Vaccinia virus (VACV) is a potent vaccine against smallpox, but its use was discontinued in the 1970s following smallpox eradication. Recently, vaccination of limited numbers of health-care workers was introduced in the USA, the UK and elsewhere in response to a perceived threat of bioterrorism with Variola virus, the aetiological agent of smallpox. Despite worldwide use of VACV as the smallpox vaccine, only recently have studies analysed the systemic immune response induced in animals (Belyakov et al., 2003; Earl et al., 2004; Xu et al., 2004) and humans (Crotty et al., 2003; Frey et al., 2003; Hammarlund et al., 2003; Amara et al., 2004; Belshe et al., 2004; Combadere et al., 2004; Greenberg et al., 2005; Pütz et al., 2005; Rock et al., 2005) by modern immunological techniques. However, the immune response at the vaccination site remains poorly understood.

Previously, an intradermal model in the mouse ear pinnae that mimics dermal vaccination with VACV was developed (Tscharke & Smith, 1999). Following vaccination, no signs of systemic illness were observed and there was little or no virus spread from the ear. Subsequently, this model was used to assess virulence of different strains of VACV, other orthopoxviruses and VACV mutants engineered to lack specific virus genes (Tscharke et al., 2002). Fig. 1(a) shows that this vaccination induced protection against challenge with all doses of VACV strain Western Reserve (WR) tested (up to $10^7$ p.f.u.). It was established previously that doses of $10^5$ p.f.u. or greater are lethal in this model (Wilcock et al., 1999; Symons et al., 2002; Fogg et al., 2004; Davies et al., 2005). Another report analysed cells that had infiltrated into VACV-infected ears by separation of the ventral and the dorsal dermal sheets and incubation of these on culture medium at 37°C for 8–10 h (Reading & Smith, 2003). Non-adherent cells that had migrated from the dermal layers were pooled with the loosely adherent cells recovered by a further incubation (20 min) in PBS containing glucose (2 mg ml$^{-1}$), but without calcium and magnesium. With this technique, called the ‘migration technique’, only up to 100 000 cells were isolated per mock-infected ear or ear early post-infection (p.i.) (Reading & Smith, 2003) (Fig. 1b), making analysis of the leukocyte population difficult. For example, lymphocyte subsets were able to be characterized only after day 7 p.i. (Reading & Smith, 2003). To increase the number of cells isolated from infected ears in order to permit more extensive lymphocyte characterization, we tried digestion with trypsin, collagenase or liberase and compared cells obtained by these methods with those obtained by migration.

The intradermal model was established with BALB/c mice infected with $10^4$ p.f.u. VACV (strain WR) (Tscharke & Smith, 1999), but lesions are bigger in C57BL/6 mice (Tscharke et al., 2002) and therefore most of the experiments were undertaken with this strain. For digestions, the ventral and dorsal dermal sheets were separated using forceps and incubated with collagenase (Sigma, crude type XI collagenase; 1 mg ml$^{-1}$, 75 min at 37°C) (Belkaid et al., 1996), trypsin (Invitrogen; 0.5–1·0 %, 30 min at 37°C) (Belkaid et al., 1998) or liberase CI (Sigma; 50 µg ml$^{-1}$, 40 min at 37°C) (Hawlisch et al., 2005); cells were then filtered using a 70 µm nylon cell strainer (Becton Dickinson). Cell numbers obtained with trypsin or collagenase were...
similar and twofold higher than those obtained with liberase (data not shown). However, trypsin treatment removed some cell-surface markers (i.e. CD4 and CD8) that were recovered after 2–4 h incubation at 37 °C (data not shown). Liberase digestion produced fewer contaminating endothelial or epithelial cells, but preferentially isolated lymphocytes and, to a lesser extent, macrophages and granulocytes (data not shown). Therefore, to enable analysis of all leukocyte populations and to avoid the problem of trypsin digestion, we selected collagenase treatment for our study.

Fig. 1(b) shows the number of cells obtained with migration or collagenase techniques after VACV infection. In mock-infected ears and up to 8 days p.i., there was an increase of >10-fold in cell recovery using collagenase digestion compared with migration and the biggest difference was on day 2 p.i. (>50-fold). Collagenase treatment yielded more than 10^6 cells per ear for each time point and this was sufficient for phenotypic analysis of the cells. Interestingly, the number of cells recovered by collagenase treatment dropped at day 11 p.i., whereas by using the migration technique, more cells were recovered at this time than earlier (Fig. 1b). Previous results using migration showed an increase in cell number, even at day 15 p.i. (Reading & Smith, 2003), when the lesion size had decreased, and when immunohistochemical detection of VACV with an antibody against the B5R protein was very weak at days 10 and 12 p.i.

