L-selectin-PNAd Interactions under Flow Conditions.

Flow Chamber Assay

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Purpose

The main purpose of the flow chamber assay is to visualize and measure interactions between flowing cells expressing a given adhesion molecule on their surface, and their receptor, either directly coated on the flow chamber lower wall or expressed on a cell monolayer.

The present protocol will focus on the interactions between L-selectin expressed on neutrophils and PNAd coated onto the plastic surface.

For neutrophil purification see Purification of human mononuclear cells and neutrophils

Materials

- Purified human PNAd (from tonsil lysates).
- 50 mM Tris pH 9.5, 0.1 % n-octyl-β-D-glucopyranosil, 0.02% sodium azide.
- 2 % Human serum albumin (HSA).
- Polysterene dishes.
- 9.5 g Hanks' balanced salts diluted in 1 L 20 mM Hepes solution. Adjust pH to 7.4.

Procedure

Coating with PNAd

1. Dissolve purified PNAd in 50 mM Tris pH 9.5, 0.1 % n-octyl-β-D-glucopyranosil, 0.02% sodium azide buffer, to the required concentration .
2. Place 10-30 µl of PNAd dilution on the center of a polysterene dish. With a permanent marker make a spot on the outer face of the dish to locate the coating area throughout the experiment.
3. Place the dish on a humid box and incubate for 1 hour at 37 ºC.
4. Wash the spot by aspirating the PNAd solution and immediately apply 20-50 µl of the Tris Buffer. Be careful not to touch the surface or leave it dry.
5. Aspirate the washing buffer and cover the area with 100-500 µl of 2% HSA diluted in Tris buffer.
6. Place the plate into the humid box and incubate at 37 ºC for 1 hour to o.n.
7. After gently washing the surface with Tris buffer, dishes can be kept at 4 ºC for several hours.

Flow chamber

1. Assemble the flow chamber to the polysterene dish, placing the coated area on the center of the chamber.
2. Set the pump connected to the outlet tube of the chamber at a refilling mode of 2 ml/min. Fill the chamber with the running buffer, Hank's buffer, making sure that no air bubbles are remainig into the chamber. To avoid bubble growth in the chamber, make sure the buffer is at room temperature and that the vacuum efficiently shields the chamber to the dish.
3. Start infusion of purified neutrophils into the chamber at high rate. Once the neutrophils are in the microscopic field, reduce the shear to 0.4 dyn/cm^2, and progressively increase it depending on the purpose of the experiment.
4. Images can be recorded onto a video tape, and analyzed off-line.

A 10X objective is recommended for visulization of neutrophils.

Expected results

Depending on PNAd coating site density stable or rolling or transient tethers (at lower densities) should be detected at shear stresses ranging between 0.8 to 1.5 dyn/cm^2.

No firm adhesion should be observed. Adherent cells could be due to unspecific interactions of neutrophils with the surface (stronger blocking conditions should be tested) or to cell activation (perform the experiment immediately after neutrophil isolation and keep neutrophils on ice).