The Matrigel Cytokine Assay

Ole Audun Haabeth (o.a.haabeth@medisin.uio.no)
Bjarne Bogen`s Lab, Centre for Immune Regulation, University of Oslo, Oslo University Hospital Rikshospitalet

Bjarne Bogen
Centre for Immune Regulation, University of Oslo, Oslo University Hospital Rikshospitalet

Alexandre Corthay
Centre for Immune Regulation, University of Oslo, Oslo University Hospital Rikshospitalet

Method Article

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Abstract

Cytokines represent a diverse group of soluble proteins that play a key role in a number of physiological processes, including regulation of both the innate and adaptive immune responses. Cytokines are generally secreted in small amounts, and are relatively unstable. Therefore, detection and measurement of cytokine local concentrations in a tissue extracellular matrix can be challenging. To investigate cytokines involved in cancer prevention by tumour-specific CD4+ T cells, we have developed a strategy consisting of embedding subcutaneously injected tumour cells in a collagen gel \(Matrigel) \[1]. Matrigel is a soluble basement membrane isolated from the Engelbreth-Holm-Swan \(EHS) tumour \[2]. This extract resembles the complex extracellular environment found in many tissues. The Matrigel is liquid at 4°C but when incubated at room temperature, the Matrigel proteins self-assemble forming a gel plug. In short, the Matrigel functions as an extracellular matrix in which infiltrating immune cells \[3] and secreted cytokines can be analyzed at various time points after s.c. injections \[1].

Reagents

• Cold sterile Phosphate-Buffered Saline \(PBS 14040-117, Gibco) • Ice-cold liquid growth factor reduced Matrigel \(356231, BD Biosciences) • RPMI 1640 cell culture medium \(61870, Gibco) • Collagenase IV from Clostridium histolyticum \(C5138, Sigma-Aldrich) • Deoxyribonuclease I from bovine pancreas \(D5025, Sigma-Aldrich) • 70 % Ethanol • Hypno/dormicum: hypnorm \(Janssen Pharmaceutica) and midazolam \(Alpharma) 7.5mg/kg. • Tryptan blue solution 0.4% \(T8154, Sigma-Aldrich)

Equipment

• 50ml centrifuge tubes \(430829, CORNING) • Scissors • Petri dish, TC dish 60 x 15 Nuncleon D SI \(150326, NUNC) • Stainless steel sieve with screens for CD-1, size 60 mesh \(CD1-1KT, Sigma-Aldrich) • 1.5ml Eppendorf tubes \(72.690.00.1 SARSTEDT) • 2ml syringe \(2015-10, BD Biosciences) • 1ml syringe \(2015-07, BD Biosciences) • 0.6mm x 25mm needle \(2015-05, BD Biosciences) • Acrodisc 25mm Syringe filter with 0.45µm HT + Tuffryn membrane \(2012-05, PALL) • Centrifuge \(HAERUS MULTIFUGE 3SR+, THERMO SCIENTIFIC) • Count slide \(87144, KOVA) • Pipettes **Note:** Before use, Matrigel should be thawed slowly at a low heat level \(e.g. overnight in the fridge at +4°C). Keep the Matrigel, the tumour cells and all the equipment cold \(+4°C). Remember that the Matrigel solution is liquid at +4°C but that it gels at room temperature.

Procedure

Injection of Tumour Cells in Matrigel : 1. Wash the cancer cells 1X in sterile ice cold PBS. 2. Count cells. Adjust to a suitable concentration, e.g. $4x10^6$/ml. 3. Anesthetize the mice with hypno/dormicum intraperitoneally or subcutaneously. 4. Mix on ice 1125µl Matrigel + 125µl tumour cells \(i.e. 10X dilution). 5. Inject, subcutaneously, carefully 250µl ice-cold tumour cell/Matrigel solution using a 1ml syringe with a 0.6mm x 25mm needle \(This will give 10^5 cancer cells per Matrigel plug if the original concentration was

