear to remain stable for decades post infection, with a 1:32 titre shown to correlate with protection[8]. To study residual immunity in the Singapore population, sera from individuals born between 1946-1984 were tested by both ELISA and a novel high throughput neutralization assay to assess the level of vaccinia-binding antibodies and neutralization. Sera obtained from individuals born prior to 1981 had high levels of binding and neutralizing activity which correlated with each other in contrast to those born after, although considerable heterogeneity was also observed.

Methods

Serum

Positive and negative control sera were obtained with informed consent (DSRB no. 2012/00917) from individuals recently vaccinated with ACAM2000 and healthy unvaccinated individuals respectively. Samples from birth cohorts ranging from 1946-1984 were obtained from anonymized residual sera stored with the Tan Tock Seng Hospital Normal Control Programme.

Recombinant chimeric antibody expression
To generate a positive control antibody standard for both serum ELISA and neutralizing titre assays, light and heavy antibody variable chain sequences were cloned into separate plasmids containing either the human light chain or IgG1 heavy chain constant region sequence respectively under the control of a pCMV promoter[9]. The recombinant antibody was then expressed transiently in HEK293 suspension culture and recovered from the harvested supernatant by Protein A purification on FPLC.

**ELISA**

10cm culture dishes seeded with a confluent monolayer of Vero E6 cells was infected with Vaccinia virus Western Reserve strain at a multiplicity of infection of 0.1 and cultured in Minimal Essential Medium supplemented with 2% foetal calf serum, penicillin/streptomycin, non-essential amino acid, sodium bicarbonate and sodium pyruvate at concentrations specified by the manufacturer (ThermoFisher). Infected cells were harvested at 48hrs post infection and pelleted by centrifugation at 350g for 5min and resuspended in 1ml of ice-cold lysis buffer[10] (50M Tris-HCl pH7.5, 150mM NaCl, 1% Triton-X, 0.1% SDS, 0.5% sodium deoxycholate) containing protease inhibitor (Roche Complete Protease Inhibitor Cocktail) and then freeze-thawed three times for complete lysis. Clarified lysate was recovered by centrifugation at 16100g for 10min and harvesting the supernatant.

Maxisorb ELISA plates (ThermoFisher) were coated with diluted lysate overnight at 4°C, washed twice with PBS, blocked with 4% skim milk in PBS for 2hrs at room temperature and washed twice again with PBS. Serum diluted in blocking agent was then added, washed thrice with PBS/0.05 % tween, followed by anti-human IgG secondary antibody diluted 1:5000 in blocking agent.

The ELISA plates were then washed thrice with PBS/0.05% tween, once with PBS and then developed with TMB for 7min and stopped with 2M sulphuric acid and read on a Biotek HTX reader at 450nm absorbance wavelength. All reagents and samples were added at 100ul/well volume for 1hr at room temperature unless otherwise indicated.
High throughput neutralization assay

25μl of diluted serum was pre-incubated with 25μl of vaccinia virus Western Reserve strain at indicated inoculum concentrations for 1hr at 37 °C in an incubator at 5% CO₂. 2x10⁴ Vero E6 cells in 50μl culture media/well were then added and incubated for 6 days. Serum, virus and cells were all diluted in Minimal Essential Medium supplemented with 2% foetal calf serum, penicillin/streptomycin, non-essential amino acid, sodium bicarbonate and sodium pyruvate at concentrations specified by the manufacturer (ThermoFisher). To determine cell survival, 100μl/well of Viral Toxglo reagent (Promega) was added and read on a Biotek HTX reader in luminescence top mode after 10min incubation.

