A recombinant measles virus expressing biologically active human interleukin-12

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Suppression of cell-mediated immunity (CMI) is well-documented during and after measles. This immunosuppression is suggested to result from decreased production of interleukin-12 (IL-12), a key interleukin for CMI. In an attempt to clearly discern the role of IL-12 in measles-induced immunosuppression, a measles virus (MV) that expresses biologically active human IL-12 was generated. This was achieved by inserting the coding sequences of the two subunits (p35 and p40) of human IL-12 separated by an internal ribosome entry site in an additional transcription unit between the H and the L genes of MV. Although the IL-12-expressing MV grew slightly slower than the normal MV, it stably maintained the inserted sequences (3.2 kb) and uniformly expressed the foreign genes after 10 passages in cell culture. These findings suggest that MV is a well-suited vector for delivery of proteins of immunogenic and therapeutic importance.

Measles virus (MV) is an important human pathogen causing acute systemic disease in nonimmune individuals. Despite the development of a successful live attenuated vaccine, measles still remains a major cause of infant mortality in developing countries (more than 1 million deaths per year around the globe are associated with MV) and continuous measles outbreaks also occur in industrialized countries (Griffin & Bellini, 1996). MV infection is accompanied by profound immune suppression resulting in increased susceptibility to secondary infections. In vivo, delayed-type hypersensitivity responses are inhibited for several weeks after acute measles (Tamashiro et al., 1987). Interestingly, measles vaccination also causes immune suppression (Hussey et al., 1996; Starr & Berkovitch, 1964). The mechanism of immune suppression is poorly understood but widely assumed to be mainly due to replication of the virus in leukocytes (Joseph et al., 1975). MV-infected lymphocytes die due to apoptosis (Addae et al., 1995; Auwaerter et al., 1996; Esolen et al., 1995). Peripheral blood lymphocytes (PBLs) isolated from measles patients show a reduced proliferation capacity in response to polyclonal stimulation (Hirsch et al., 1984; Whittle et al., 1978) possibly due to a cell cycle arrest in the end of the G1 phase (McChesney et al., 1987, 1988). Even uninfected PBLs were shown to exhibit decreased proliferation in presence of cells that express measles glycoproteins (Niewiesk et al., 1997; Schröder et al., 1996). In addition, MV infection also influences the maturation, survival and functioning of dendritic cells (DCs) (Fugier-Vivier et al., 1997; Grosjean et al., 1997; Schnorr et al., 1997).

During and after measles, the immune response is polarized towards Th2 responses (antibody-mediated immunity) (Griffin & Ward, 1993; Ward et al., 1991). This polarization is suggested to be partly due to the decreased ability of macrophages, monocytes and DCs to produce IL-12 (Fugier-Vivier et al., 1997; Karp et al., 1996) [Note, however, that in one study slightly increased IL-12 production after MV infection of DCs was found (Schnorr et al., 1997).] IL-12, a heterodimer of p35 and p40 protein subunits, is principally produced from monocytes, macrophages and DCs and is a key interleukin that directs the differentiation of uncommitted Th0 to Th1 cells which contribute towards cell-mediated immunity (Macatonia et al., 1995; Trinchieri, 1998).

To define the role of IL-12 in MV-induced immunosuppression, we set out to express IL-12 from MV in order to compare the immunological effects of this virus with those of the normal virus. To construct a recombinant MV that can express biologically active human IL-12, our system for rescue of MV from cloned DNA was used (Radecke et al., 1995). In MV, as in all members of the order Mononegavirales, the sequential transcription of genes results in a polar accumulation of viral mRNAs (Cattaneo et al., 1987), generally reflected by a similar gradient of relative amounts of the corresponding proteins. To avoid an MV transcription-dependent bias in the relative amounts of p35 and p40, the expression of the two subunits of IL-12 was designed to occur from the same mRNA. To achieve this and to ensure a relatively low-level expression of IL-12, the p35 and p40 coding sequences were cloned into...
Fig. 1. Cloning human IL-12 in p(+)MVNSe antigenomic MV plasmid. ORFs of MV and hIL-12 genes are shown as rectangles labelled with letters: N, nucleocapsid; P, phosphoprotein; M, matrix; F, fusion; H, haemagglutinin; L, large protein of MV; p40 and p35 are the two subunits of hIL-12. Grey rectangles denote nontranslated regions and vertical bars denote the nontranscribed intergenic trinucleotides. The triangle indicated as artificial intergenic region (aigr) consists of gene termination, intergenic and gene start sequences followed by unique cloning sites. IRES denotes internal ribosome entry site sequences of EMCV derived from pTM1 (Moss et al., 1990). Plasmid names together with total size in base pairs (bp), MV antigenomic nucleotide numbers (based on EMBL accession no. Z66517) and restriction sites are as indicated. T7, T7 RNA polymerase promoter; δ, hepatitis delta virus ribozyme; T, T7 RNA polymerase terminator.

an additional transcription unit between the H and the L genes of MV (see Fig. 1) such that the expression of the two coding regions is mediated by a bicistronic mRNA in which these coding regions are separated by the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) (Moss et al., 1990).

