INVITTOX Protocol No. 71

THE FLUORESCEIN LEAKAGE TEST

Damage caused by the test compound to the tight junctions in MDCK monolayers is determined by the amount of fluorescein which leaks through the cell layer and is an indication of potential to cause eye irritancy.

Contact

Dr Richard Clothier
FRAME Alternatives Laboratory
Department of Human Anatomy and Cell Biology,
Queen's Medical Centre
NOTTINGHAM NG7 2UH, UK
Tel: +44-115 9709432
Fax: +44-115 9709732

NOTE
The protocol presents the standard operation procedure used in the Home Office UK/EEC Validation Study for Alternatives to the Draize Test. It should be noted that this protocol might need to be modified in light of experience gained in the study.

Rationale

This method is based on that of Tchao (1988). The loss of trans-epithelial impermeability of a confluent monolayer of Madin Darby Canine Kidney (MDCK) cells through the effect of irritant materials is measured by the leakage of fluorescein across the cell monolayer.

The integrity of trans-epithelial permeability is a major function of an epithelium such as that found in the conjunctiva, the corneal epithelium and the skin. Trans-epithelial permeability is controlled by various inter-cellular junctions. The zonular occludens or tight junctions form a seal at the apical side of the epithelium and effectively prevent the passage of most molecules except water and some inorganic ions. The maculae adherens or desmosomes provide the strength in cell to cell connection as well as being permeability barriers.

In eye irritancy, an initial physiological event may be the loss of the impermeability of the corneal epithelium and the conjunctiva, allowing the irritant access to the underlying stroma (maybe causing the corneal opacity). The tight junctions are disrupted and desmosomal junctions separate, leading to loss of cell contact and possible cell detachment.

Various epithelial cell types in culture, including the established MDCK cell line, also develop these junctions and, like the corneal epithelium, in confluent monolayers can form an impermeable barrier to most chemicals, including the non-toxic dye, fluorescein, which is also used to assess the loss of corneal epithelium in humans. Using MDCK cells grown to confluence on porous filters, chemically induced loss of trans-epithelial

impermeability is determined by measuring the "leakage" of fluorescein through the cellular layer. Thus, this assay models the disruption of the integrity of the corneal epithelium by chemicals which may be potentially irritant to the eye.

Basic Procedure

MDCK cells are maintained in culture and exposed for a short period, such as one minute, to five fixed concentrations of test compound. The amount of damage caused to the tight junctions is determined by the amount of fluorescein which leaks through the cell layer over a set period of time. This is compared to two controls: the amount of fluorescein which leaks through an untreated confluent monolayer, referred to as 0% leakage or the blank; and the amount of fluorescein which leaks through an insert on which there are no cells, referred to as maximum leakage. The percentage of leakage and therefore amount of damage to the tight junctions is expressed, relative to these controls, for each of the set concentrations.

Critical Assessment

One of the advantages of this test is that it may be used to assess acute effects as well as to assess recovery of the cell monolayer from damage. Fluorescein leakage may also be assessed using multi-layers such as those currently being developed as 'skin equivalents'.

There are certain limitations with the assay, mainly concerning the solubility of the compound to be tested.

During testing procedures, in particular during the washing/aspiration cycles the membranes can be damaged if care is not taken, giving false results. This can be determined by examining the inserts before the beginning and the end of the initial procedures, prior to the recovery phase and when retesting after recovery. At the end of the assay the filters should be closely examined for damage.

