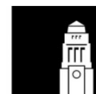

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Delivery of Oncolytic Reovirus by Cell Carriers

Elizabeth J. Ilett

Leeds Institute of Medical Research at St James'

St James' University Hospital

Leeds

LS9 7TF

e.ilett@leeds.ac.uk

Abstract

Oncolytic virus therapy is a rapidly expanding branch of cancer immunotherapy and represents a genuine opportunity to improve currently available treatment options. However, as single agents oncolytic viruses have shown only moderate clinical benefit and many challenges remain before their full potential is realized. Central to this is the efficient delivery of the virus to the tumour site and potentiation of the anti-tumour immune response. This chapter describes the loading of oncolytic reovirus onto monocytes which act as carriers for delivery of the virus to the tumour site and, as antigen presenting cells, may also thereby potentiate the development of an adaptive anti-tumour immune response.

Key words

Cell carriage, immune cells, reovirus, cancer immunotherapy, antibodies

1. Introduction

Oncolytic viruses (OVs) are those that preferentially infect and lyse tumour cells while sparing normal tissues. They were first developed as direct cytotoxic agents(1-3) but it is

now known that their anti-tumour activity also depends to a large extent on activation of the immune system(4, 5). The best route of delivery for OVs is not yet known; the route designed to maximize oncolysis may not best facilitate immune-mediated tumour clearance. As of 2018, the only FDA-approved OV (T-VEC) is administered by direct injection into the tumour (melanoma). This route ensures delivery of the full viral dose to the tumour tissue but the degree of anti-tumour immunity generated may be sub-maximal; observed responses to T-VEC were 15% in visceral metastases versus 64% in directly injected lesions(6). Perhaps more important, the use of intra-tumoural administration restricts the use of OV to those tumours that are readily accessible and many are not. Systemic delivery is safe, broadly applicable in a clinical setting, and more suitable for targeting visceral or widespread metastatic disease. However, virus delivered into the bloodstream faces inactivation by several mechanisms including scavenger cells in the blood or reticuloendothelial organs and immune molecules such as complement and virus-specific antibodies. Thus the virus faces rapid neutralization before it can ever reach the tumour. One method that has been investigated to protect the OV within the bloodstream is to load the virus onto or into carrier cells. Various cell types have demonstrated efficacy as carriers for OV delivery to tumours including, transformed cells, endothelial cells and immune cells(7-10). The benefit of using immune cells is the potential for a dual attack on the tumour. For example, using tumour-specific T cells for direct delivery of an oncolytic agent theoretically ensures specific delivery of the virus to the tumour target and, in addition, benefits from the cytotoxic potential of the carrier cell(11). Although expanding endogenous tumour-specific T cells from patients is still problematic, chimeric antigen receptor (CAR) T cells represent an attractive possibility as viral carriers(12). Alternatively, using antigen

presenting cells such as dendritic cells (DC) or monocytes as the viral carriers may enhance the development of an adaptive anti-tumour immune response(10, 13).

More recently, it has been shown that virus-specific neutralizing antibodies are not always a barrier to systemic OV delivery and tumour therapy. Reovirus, a non-enveloped, double stranded RNA virus, is taken up more efficiently by monocytes in the presence of anti-viral antibodies(14); this is dependent on antibody receptors expressed by these cells. Furthermore, while antibody-neutralized reovirus or Coxsackievirus are unable to infect and kill tumour cells directly, when loaded onto human monocytes these viruses can be delivered to tumour cells in a functional/replicative form resulting in cell lysis(15). The protocol for loading monocytes with reovirus or with reovirus-antibody complexes is described below. The same protocol is effective for the loading of other immune cell subtypes following their isolation by magnetic-activated cell sorting (MACS); T cells, DC and natural killer (NK) cells have all been loaded in this manner. After viral loading, the carrier cells can be co-cultured with tumour targets for 72-96 h to elicit tumour cell death.

2. Materials

2.1 Preparation of human cells

1. Human buffy coat/leukapheresis cone/fresh blood

2. PBS

3. Lymphoprep density gradient medium

4. Pasteur pipettes

5. human anti-CD14 MicroBeads or the Pan Monocyte isolation kit (*see* Note 1)

6. MACS Manual Separator

7. Magnetic cell separation LS columns

8. MACS buffer: PBS containing 1% (v/v) FBS and 2mM EDTA

2.2 Preparation of murine cells

1. Dissection equipment: scissors, tweezers, scalpel

2. Sterile 70% ethanol

3. Complete RPMI: supplement RPMI with 10% (v/v) FBS, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, Penicillin 100 U/ml, Streptomycin 100 μg/ml.

