Decades after smallpox was eradicated and vaccination discontinued, the level of residual immunity in today’s population is largely unknown. This study describes an epidemiological assessment in Italians of antibodies against the intracellular mature virus (IMV) and extracellular envelope virus (EEV) forms of *Vaccinia virus*. Serum samples \((n = 642)\) were taken in 1993 and 2003 from people between 11 and 102 years old. Most citizens > 27 years old were positive for antibodies to IMV and EEV. These antibodies were long-lasting and similar titres were present in citizens between 30 and 100 years old. Serum samples from 1993 and 2003 displayed very similar EEV- and IMV-specific antibody titres. By using these data and demographic considerations, it was predicted that, in 2003, 46% of the Italian population were positive for both IMV and EEV, 42% were negative for both and 12% were positive for one antigen.

*Variola virus*, the causative agent of smallpox, was eradicated in 1977 after widespread vaccination with *Vaccinia virus* (VACV) (Fenner *et al.*, 1988). In Italy and many other European countries, smallpox vaccination used predominantly VACV strain Lister/Elstree. Vaccination was compulsory in Italy until 1976 (Tagarelli *et al.*, 2004) although, after 1974, public-health services were less stringent in their vaccination policy. Mass smallpox vaccination was abandoned in most European countries in the early 1970s and production of the vaccine was discontinued in the early 1980s.

Understanding of the immune responses induced after vaccination with VACV is incomplete and concern about the deliberate release of *Variola virus* (Smith & McFadden, 2002) has prompted investigation of how much immunity remains in populations today. It was generally accepted that vaccination would protect most vaccinees for 5–10 years (Fenner *et al.*, 1988), although, the World Health Organization (WHO) recommended revaccination every 3 years for those in areas where smallpox was endemic. Recent studies have shown that humoral (Crotty *et al.*, 2003; Frey *et al.*, 2003; Gallwitz *et al.*, 2003; Hammarlund *et al.*, 2003; Hatakeyama *et al.*, 2005; Viner & Isaacs, 2005) and cellular (Hammarlund *et al.*, 2003; Amara *et al.*, 2004; Combadiere *et al.*, 2004; Kennedy *et al.*, 2004) forms of immunity are long-lived after VACV immunization, but it is unknown whether this remaining immunity will protect against smallpox. Although analysis of historical data suggests that protection after vaccination might last for several decades (Hanna & Baxby, 2002; Eichner, 2003a), the last smallpox fatality, in Birmingham, UK, in 1978, occurred despite the patient having been vaccinated twice previously, once as a child and once 12 years before contracting the disease (Shooter, 1980).

VACV produces two morphologically and antigenically distinct infectious forms of virus, called intracellular mature virus (IMV) and extracellular envelope virus (EEV) (Smith *et al.*, 2002). Most infectious virus particles remain in the cell as IMV until cell lysis; this form is very stable and so is likely to be responsible for host-to-host spread. EEV is wrapped by a second lipid envelope containing several virus proteins. It is released before cell lysis and is better-adapted for spread within the host because it is relatively resistant to destruction by complement (Vanderplasschen *et al.*, 1998) and neutralization by antibodies (Abs) (Vanderplasschen *et al.*, 1997; Law & Smith, 2001). Studies in animal models showed that anti-EEV Abs are more important than anti-IMV Abs for protection against poxvirus challenge *in vivo* (Appleyard *et al.*, 1971; Boulter & Appleyard, 1973; Fogg *et al.*, 2004; Law *et al.*, 2005). These findings emphasize the importance of measuring responses to EEV as well as, or instead of, those against IMV when analysing Abs induced by VACV vaccination.

Several virus-encoded glycoproteins are present on the EEV surface (Smith *et al.*, 2002) and two of these, B5R and A33R, are important targets for protective immune responses in animal models (Galmiche *et al.*, 1999; Hooper *et al.*, 2003;
Fogg et al., 2004; Pulford et al., 2004). B5R is the major target of EEV-neutralizing Abs induced after VACV immunization in rabbits (Law & Smith, 2001; Law et al., 2005) and in humans (Bell et al., 2004; Law et al., 2005) and, recently, mAbs that neutralize EEV by binding to B5R were described (Aldaz-Carroll et al., 2005). In addition, the EEV-neutralizing activity of sera correlated with their protective efficacy against intranasal VACV challenge in mice (Galmiche et al., 1999; Law et al., 2005).