References

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Procedure Details

Equipment

Incubator - 37°C, humidified, 5% CO_2/95% air.
Tissue culture flasks
24-well tissue culture plates
Inserts, for 24 well plates, Millicell-HA, 12mm, 0.45m pore size - Millipore Ltd

N.B. Anocell 10 inserts (Whatman) can also be used)

Cytofluor fluorescence measurement system, excitation 485 nm, emission 530 nm

Cell Line

MDCK (CB997):
These are obtainable from the European Collection of Animal Cell Cultures (ECACC), PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury SP4 0JG, Wilts, UK
ECACC Number 84121903

Materials

Hank's balanced salt solution (HBSS), without phenol red, 500 ml, no. 041-04025M - Gibco Ltd or from powder, without phenol red, no. H1387 -

Sigma Chemical Co. Ltd

Trypsin-EDTA (0.05% w/v Trypsin, 0.02% w/v EDTA), 1X prepared in modified Puck's saline, 100ml, no. 043-05300H - Gibco Ltd

Dulbecco's Modified Eagle's medium/Nutrient Mix F12 (1X concentrate with L-Glutamine, and containing 15mM HEPES), 500 ml, no. 041-01330M - Gibco Ltd

Foetal calf serum (FCS), heat inactivated, 500 ml, no. 013-06290M - Gibco Ltd., UK

Streptomycin sulphate, Sigma no. S6501 - Sigma Chemical Co. Ltd

Benzylpenicillin (Penicillin G, sodium salt), Sigma no. PEN-NA - Sigma Chemical Co. Ltd

Amphotericin B, solubilised (Fungizone), Sigma no. A 9528 - Sigma Chemical Co. Ltd

Light mineral oil, Sigma no. M3516 - Sigma Chemical Co. Ltd

Brij 35, Aldrich no. 85,836-6 - Aldrich Chemical Co. Ltd

Fluorescein, disodium salt, water-soluble, Aldrich 16,630-8 - Aldrich Chemical Company Ltd, or Fluorescein, disodium salt, water-soluble, Sigma F6,377 - Sigma Chemical Co. Ltd

Make up the following:

Dulbecco's supplemented medium
Composition per litre:
900 ml Dulbecco's MEM/Nutrient Mix F12
100 ml FCS
100 mg streptomycin sulphate
100,000 IU benzylpenicillin
1 mg amphotericin B

Store at 4^{o}C. It is recommended that the medium be used within two weeks.

Fluorescein solution, 0.01% (w/v)
Dissolve 50 mg fluorescein in 500ml HBSS.

This solution may be stored at room temperature. It is recommended that the fluorescein solution is stored in the dark, since it may lose its fluorescence within one week under exposure to light.

N.B. If using Anocell inserts, make up a 0.02% fluorescein solution.

Test compounds
All compounds to be tested are made up in sterile HBSS from a stock

solution, at 5 set concentrations: 1, 25, 100, 250 mg/ml, and a neat or a saturated solution. When testing a solid material a very high concentration, 750 mg/ml, should also be included. This concentration of material may have to be pipetted using a positive displacement pipette. A fresh stock solution should be prepared for each experimental run and used within 30 minutes of preparation. Dilute both solid and liquid test materials on a weight per volume basis. Make up enough test material for 3 inserts per concentration tested. One test material is tested per 24-well plate. If the toxicity is found to be between 25 and 100 mg/ml, the following concentrations should be tested: 1, 25, 50, 75, 100 mg/ml. However, if the toxicity is below 1 mg/ml, the following concentrations should be tested: 0.01, 0.1, 0.25, 1, and 10 mg/ml.

Use of mineral oil
Test the solubility of the chemical at 250mg/ml in HBSS prior to testing in the FL test. If the chemicals forms a stable suspension or emulsion, over 30 minutes, at this concentration HBSS can still be used as the solvent. However, if the material is found to be insoluble in the HBSS at this concentration light mineral oil should be used as the solvent.

N.B. Once a concentration range has been found which includes the FL20 value, the same range of concentrations should be tested twice more. This means that the FL20 value is determined from three definitive experimental runs. A different flask of cells is required for each definitive experimental run.

Positive control
100 mg/ml Brij 35. This concentration of material should give approximately 30% damage to the cell layer. Results are acceptable if twenty to forty percent damage is seen, if not the results should be discarded.