4. Mouse monocyte isolation kit (bone marrow)

5. MACS Manual Separator

6. Magnetic cell separation LS columns

7. MACS buffer: PBS containing 1% (v/v) FBS and 2 mM EDTA

2.3 Loading immune cells with reovirus

1. Complete medium as required for target cells

2. Complete RPMI

3. Reovirus

4. Anti-reoviral antibodies (optional, for generating antibody-neutralized reovirus complexes)

3. Methods

3.1 Preparation of cells

3.1.1 Preparation of human PBMC by density gradient separation

Carry out all steps at room temperature.

1. Dilute blood sample. Fresh blood and buffy coats should be diluted 1:1 with PBS, leukapheresis cones should be diluted with PBS to a final volume of 50 ml.
2. Layer blood onto lymphoprep at a ratio of 2:1, blood to lymphoprep. Add 15 ml lymphoprep per 50 ml Falcon tube and carefully layer 25 ml diluted blood on top.
3. Centrifuge at 800 g for 25 min with minimum acceleration and brake.
4. Remove white buffy layer gently with wide tipped Pasteur pipette. Combine 2 buffy layers into 1 and add PBS to 50 ml.
5. Centrifuge at 400 g for 10 min.
6. Discard supernatant and flick tube to loosen pellet. Combine cell pellets, add PBS to 50 ml and centrifuge at 300 g for 5 min.
7. Discard supernatant, re-suspend cell pellet in 50 ml PBS and make a 1 in 10 dilution for cell counting. Count PMBC.
8. Place the required number of PBMC into a fresh 50 ml Falcon tube, make volume up to 50 ml and centrifuge at 300 g for 5 min (*see* Note 2).
9. Discard supernatant. Cells are now ready for selection (*see* Note 3).

3.1.2 Preparation of murine bone marrow cells

1. Dissect out mouse hind legs removing as much muscle as possible and place in medium, on ice.
2. Put bones in 70% ethanol for 1 minute.

3. Put bones into fresh complete RPMI in 10 cm dish and separate and strip tibias and femurs, then remove both ends of the bones with a scalpel.
4. Transfer into fresh complete RPMI in a new dish and using a 21 G needle and 10 ml syringe. Pull up a few ml of medium and flush the marrow out each of bone into the dish.
5. Transfer cells and medium to a 50 ml Falcon tube and pipette up and down repeatedly to dissociate the cells. Pass through a 40 μm cell strainer into a fresh tube.
6. Add PBS to a final volume of 50 ml, take an aliquot of cells for counting and centrifuge the remainder at 400 g for 5 min, minimum brake (*see* Note 4).
7. Discard supernatant. Cells are now ready for selection.

3.2 Selection of Immune Cells (monocytes)

3.2.1 Human monocytes

1. Re-suspend the PBMC in the required volume of cold MACS buffer according to the manufacturer's instructions and add the appropriate volume of CD14 MicroBeads (*see* Note 5).
2. Incubate at 4°C for 15 min.
3. Add 10 ml cold MACS buffer and centrifuge cells at 300 g for 5 min. Discard the supernatant, re-suspend the cells in an appropriate volume of MACS buffer according to the manufacturer's instructions and add to a prepared LS column (*see* Note 6).
4. Apply cell suspension and allow to run through.
5. Rinse through 3 times with 3 ml MACS buffer.

6. Add 5 ml of MACS buffer to the column, immediately remove from the magnet and flush positive cells out into a fresh 30 ml Universal tube or 15 ml Falcon tube using the plunger.
7. Count CD14 cells. Verification of cell purity should be carried out by FACS analysis. The cells are now ready for loading with reovirus (*see* Note 7).

3.2.2 Murine monocytes

1. Re-suspend cells in 175 μl MACS buffer per 5 x 10^7 cells.
2. Add 25 μl FcR blocking reagent and mix well.
3. Add 50 μl antibody cocktail per 5 x 10^7 cells, mix well and incubate at 4°C for 5 min.
4. Add 10 ml MACS buffer per 5 x 10^7cells and centrifuge at 300 g for 10 min.
5. Re-suspend cells in 400 μl MACS buffer per 5 x 10^7 cells, add 100 μl microbeads per 5 x 10^7 cells and incubate at 4°C for 10 min.
6. Apply this solution directly to a prepared LS column and allow to run through.
7. Rinse through with 3 x 3 ml MACS buffer and collect eluate containing the untouched monocytes.
8. Count the monocytes. Verification of cell purity should be carried out by FACS analysis. The cells are now ready for loading with reovirus (*see* Note 8).