Plasmid pBSIL-12 (Hemmi et al., 1998) was digested with NotI/XhoI and blunted, and the resulting 2306 bp fragment was ligated into the NruI site of peFHaigrL (peFHL is described in Schmid et al., 1992) to obtain peFHLIL-12 (note that NruI cut the recognition sequence TCGCGA after the first four bases, TCGC) to obtain peFHLIL-12. A PacI–SpeI fragment of peFHLIL-12 was replaced in p(+)MVNSe [containing the antigenomic MV tag-Edmonston B sequence, slightly modified from p(+)MV (Radecke et al., 1995) to exhibit unique NarI and SpeI sites; L. Hangartner and others, unpublished data] to obtain plasmid p(+)MVIL-12.

The virus rescue was carried out as described by Radecke et al. (1995). Briefly, 5 µg each of p(+)MVNSe or p(+)MVIL-12 together with 100 ng of pEMC-La (specifying mRNA for the MV L protein) were transfected into 293-3-46 cells using the calcium phosphate method. Three days after transfection, three to four syncytia developed from both DNA constructs and were picked and transferred to 35-mm-dish Vero cell cultures which were subsequently expanded to 175 cm² dishes. Infections were harvested at 80–90% CPE by scraping the cells into 3 ml OptiMEM I (GIBCO BRL) followed by one round of freezing and thawing. This virus preparation was designated as MVIL-12 passage 1 (P1) virus. Single syncytia derived from infection of Vero cells by passaged viruses were screened for IL-12 expression by immunofluorescence using goat polyclonal anti-human IL-12 antibodies (R&D Systems) followed by anti-goat FITC-conjugate (Fig. 2a). No fluorescent signal was detected in syncytia induced by MV.

The expression kinetics of IL-12 were monitored in Western immunoblots. Monolayers of Vero cells grown in six-well plates were infected with MVIL-12 or MV. The cells were harvested after 8, 12 and 24 h post-infection (p.i.) on ice in lysis buffer (50 mM Tris–HCl, pH 8, 62.5 mM EDTA, 1% NP-40, 0.4% deoxycholate) supplemented with a cocktail of protease inhibitors (pefabloc 1 mg/ml, leupeptin 10 µg/ml, pepstatin 10 µg/ml, aprotinin 1 µg/ml and PMSF 100 µg/ml). The clarified lysates were mixed with equal volumes of PAGE sample loading buffer, boiled for 5 min and subjected to SDS–12% PAGE. The proteins were blotted onto Immobilon-P membranes (Millipore). The IL-12-specific p35 and p40 protein
subunits were revealed using goat anti-human IL-12 antibodies as first antibody and rabbit anti-goat HRPO conjugate as second antibody according to the ECL protocol (Amersham). The expression of p35 and p40 subunits gradually increased from 8 to 24 h.p.i. (Fig. 2b) in parallel with the advancing virus infection in monolayers reflected by increase in the CPE (not shown). These protein bands were not observed in the MV-infected Vero cell lysates.

In view of the gene expression gradient in MV, insertion of an additional gene is expected to result in slower replication of the virus. This effect has already been observed in our laboratory using marker genes such as green fluorescence protein, β-galactosidase and chloramphenicol acetyltransferase. Such viruses grow slightly slower and attain somewhat reduced titres in comparison with normal MV (L. Hangartner and others, unpublished results). Similarly, the replication of MVIL-12 was delayed by 12 h and yielded a final titre of $6.05 \times 10^6$ TCID$_{50}$/ml as compared to that of MV ($6.7 \times 10^6$ TCID$_{50}$/ml; Fig. 2c). The slower replication of MVIL-12 was also reflected in the reduced plaque size. MVIL-12 produced plaques with an average size of 0.48 mm as compared to MV which produced plaques of 0.93 mm (not shown).