N.B. Physical methods for aiding solubilization can cause degradation of the test chemical and so must be kept within certain guidelines:

(i) Vortexing the solvent with the chemical should be done for up to a maximum of 10 minutes.
(ii) Sonication can only be done for up to a maximum of 10 minutes, as prolonged sonication heats the solution which may cause degradation of the test material.

All glassware, solutions etc. are sterile and all procedures are carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

METHOD

Cell maintenance and culture procedures
Stocks of MDCK cells can be stored in sterile screw-top vials in liquid nitrogen or -80°C freezer, following suspension in FCS containing 5% DMSO as a cryoprotective agent. The cells may be centrifuged for short periods, i.e. 5 minutes, at low speeds (approximately 100 x g.), prior to making up this suspension, the concentration of which is approximately 1 x 10^6 cells/ml. Cells are frozen down in 1 ml aliquots.

When required the cells should be rapidly thawed at 37°C in a water bath, suspended in fresh medium and centrifuged for 10 minutes at approximately 100 x g. Then the pellet of cells is resuspended in 25 ml of fresh medium and placed in a sterile tissue culture flask. Once the cells have attached over night, the medium should be replaced i.e. approximately 25 ml in a 75 cm^2 tissue culture flask.

When the cells are 70-80% confluent, they should be removed from the flask by trypsinisation:
Decant the medium and rinse the cultures twice with PBS at 37°C.
Add 1 ml of Trypsin-EDTA and incubate at 37°C for approx. 10 min.
Give the flask 2 or 3 sharp taps to detach the cells into a single suspension. (If at this time no cells detach, the flask is left for a longer time until they do.)
Add approximately 10 ml culture medium to prevent enzymatic damage.
Centrifuge the cells for 5 minutes at 1000 x g.
Resuspend the pellet in a known volume of fresh medium.

Dilute the cell suspension with medium at 37°C, to give a final concentration of 4 x 10^5 cells/ml, i.e. ~2 x 10^5 cells/insert.

N.B. Cells should always be passaged from sub-confluent flasks

When the cells are ready the assay is set up as follows (Day 0):
Aliquot 400µl of fresh medium into each well of a 24-well plate.
Carefully place an insert into each well.
Aliquot 400 µl cell suspension into 21 of the inserts.
Aliquot 400 µl culture medium alone into the remaining 3 inserts.
Agitate the plate gently to evenly spread the cell suspension.

N.B. If the plates are not agitated gently the cells may rise up and spread up the sides of the insert.

The cells are grown to confluence over 96 hours. Replace medium in the wells and in the inserts 72 hours (Day 3) after the cells are initially seeded.

b) Treatment of monolayer with test chemical
Once confluent, the cells are ready for treatment. (Day 4)

Plate lay-out:

M	M	M	B	B	B
P	P	P	T1	T1	T1
T2	T2	T2	T3	T3	T3
T4	T4	T4	T5	T5	T5

where:
M = maximum leakage of fluorescein through the membrane, with no cells.
B = blank, 0% leakage through the intact cell monolayer.
P = positive control on the tight junctions. 100 mg/ml Brij 35.

T1-5 = concentrations of test compound (1 being the lowest).

Insert	Concentration (mg/ml)		
T1	1	1	0.01
T2	25	25	0.1
T3	100	50	0.25
T4	250	75	1
T5	neat or saturated solution	100	10

Make up test materials as previously outlined under Test chemicals.
Gently aspirate the medium from the inserts, without disturbing the monolayer.
Add 200 µl of test compound (3 inserts per concentration) to inserts T1-5.
Add 200 µl sterile HBSS to control inserts, i.e. 3 inserts labelled B which contain cells; and 3 inserts labelled M, which contain no cells.
Add 200 µl of the positive control, Brij 35 (100 mg/ml), to 3 inserts labelled P.
After 1 minute exposure, remove the test compound, positive control, etc., by aspiration.
Wash the cell monolayer twice with 400 µl sterile HBSS.

c) Determination of permeability to fluorescein
The percentage of fluorescein which leaks through the cellular monolayer gives a quantitative indication of damage to the tight junctions.