An epidemiological study of EEV-specific Abs decades after smallpox vaccination has not been undertaken. In this study, we have addressed this in the Italian population and measured the EEV and IMV Ab titres by using ELISAs specific for recombinant B5R protein and VACV-infected cell lysate, respectively (Law et al., 2005). ELISA Abs against VACV have been shown to correlate with the presence of IMV-neutralizing Abs (Hammarlund et al., 2003; Hatakeyama et al., 2005), whereas the Ab response to purified B5R 42 kDa glycoprotein, produced in CHO cells (Law et al., 2005), correlates very well with EEV-neutralizing activity (M. M. Pu¨tz, C. M. Midgley, M. Law & G. L. Smith, unpublished data) in human serum after VACV immunization. Anonymous serum samples from routine laboratory testing in 1993 (n = 119) (‘1993 group’) and between 2001 and 2003 (n = 523) (‘2003 group’) were collected at the Central Laboratory in the General Hospital of Siena, Italy, and were stored at −20 °C. Samples from subjects known to have an immunosuppressive or acute infectious disease and from subjects who had undergone a recent blood transfusion were excluded. Subjects ranged from 11 to 102 years of age at the time of sample collection and their vaccination status was unknown.

To address what proportion of the Italian population contained Abs to EEV and IMV and to address whether the titres of these Abs varied with age, we used the ‘2003 group’ of serum samples. Ninety-six-well plates were coated overnight with 100 ng purified B5R or BSA (negative control) per well, or UV-inactivated VACV-infected cell lysate corresponding to 10^5 p.f.u. per well. Linear-regression plots were determined for each serum sample and end-point titres were defined as dilutions corresponding to twice the mean OD values obtained with BSA. A control serum from an individual vaccinated twice (anti-B5R titre, 1 : 544; anti-VACV titre, 1 : 2024) was used to normalize results between plates and assays and the 60 serum samples from subjects aged 11–20 years were used as a negative-control group. Cut-off titres defining seropositivity/seronegativity were calculated for B5R (1 : 75) and for VACV (1 : 364) as three times the geometric mean titre (GMT) obtained in this group; these values generated 100 % specificity.

In the ‘2003 group’, serum samples from the 11–20-year-old group were all negative for Abs against B5R and VACV cell lysate, but Abs to both antigens were detected in most older subjects > 25 years after vaccination with VACV was discontinued in Italy (Table 1, Fig. 1). These findings show that EEV-specific Abs persist long after smallpox vaccination and are in accordance with the longevity of Abs against IMV described previously (el-Ad et al., 1990; Frey et al., 2003; Gallwitz et al., 2003; Hammarlund et al., 2003). In the

Table 1. Proportion of serum samples positive for B5R, VACV or both antigens by ELISA