Although <30% of total cells recovered from mock-infected ears by collagenase treatment were leukocytes [detected by CD45 mAb–fluorescein isothiocyanate (FITC); Serotec], compared with around 80% with the migration technique, the total numbers of macrophages and lymphocytes were >50- and >60-fold-increased with enzymic digestion (Fig. 1c). At day 5 p.i., leukocytes (CD45^+) represented 70–90% of total cells recovered with either technique (data not shown). In addition to different numbers of cells being recovered by the two techniques, the cells recovered showed different kinetics of infiltration in the ears. With collagenase treatment, macrophages, which play an important role in the innate response and were detected with F4/80–Tri-Color mAb (Caltag), peaked at day 5 and then declined to levels found in mock-infected ears by day 11. Similarly, effectors of the adaptive immune response, such as T cells [detected with anti-CD3–phycoerythrin (PE)–Cy5 mAb; BD Pharmingen], recovered by collagenase treatment, peaked at day 8 and then declined. In contrast, with migration, both macrophages and T cells were maximal at day 11 (Fig. 1c). Similar results were observed with the BALB/c mouse strain (data not shown). Isolation of leukocytes by collagenase treatment enabled the beginning and end of the immune innate response and the subsequent adaptive response to be followed. Moreover, when cells were isolated by collagenase treatment, the kinetics of cell infiltration followed the kinetics of the innate and adaptive immune response more closely. In contrast, the immune-cell infiltration observed with the migration technique continued even after the lesion had healed (Tscharke et al.,

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**Fig. 1.** (a) Weight loss of groups (n=2–5) of BALB/c mice infected intranasally with the indicated doses of VACV WR or mock-infected. Twenty-eight days after intradermal immunization or not (‘No immuni.’ group) with 10^4 p.f.u. VACV strain WR in both ears, the mice were challenged intranasally and weighed daily. (b, c) Cell isolation by migration and collagenase techniques after intradermal infection of C57BL/6 mice with 10^4 p.f.u. VACV WR. (b) Total numbers of viable cells as determined by trypan blue exclusion. (c) Total number of T cells (CD3) and macrophages (MØ) obtained with migration (‘migr’) or collagenase (‘coll’) techniques. Data are means ± SEM of cell count by ear (n>6 except for mock-infected ear with migration technique, where n=1).
These results suggested that the collagenase technique is more appropriate than the migration technique to study the kinetics of leukocyte populations after viral infection, and the large number of cells obtained enabled functional analyses of these cells.

Leukocyte populations present in the VACV-infected ear and obtained with the collagenase technique were analysed further (Fig. 2). Resident macrophages constituted the major subpopulation of leukocytes in the mock-infected ear and early after VACV infection (Fig. 2a, b), as reported previously by in situ analysis of the mouse ear dermis (Dupasquier et al., 2004). However, later after infection, the percentage of macrophages decreased (Fig. 2a) and their absolute number declined after day 4 p.i. (Fig. 2b). The proportion of granulocytes (detected by high expression of Ly6G–PE; BD Pharmingen) increased slightly by day 2 p.i. (Fig. 2a) and continued to increase up to day 10 p.i., followed by a decline. The percentage of lymphocytes continued to increase up to day 17 p.i. (Fig. 2a), when the lesions had mostly disappeared and most leukocytes were lymphocytes (Fig. 2a, b). To detect antigen-presenting cells (APCs), cells were stained with anti-CD11c–PE and anti-major histocompatibility complex class II mAb–FITC (both from BD Pharmingen), which recognize both dermal dendritic cells and Langerhans cells. The number of these cells increased only slightly after infection and the proportion of APCs was similar to the percentage found in situ in mouse ear (Dupasquier et al., 2004). Overall, these data suggest that collagenase treatment enabled the kinetic response of the different immune populations to be evaluated after viral infection.

Further analysis of lymphocyte subsets studied recruitment of CD4+ and CD8+ cells (Fig. 2c, d). Among the T cells, TCRγδ cells are abundant in murine skin (Asarnow et al., 1988). These cells are considered a first line of defence and play a role in the control of viral infection (Ninomiya et al., 2000; Wang et al., 2003), including after intraperitoneal infection with VACV (Selin et al., 2001). Up to day 4, these cells (detected with anti-TCRγδ–PE mAb; BD Pharmingen) were the major population of lymphocytes (Fig. 2c, d). Previously, the lymphocyte populations have been studied only after day 7 p.i., due to the limited number of cells obtained, and TCRγδ T cells were a minor population in the VACV-infected ear (Reading & Smith, 2003). The percentage of these cells did not increase after VACV infection (Fig. 2d), but the absolute cell number increased up to 4 days p.i.