4 x 10^6/ml). **Preparation of Matrigel samples for extracellular matrix cytokine detection and flow cytometry analysis:** **Note:** During the whole procedure, keep samples on ice as much as possible. 1. Sacrifice the mice 2. From each mouse, dissect carefully the Matrigel plug and transfer it into a 50ml tube with 1ml cell medium RPMI1640 supplemented with 0.3mg/ml DNase and 1mg/ml collagenase IV. 3. Cut the Matrigel plug in small pieces using sterile scissors. 4. Put sample on ice. 5. Clean dissection equipment and scissors between each mouse. 6. Go back to step two until all Matrigel plugs are collected. 7. Place the tubes in a water bath for 30 minutes at 37°C. 8. After 30 minutes, place the samples on ice and keep them on ice as much as possible. 9. Mesh the Matrigel samples through a stainless-steel sieve onto the lid of a 5cm Petri dish, and transfer the solution with a pipette to a 1.5ml Eppendorf tube. 10. Clean and burn mesh, using 70% ethanol, between each Matrigel sample. 11. Centrifuge the tubes for 7 minutes 300g. 12. Remove the supernatant with a pipette and transfer to a clean 1.5ml Eppendorf tube. 13. Prepare the cell-pellet for flow analysis. 14. Filter the supernatant through a 0.45µm filter. 15. Aliquot the samples in 1.5ml Eppendorf tubes \(e.g. 50µl per tube). Freeze immediately at -70° C or below and keep samples frozen until analysis with ELISA or luminex technology. **Note:** After incubation I _step 7_, you will still observe some Matrigel clumps in the sample. These clumps are easily meshed through the stainless sieve. After meshing \(_step 9_) you will loose the rest of the Matrigel collagen fibers in centrifugation \(_step 11_) and filtering \(_step14_). Cytokines in Matrigel extracellular matrix are diluted approximately five times in _step 2_ \(1ml RPMI + ~250µl Matrigel plug). Final concentration is therefore found by multiplying observed concentration with 5. A starting volume of 1ml RPMI + Matrigel plug will typically give 400-700µl filtered supernatant.

Timing

Injections: -1-2 hours \(for 24 Matrigel plugs) **Collection of Matrigel plugs:** -usually takes 1-2 hours depending on the number of mice. Generally, you can expect to spend approximately 5 minutes on each mouse. **Preparation of the Matrigel samples from water-bath to freezer:** -takes 2-3 hours depending on the number of samples. You can expect to use 3-4 minutes on meshing one sample + cleaning between each sample.

Troubleshooting

Air bubbles in the Matrigel: a) As Matrigel is a low viscosity liquid, you should be very careful when mixing it with your cells and injecting it in the mice. b) Treating it to roughly may lead to air bubbles in the mixture. These air bubbles are very difficult to get rid of once inside the Matrigel solution in the syringe. Air bubbles in the syringe will give you an incorrect injection volume, and may cause the Matrigel to disintegrate once inside the mouse. c) We have experienced that if you thaw the Matrigel overnight at 4°C, it will be easier to handle. **Clogging of the needle:** a) If the Matrigel/cell mixture reaches room temperature while inside the syringe, it will clog your needle. Keep the syringe and needle on ice before use. **Very low levels of cytokines:** a) Cytokines are relatively unstable and generally have a short half-life. To prevent degradation of cytokines in your samples, keep samples on ice as much as possible

during preparation. This reduces necrotic cell death and the subsequent release of proteases that may degrade the cytokines. b) Aliquot the samples before freezing to avoid a reduction in the level of cytokines due to repeated freezing and thawing. c) When samples are ready, freeze immediately at -70°C or below.

References

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Figures

Figure 1

Example of comparison of cytokine levels in Matrigel. Tumour-specific mice where injected with either 105 MOPC315 (n=6) or 105 Antigen-loss (Ag-loss) MOPC315 (n=6) myeloma cells in Matrigel. Samples where prepared using the Matrigel cytokine Assay, and the concentration of cytokines measured in Matrigel using the luminex technology. N.D. not detected.