Results

For maximal detection of anti-vaccinia antibodies in sera, vaccinia-infected cell lysate was used as the binding antigen instead of a single viral protein. To optimize the ELISA for maximum sensitivity, various dilutions of clarified lysate was tested using the anti-vaccinia IgG1 antibody H3-41[11]. 1:150 dilution was found to give the maximum signal and used for all subsequent ELISAs (Supp Fig. 1A). However, testing with negative control sera from unvaccinated patients indicated significant background signal against lysate, with approximately 4-fold higher signal in infected lysate compared to uninfected lysate across a range of serum dilutions (Supp Fig. 1B). As expected, a signal-to-noise ratio above 5 were observed in a positive control sample from a recently vaccinated individual. On this basis, we elected not to use raw absorbance values as a cut-off but a signal-to-noise ratio of 5 instead to determine the endpoint titre. Serum samples were grouped into 12-year cohorts (1946-1957, 1958-1969, 1970-1981) and tested for anti-vaccinia IgG. 3 individuals born post-1981, the year in which smallpox vaccination was halted in Singapore, were grouped as a separate unvaccinated cohort. As expected, the unvaccinated cohort had no detectable vaccinia IgG. However, there were also individuals in the vaccinated cohorts with no detectable IgG. Interestingly, there was a marked increase in geometric mean titre with age, with a statistically significant difference between the 1970-1981 and 1946-1957 cohort (Fig. 1A).
To test for neutralization activity, we decided to develop a cell viability based 96-well plate assay, which provides a quantitative luminescence readout for higher throughput and reproducibility as compared to the traditional plaque reduction assay. To optimize the assay for maximum sensitivity, the anti-vaccinia antibody M12B9 was selected for use as an antibody standard due to its potent in vitro neutralizing activity [12]. M12B9 was expressed as a chimeric recombinant mouse-human IgG1 antibody, diluted into unvaccinated control sera and tested for neutralizing activity across a range of viral inoculum concentrations. 50PFU was found to provide highest sensitivity, with an 50% neutralization titre (NT50) value of approximately 0.33ug/ml, and was used for the subsequent assay with serum samples (Supp Fig 1C). As observed with the ELISA, the unvaccinated cohort had very low or undetectable NT50 titres while the other three vaccinated cohorts had a higher geometric mean titre above 32, which was statistically significant for the 1946-1957 and 1970-1981 cohorts (Fig 1B). Unlike the ELISA IgG titres however, no age-associated increase was observed.

To determine if anti-vaccinia serum IgG was the primary determinant for the neutralization effect in serum, the relationship between ELISA endpoint and NT50 titres was tested using Spearman rank-order correlation coefficient for the three vaccinated cohorts. Interestingly, there were several sera across all three vaccinated cohorts with detectable neutralizing activity up to an approximate titre of 1:128 that had no detectable vaccina-binding IgG (Fig 2A). Nonetheless, there were significant correlations for all three cohorts. Log-log regression indicated that an ELISA titre of 1:80 was equivalent to the protective NT50 titre of 1:32. However, due to the wide variation in NT50 titre, an ROC curve was plotted to determine the appropriate ELISA titre cut-off for maximal specificity with reasonable sensitivity, with a cut-off of 1:912 providing 85% specificity and 60% sensitivity for individuals with NT50 titre greater than 1:32 (Fig 2B).
Discussion

In this study, we show that, as reported by other studies, vaccinated cohorts retain significant residual humoral immunity[5,6,13]. However, there were still seronegative samples that comprised a total of 28 out of the 57 presumably vaccinated individuals. Out of these, all except 4 had neutralizing activity, which may be due to non-IgG antibodies, antibodies against epitopes absent from the lysate or serum immune factors that impeded viral replication. These seronegative samples may also be due to individuals responding poorly to vaccination or whose antibody levels had waned over time[14]. Conversely, higher titres might be due to boosting from repeat vaccinations, as Singapore National Childhood Vaccination Programme had routinely used a two-dose regimen given at two-months and six years of age that covered approximately 90% or more of each cohort until halted in 1981[3]. Multiple studies have shown that there are small but statistically significant increases in ELISA titres among individuals with multiple vaccination that persist decades after the last vaccination [5,6]. Such heterogeneity in vaccine longevity had been observed for in prior cohort studies with some individuals having low or no detectable antibodies despite overall high cohort mean titres [7,13]. It therefore cannot be assumed that all vaccinated individuals remain protected in the event of exposure and that a test for humoral immunity would be useful to determine whether re-vaccination is required. We also cannot rule out inclusion of unvaccinated individuals in this study due to anonymization of the serum samples.