NK cells are another effector population of the innate response that was reported to be involved in the response to VACV infection (Bukowski et al., 1983; Kennedy et al., 2000), but few studies have addressed NK recruitment after VACV infection (Natu & Welsh, 1987; Prlic et al., 2005). In this study, usually <1% of lymphocytes in mock-infected ears were NK cells (detected with NK1.1–FITC mAb; BD Pharmingen) (data not shown), but the percentage and absolute number of NK cells increased and peaked at day 7–10 p.i. NK activity against K562 target cells, detected with
a $^{51}$Cr-release assay, was observed 6 days p.i. (data not shown). The changes in the numbers of TCRγδ and NK cells were not described previously because these lymphocyte populations were followed only after day 7 p.i. (Reading & Smith, 2003), by when these cell populations had already decreased (Fig. 2c, d).

Compared with other lymphocytes, the infiltration of cells from the adaptive immune response, such as CD4$^+$ and CD8$^+$ T cells, was later and more pronounced, especially on days 7–10 p.i. (Fig. 2c, d). The percentage of CD4$^+$ T cells (detected with anti-CD4–Tri-Color mAb; Caltag) still increased at day 17 p.i. (Fig. 2c), whereas the majority of the lesions had resolved at that time. In contrast, the percentage of CD8$^+$ T cells (detected with anti-CD8–PE–Cy5 mAb; BD Pharmingen) decreased after day 7 p.i. (Fig. 2c). The absolute number of both T-cell subsets declined after day 10 p.i. (Fig. 2d). Cytotoxic T-lymphocyte activity against EL4 cells infected at 10 p.f.u. per cell with VACV strain WR was detected by using a $^{51}$Cr-release assay by day 7 p.i. (data not shown), as reported with BALB/c mice (Reading & Smith, 2003). Depletion of lymphocyte subsets showed that both CD4$^+$ and CD8$^+$ T-cell-mediated immunity contribute to protection against intraperitoneal infection with VACV (Xu et al., 2004).

An expansion of T cells expressing NK-cell markers was reported in the lungs of influenza virus-infected mice (Kambayashi et al., 2001) and in the spleen of lymphocytic choriomeningitis virus-infected mice (Slifka et al., 2000).

These cells are usually referred to as ‘NK cells’. The proportion of T cells expressing NK1.1, used to quantify the NK cells, was very low (<1%) in mock-infected ears and did not increase after VACV infection (data not shown). The presence of another NK-cell marker, DX5, which is also expressed on all murine NK cells, was detected on only a few CD3$^+$ T cells in mock-infected ears (Fig. 3a) and the majority of these cells co-expressed NK1.1 (data not shown). However, during VACV infection, a population of T cells expressing DX5 (but not NK1.1) emerged (Fig. 3a, b) and underwent enormous expansion (>400-fold) that was the highest among all of the cell populations studied (Fig. 3b). At day 9 p.i., 67 ± 7% of these DX5$^+$ CD3$^+$ cells were CD4$^+$ and 28 ± 7% were CD8$^+$ (data not shown). After day 10 p.i., the percentage and number of these cells decreased (Fig. 3b) in parallel with the reduction in lesion size (Tscharke et al., 2002). CD3$^+$ DX5$^+$ T cells were associated with activation marker (CD69) and subsequent cell death in influenza virus infection (Kambayashi et al., 2001) and the results presented here suggest that NKT cells could play an important role in the control of VACV infection.

VACV was used to eradicate smallpox, and VACV recombinants expressing foreign antigens were proposed as vaccines against other infectious diseases (Smith et al., 1983). The intradermal ear-infection model mimics dermal vaccination because only a local lesion develops (Tscharke & Smith, 1999) and immunity against VACV challenge is induced (Fig. 1a). In this report, the different leukocyte populations infiltrating into the VACV-infected ear are described. VACV encodes numerous proteins that interfere with the host response to infection (Smith et al., 1997; Moss & Shisler, 2001) and their study is relevant to the development of recombinant VACV vaccines. The local immune response reported here could be used as a reference to test the effects of these different immunomodulatory proteins. Moreover, this intradermal model could be suitable to study the kinetics of the immune response in other viral infections.

**Acknowledgements**

This work was supported by grants from The Wellcome Trust. G. L. S. is a Wellcome Trust Principal Research Fellow.

**References**