<table>
<thead>
<tr>
<th>Age group</th>
<th>n</th>
<th>B5R+</th>
<th>VAC+</th>
<th>B5R+VAC+</th>
<th>B5R−VAC−</th>
<th>Demographic contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Samples from 2003</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;11 years</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>10:3</td>
</tr>
<tr>
<td>11–20 years</td>
<td>60</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>60 (100)</td>
<td>10:2</td>
</tr>
<tr>
<td>21–30 years</td>
<td>60</td>
<td>15 (25)</td>
<td>16 (27)</td>
<td>15 (25)</td>
<td>44 (73)</td>
<td>13:5</td>
</tr>
<tr>
<td>31–40 years</td>
<td>66</td>
<td>44 (67)</td>
<td>45 (68)</td>
<td>42 (64)</td>
<td>19 (29)</td>
<td>16:1</td>
</tr>
<tr>
<td>41–50 years</td>
<td>65</td>
<td>55 (85)</td>
<td>59 (91)</td>
<td>53 (82)</td>
<td>4 (6)</td>
<td>13:5</td>
</tr>
<tr>
<td>51–60 years</td>
<td>58</td>
<td>38 (66)</td>
<td>46 (79)</td>
<td>37 (64)</td>
<td>11 (19)</td>
<td>12:5</td>
</tr>
<tr>
<td>61–70 years</td>
<td>68</td>
<td>35 (52)</td>
<td>52 (76)</td>
<td>33 (49)</td>
<td>14 (21)</td>
<td>11:4</td>
</tr>
<tr>
<td>71–80 years</td>
<td>58</td>
<td>40 (69)</td>
<td>53 (91)</td>
<td>40 (69)</td>
<td>5 (9)</td>
<td>8:6</td>
</tr>
<tr>
<td>81–90 years</td>
<td>60</td>
<td>33 (55)</td>
<td>52 (87)</td>
<td>31 (52)</td>
<td>6 (10)</td>
<td>3:4</td>
</tr>
<tr>
<td>&gt;90 years</td>
<td>28</td>
<td>18 (64)</td>
<td>21 (75)</td>
<td>16 (57)</td>
<td>5 (18)</td>
<td>0:6</td>
</tr>
<tr>
<td><strong>Italian population</strong></td>
<td>48</td>
<td>(57)</td>
<td>(46)</td>
<td>(42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Samples from 1993</strong></td>
<td>39</td>
<td>32 (82)</td>
<td>32 (82)</td>
<td>32 (82)</td>
<td>7 (18)</td>
<td></td>
</tr>
<tr>
<td>31–40 years</td>
<td>40</td>
<td>27 (68)</td>
<td>34 (85)</td>
<td>26 (65)</td>
<td>5 (13)</td>
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<tr>
<td>41–50 years</td>
<td>40</td>
<td>27 (68)</td>
<td>34 (85)</td>
<td>26 (65)</td>
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<tr>
<td>51–60 years</td>
<td>40</td>
<td>25 (63)</td>
<td>26 (65)</td>
<td>25 (63)</td>
<td>4 (10)</td>
<td></td>
</tr>
</tbody>
</table>
‘2003 group’, 278 samples (53 %) were seropositive for B5R and 344 (66 %) were positive for VACV (Table 1). When seropositivity levels obtained for B5R and VACV in the various age groups were compared by using a Wilcoxon signed-rank test (SPSS 12.0; SPSS Inc.), a significantly higher seropositivity rate was observed for VACV ($P = 0.0078$). The majority of subjects negative for both antigens were <30 years of age. In the group of 21–30-year-olds, only 15 (25 %) or 16 (27 %) subjects were either B5R- or VACV-seropositive, respectively, and 15 (25 %) were seropositive for both antigens. The youngest subject to test positive for both antigens was 28 years old (Fig. 1a, b) and was probably vaccinated at the very end of the smallpox-vaccination campaign in Italy, 1976–1978.

The percentage of subjects positive both for B5R and VACV was in the range 49–64 %, except for the group of 41–50-year-olds (82 %). An equally high percentage of ‘double positives’ was also observed in the serum samples of the 31–40-year-olds, which were collected in 1993 and which are from the same decade of birth (Table 1, decade of birth 1953–1962). Surprisingly, the different age groups (>30 years) showed similar Ab levels (Fig. 1a, b) and GMTs (Fig. 1c) against VACV and the same was true for B5R. Linear covariation analysis using a two-tailed Spearman correlation test demonstrated no correlation between the age of subjects and their B5R-specific ($r = -0.037$, $P = 0.46$) or VACV-specific ($r = 0.082$, $P = 0.10$) titres. To address this further, GMTs obtained in the various 10-year cohorts (>30 years) were compared by using a non-parametric, unpaired, two-tailed Mann–Whitney U test and a Bonferroni adjustment of the significance level was applied for multiple comparisons. No significant differences were observed when these age groups were compared with each other (21 tests, $P > 0.0024$), except for the group of subjects aged 41–50 years, who displayed a higher anti-B5R GMT than the subjects aged 61–70 years ($P < 0.001$). Similarly, the Ab GMTs in the serum samples collected in 1993 in age groups 31–40, 41–50 and 51–60 years were not significantly different (Fig. 2) (three tests, $P > 0.017$).