To determine the stability of IL-12 expression, the progeny virus of a representative syncytium was serially passaged 10 times in Vero cells using an m.o.i. of 0.01, resulting in a final amplification factor of $2.56 \times 10^9$. The concentration of heterodimeric IL-12 secreted in the culture supernatants was estimated using an ELISA kit containing a capture antibody that recognizes only the IL-12 heterodimer and not the individual subunits of the heterodimer (R&D Systems). The viruses at P3, P7 and P10 secreted $10^6$ 1 6, $9.78 \times 10^6$ and $1.059 \times 10^6$ pg IL-12 per ml of culture supernatant from 10$^6$ cells using an m.o.i. of 0.1 (Fig. 3a). Increasing amounts of IL-12 were secreted in response to increasing m.o.i. used to infect the cells (Fig. 3b), confirming that IL-12 secretion is a function of MVIL-12 replication. Additionally, immunofluorescence assays showed IL-12-specific signals in more than 95% of plaques even after 10 passages (results not shown). These observations demonstrate that MV can accommodate complex IRES sequences in the context of additional coding sequences encompassing a total size of 2-3 kb. Moreover, the inserted genes are stably maintained and expressed uniformly after virus amplification of more than $10^{20}$ in cell culture.

The biological activity of the secreted IL-12 was ascertained by induction and secretion of IFN-γ from PBMCs. Monocyte-depleted PBMCs (2 x 10$^6$ cells/ml in 200 µl RPMI) were incubated with MVIL-12 supernatants containing 50, 250 or 500 pg/ml IL-12 or recombinant IL-12 (R&D Systems) in the presence of 3 µg/ml PHA (Sigma; Anderson et al., 1997). After 48 h the culture supernatants were collected to measure IFN-γ using a commercially available ELISA kit (R&D Systems). MV- and mock-infected Vero cell supernatants served as controls. Fig. 3(c) summarizes the results of this assay. Using 250 pg/ml recombinant IL-12 induced the production of 151 pg/ml IFN-
whereas IL-12 secreted from MVIL-12 induced 116 pg/ml IFN-γ. In supernatants of mock- or MV-infected cells, IFN-γ values of 18 and 22 pg/ml, respectively, were detected whereas the basal level of IFN-γ secretion from untreated PBMCs was 38 pg/ml. Note that PBMCs depleted of monocytes by plastic adherence might have contained contaminating DCs resulting in basal levels of IFN-γ secretion. When the assay was performed in the presence of anti-IL-12 antibody (10 µg/ml) to neutralize the IL-12, the level of IFN-γ secretion was reduced to control values. This confirmed that the IL-12 secreted from MVIL-12 was biologically active and specifically induced the secretion of IFN-γ from PBMCs in a dose-dependent fashion.

The effects of MVIL-12 on monocytes and DCs remain to be investigated. Experiments are under way to determine: (i) whether this virus can restore IL-12 production from monocytes, macrophages and DCs to normal levels; (ii) whether there is any alteration in the production of other cytokines; (iii) whether maturation and other functions of monocytes and DCs are affected; and (iv) whether the proliferative defects, apoptosis, virus spread or release and cytopathogenicity vary in PBMCs infected with MVIL-12 from those infected with MV. Considering the involvement of an alternative receptor for cell entry of wild-type MV (Bartz et al., 1996; Lecouturier et al., 1996; Murakami et al., 1998) as compared to the Edmonston B strain (used in our studies), it would also be interesting to determine the role of IL-12 in infection with recombinants expressing H protein derived from a wild-type strain. Moreover, it will be essential to investigate the effect of MVIL-12 on the overall immune response and its pathogenicity in in vivo studies. Such experiments can be done in monkeys where human IL-12 is known to be functional (Hoffman et al., 1997).

At present, our studies clearly demonstrate that MV can express foreign proteins, preserving their biological activities, without a severe deterioration of replication efficiency. Thus, MV appears well suited as a vector for delivery of proteins for immunogenic and therapeutic purposes. In a parallel study we show that MV can be used as a vaccine vector that can simultaneously immunize mice against MV as well as hepatitis B virus (HBV) surface antigen (M. Singh and others, unpublished results).
In addition to its potent immunomodulatory roles, IL-12 also seems to possess other biological properties such as antitumour activity (Brunda et al., 1996). Phase I clinical trials using recombinant human IL-12 show partial recovery of patients with advanced malignancies (Atkins et al., 1997). However, some of the individuals who received higher doses of IL-12 showed mild side-effects which are in principle typical of IFN-γ side-effects (Ryffel, 1997). IL-12 can also inhibit the replication of certain pathogens such as HBV and malaria parasites (Cavanaugh et al., 1997; Hoffman et al., 1997). A favourable outcome of in vivo studies on MVIL-12 would be encouraging to explore the possibility of using MVIL-12 itself as an anti-measles vaccine devoid of immunosuppressive activity. Alternatively, MVIL-12 enriched with additional transcription units encoding the proteins of other pathogens (such as HBV surface antigen) may simultaneously protect vaccinees against MV and other pathogens.

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References


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