Nonetheless, our study indicates that overall population immunity remains high, with each cohort mean titre as well as approximately 50% of individual NT50 titres exceeding 1:32, established in earlier prospective studies to confer protection against smallpox [8]. While the assays used to measure neutralization have changed, Vaccinia Immune Globulin was shown to have higher neutralization efficacy against both variola and monkeypox virus as compared to vaccinia itself[15]. This suggests that that titres measured using vaccinia would be equally valid for protection against other orthopoxviruses and hence some older Singaporeans remain protected. While we did not assay for cellular immunity, there is also evidence that T-cell responses are also sustained in a similar
manner[13]. However, the correlates of protection for cellular immunity have not been well characterized and it is unclear what level of T cell response would be protective.

Interestingly, while the mean neutralization titres do not alter significantly across all three vaccinated cohorts, there is an increase in overall antibody titres in the oldest vaccinated cohort. This is the only cohort likely to have been exposed to smallpox, the last case having occurred in Singapore in 1959, suggesting that natural smallpox infections may generate a stronger immune response compared to vaccination[3]. We also showed that there is a strong correlation between anti-vaccinia IgG levels and neutralizing activity in the sera for all vaccinated cohorts (Fig 2A). While there were multiple samples with neutralizing but no binding activity, only 2 samples had binding but no neutralizing activity, possibly due to targeting of non-immunoprotective epitopes. That suggests that the presence of anti-vaccinia antibodies would be sufficient as a surrogate marker for humoral protection. ROC analysis indicated that it was possible to identify immune individuals (NTS0>1:32) with high specificity so as not to incorrectly identify those with insufficient neutralizing titre. This would remove the requirement to conduct neutralization assays, which have a much longer turnaround time compared to rapid serological assays, when accessing the need for re-

vaccination.

In summary, we showed that residual smallpox immunity can be retained for at least four decades since immunisation and possibly up to almost eight decades assuming vaccination during childhood. As this study utilized samples from a serum bank, it should be more representative of the overall population immunity as compared to studies that specifically recruit only vaccinated individuals. However, as samples were solely selected based on birthdate and were anonymized without any accompanying vaccination information, this precluded exact determination of vaccination number and interval and thus a more precise evaluation of duration of immunity.
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1 References


**Figure Descriptions**

Fig. 1 (A) Anti-vaccinia IgG levels of sera taken from 3 vaccinated (1970-1981, 1958-1969, 1946-1957) and unvaccinated (1982-1984) birth cohorts as measured by ELISA endpoint titre. Duplicate 4-fold dilutions of sera were tested against infected and uninfected cell lysate and endpoint titres determined by linear interpolation of signal-to-noise values at dilutions above and below the cut-off of 5. Samples without binding activity are plotted on the LOD line. The line indicates the geometric mean titre of each cohort with bars indicating 95% CI. (* p<0.05 using Dunn’s multiple comparison test). (B). Neutralizing activity of sera taken from same cohorts based on NT50 titre. Duplicate 3-fold dilutions of sera were incubated with 50 PFU of vaccinia virus and NT50 titres calculated by fitting a normalized dose-response curve. Samples neutralization activity below the cut-off are plotted on the LOD line. The line indicates the geometric mean titre of each cohort with bars indicating 95% CI. (* p<0.05, ** p<0.01 using Dunnett’s multiple comparison test).

Fig. 2 (A). Correlation of ELISA endpoint titre with NT50 titre. All three vaccinated cohorts (birth years indicated) showed significant correlation between the presence of anti-vaccinia IgG in sera and its neutralizing activity, r values indicate Spearman correlation coefficient (* p<0.05, *** p<0.001). Samples without detectable IgG or neutralizing activity are plotted on the axis at the limit of detection (LOD). The log-log regression line is indicated with the dotted lines indicating the corresponding 1:80 ELISA endpoint titre for the protective 1:32 NT50 titre. (B) ROC analysis of ELISA
titre, using samples with NT50 >1:32 classified as positives and remainder as controls. Using a cut-off of 1:912 (indicated by large dot), provided 85% specificity and 60% sensitivity.