All these data suggested that the Ab titres were long-lived. To investigate this further, additional serum samples that had been collected in 1993 were tested and compared with the GMTs of the ‘2003’ samples (Fig. 2). The GMTs from cohorts of the same decade of birth had very similar B5R- and VACV-specific titres in 1993 and, 10 years later, in 2003 ($P > 0.05$). The only significant difference was that, surprisingly, the VACV-specific titre for people born between 1953 and 1962 was higher in 2003 than 1993 ($P < 0.05$). This might suggest that the size of the cohort analysed from 1993 was too small to prevent a bias being introduced through sampling.
A comparison of the B5R- and VACV-specific ELISA titres (Fig. 1d) showed a reasonable correlation (two-tailed Spearman correlation test, \( r = 0.760, P < 0.001, n = 642 \)), in that individuals with higher titres for B5R also had higher titres for VACV. Overall, the B5R-specific ELISA generated fewer positives than the VACV-specific ELISA (Table 1). This is probably because the anti-VACV ELISA detects Abs to many different VACV antigens and these are predominantly IMV or non-structural, because the VACV-infected cell lysate used contains very little EEV-specific antigenicity (Law et al., 2005). In contrast, the EEV titre is measured by a single antigen, which was produced in a mammalian-cell expression system (Law et al., 2005). Vaccinia immune globulin (VIG) from vaccinated humans reacts predominantly with three immunodominant antigens: H3L, an IMV envelope protein, and D13L and A10L, two viral core proteins (Davies et al., 2005). Our observations suggest that IMV antigens also account for most of the reactivity measured by VACV-specific ELISA (unpublished data). Findings by Davies et al. (2005) that VACV immunization of humans induced no Abs against B5R in human VIG contrast with our data (Law et al., 2005) and might be due to use of an inappropriate B5R protein that was Unglycosylated and/or misfolded due to its expression in Escherichia coli.

When taking into account the respective proportions of the different age groups in the Italian population, it is predicted that the 267 samples from the '2003 group' that contained Abs against both B5R and VACV correspond to 46 % of the Italian population (Table 1). Similarly, the 168 'double-negative' samples correspond to 42 % of the total population. The remaining 12 % were positive for one antigen only. The GMTs and the amount of seropositive subjects observed for both B5R and VACV were very similar in age groups over 30 years old. The group aged 41–50 years displayed the highest percentage of 'double positives' in 2003; this may be due to a high proportion of people born between 1953–1962 receiving follow-up vaccinations during their compulsory schooling in the course of the intensified immunization programme launched by the WHO in 1967. Secondary immune responses after vaccination occur more rapidly (McCarthy et al., 1958; Frey et al., 2003; Greenberg et al., 2005) and induce higher levels of remaining Abs (Frey et al., 2003; Hammarlund et al., 2003; Greenberg et al., 2005; Viner & Isaacs, 2005) than primary vaccination. Further boosting (more than two vaccinations) had little effect on long-term Ab levels (Hammarlund et al., 2003; Viner & Isaacs, 2005).

Accurate knowledge of the residual immunity in a population is important when evaluating the potential of Variola virus to spread in the human population if it were introduced (Gani & Leach, 2001) or policy options for controlling a potential outbreak (Eichner, 2003b; Ferguson et al., 2003). In another study, 1 year after revaccination with the Lister vaccine, we observed levels (95 % confidence intervals) for B5R- and VACV-specific Abs of 142–578 and 2084–5003, respectively (M. M. Pu¨tz, C. M. Midgley, M. Law & G. L. Smith, unpublished data). If these titres are compared with the titres detected in samples from the Italian population in 2003 and those samples that are within these ranges (or higher) are counted, only 91 of the Italian samples, corresponding to 15 % of the Italian population, are within the ranges for both antigens. However, no conclusions can been drawn about the protective immunity from the presence or absence of Abs against B5R and/or VACV, because the exact contribution of neutralizing Abs in the mechanism of protection is unknown. In the absence of any vaccination records, it is impossible to determine what proportion of previously vaccinated subjects lost their Ab immunity over time. A recent report suggested that the capacity to neutralize EEV had diminished by 20 years after vaccination (Viner & Isaacs, 2005). Except for some military personnel, health-care workers and laboratory staff who have received vaccination recently, people 27 years of age (29 % of the population) have never been vaccinated and would be susceptible to smallpox.
In summary, this study describes the epidemiological prevalence of EEV- and IMV-specific Abs in the Italian population. Regardless of the fact that Abs induced against both infectious forms of VACV are long-lived, a substantial proportion of the population has never been vaccinated or has only very low Ab levels. High levels of protection might only be achieved through revaccination and, therefore, there is a need for a new and safer vaccine against smallpox.

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