3.3 Loading immune cells with reovirus

1. Place the required number of immune cells into a 15 ml Falcon tube (*see* Note 9).
2. Add 10 ml of PBS to the tube, centrifuge cells at 300 g for 10 min and discard supernatant.

3. Add reovirus at the required multiplicity of infection (MOI) to the cells in the residual volume of PBS (*see* Note 10).
4. Incubate for 3-4 h at 4°C.
5. Add 10 ml PBS to the tube, centrifuge at 300 g for 10 min and discard the supernatant.
6. Repeat the PBS wash twice more to ensure complete removal of unbound reovirus.
7. Gently re-suspend the cell pellet in the required volume of complete RPMI and add the loaded carrier cells to the tumour targets (*see* Note 11).
8. Monitor tumour cell death.

3.4 Preparation and loading of reovirus-antibody complexes

Reovirus-neutralizing antibody complexes (reoNAb) have been generated using (i) whole serum from clinical trial patients receiving Reolysin® therapy or (ii) reovirus-specific mouse monoclonal antibodies(15).

1. To form the reoNAb complexes, incubate the reovirus with a neutralizing volume of the serum/antibody in a 500 µl eppendorf and incubate at 37°C for 2-3 h (*see* Note 12).
2. After incubation, store the reoNAb at 4°C (*see* Note 13).
3. ReoNAb loading onto immune cells is performed in exactly the same manner as described above for reovirus.

4. Notes

1. If the loaded monocytes are to be processed for electron microscopy the use of negative selection to isolate untouched monocytes is preferable as this avoids the

problem of bead debris in the preparation which can produce artefacts in the microscopy images.

2. Estimate the number of PBMC required from the expected frequency of the cell subset found within the whole PBMC population e.g. monocytes make up approx. 10-20% of human PBMC, T cells up to 70% and NK cells 5-10%. DC are rare (1-2%) but myeloid-derived DC can be obtained by culturing isolated monocytes in medium containing 800 U/ml human recombinant GM-CSF and 500 U/ml human recombinant IL-4 for 5-6 days. Anticipate up to 30% cell loss to occur during the culture period. The cells will have an immature DC phenotype and can be used for viral loading or matured with a suitable stimulus prior to use. We routinely loaded 2×10^6 carrier cells with reovirus for each condition required.
3. Cell separation is best carried out using fresh samples rather than frozen. However, buffy coats and leukapheresis cones can be stored at room temperature overnight prior to PBMC preparation.
4. Typical count of total BM cells = $4 - 5 \times 10^7$ cells per mouse.
5. For positive selection of monocytes using CD14 MicroBeads, it is possible to obtain efficient isolation using only 5 μl of the beads per 10^7 cells rather than the recommended 20 μl. We have only verified this for Miltenyi CD14 MicroBeads.
6. 2 LS columns are required per whole buffy coat/leukapheresis cone
7. If required, isolated cells can be cultured overnight in RPMI/10% FBS/2 mM L-glutamine prior to loading with virus.
8. If DC are required the monocytes can be cultured in RPMI complete medium (no Pen/Strep) containing 20% L929 conditioned medium as a source of GM-CSF. The cells should be viewed daily and fresh medium added as required. At day 5 - 6 the

cells will have an immature DC phenotype. They can then be used for viral loading or matured with a suitable stimulus prior to use.

9. 15 ml polypropylene Falcon tubes give more efficient retrieval of cells following viral loading than 30 ml Universal polystyrene tubes. Fewer than 2 x 10^6 cells per tube is not recommended as the cell pellet is easily lost during the post-loading wash steps.
10. MOI ranging from 1-10 with respect to the target cells have been used successfully(10, 13, 15). Coxsackievirus A21 has also been loaded efficiently using this protocol(15).
11. Tumour target cells should be seeded into multi-well plates 24 h prior to the addition of the carrier cells. Routinely 3 x 10^5 melanoma cells were seeded into wells in 6-well plates. Immediately prior to adding the loaded carrier cells, the medium in the wells should be removed and replaced with 2 ml fresh complete medium. Virus-loaded carrier cells were then added at a 4:1, 2:1 or 1:1 ratio, carrier cells to targets, in a volume of 500 - 700 μl.
12. The amount of serum/antibody required should be determined by a standard neutralization assay on L929 cells and confirmed by the absence of cell death in target cells treated directly with reoNAb. In general, a reovirus to serum ratio of 1:10 was used for the formation of reoNAb complexes; this was in excess of the volume that was found to be completely neutralizing for all sera tested.
13. Pre-formed reoNAb complexes can be stored at 4°C for up to 12 h prior to use.

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