Remove inserts which have been washed to a fresh 24-well plate, each well containing 400 µl warm HBSS.
Into each insert aliquot 400 µl fluorescein solution.
Leave at room temperature for 30 minutes.
Carefully remove inserts, using tweezers, from each well and visually check each filter for any damage which may have occurred during handling. Record any damage which may be seen.

At the end of the 30 minute incubation with fluorescein solution, and after removal of the insert, read the resulting fluorescein solution using a Cytofluor (excitation filter 485nm, emission filter 530nm).

From the fluorescence intensity values obtained, the percentage of fluorescein leakage can be determined for (the fixed concentrations) in comparison to the leakage of fluorescein across the filter of the insert alone (i.e. maximum leakage).

N.B. The sensitivity of the Cytofluor is set so that there is the highest numerical difference between the maximum fluorescein leakage (insert with no cells) and the minimum fluorescein leakage (insert with untreated confluent cell monolayer). The maximum fluorescein leakage value should not be greater than 9999.

TREATMENT TIME-TABLE

DAY	Protocol

		EXPT. 1	EXPT. 2	EXPT. 3	EXPT. 4
0	Monday	seed inserts			
1	Tuesday				
2	Wednesday				
3	Thursday	refeed MDCK monolayers			
4	Friday	treat monolayers with test chemical	seed inserts		
5	Saturday				
6	Sunday				
7	Monday		refeed MDCK monolayers	seed inserts	
8	Tuesday		treat monolayers with test chemical		
9	Wednesday				
10	Thursday			refeed MDCK monolayers	
11	Friday			treat monolayers with test chemical	seed inserts, etc...

For each experimental run six chemicals may be tested by one person. The time-table allows one person to test all 60 chemicals within a 5 week period.

Results

Calculate:
The mean maximum leakage fluorescence intensity = x

The mean 0% leakage fluorescence intensity = y
(this can also be considered as the background leakage or blank)

100% leakage (by subtracting the 0% leakage from the maximum leakage),

i.e. x - y = z

The percentage leakage for each fixed dose. Simply mean the fluorescence intensity readings, subtract the 0% leakage and divide by the 100% leakage.

%FL = [(m-y)/(z)] *x 100%*

where:
m = the mean fluorescence intensity of the concentration involved.
%FL = The percent of the fluorescein which leaks through the cell layer.

To calculate the concentration of chemical which causes 20 and/or 50% damage to the cell layer.

FL_D = [(A-B)/(C-B)]x(M_C - M_B) + M_B

where:
D = % inhibition.
A = % damage (20 or 50% fluorescein leakage)
B = % fluorescein leakage < A
C = % fluorescein leakage > A
M_C = Concentration (mg/ml) of C
M_B = Concentration (mg/ml) of B

If the toxicity is found between 250mg/ml and the undiluted sample, you should be aware, from the weight/volume calculation of the dilution, what the weight is of 1 ml of undiluted material. Hence the apparent concentration of 1 ml of undiluted material should be used in the calculation of the result of an FL20 value.

It should also be noted that the toxicity curves may not be linear or increasing throughout the full range of concentrations tested. For example, the percentage fluorescein leakage seen with undiluted test material could be lower than that for 100 or 250mg/ml - the FL20 might be 75 mg/ml with the 250 mg/ml and undiluted material giving 15% and 5% respectively.

If the FL20 is not reached at any concentration inclusive of the undiluted material, then the maximum percentage fluorescein leakage should be quoted along with the concentration of chemical causing this, for example, 100mg/ml (FL 18.5%), or undiluted sample with calculated 955mg/ml (FL 3.2%).

Acceptance of results

Control values
Because of the individuality of each Cytofluor it is suggested that a sensitivity is used which will give a maximum fluorescein leakage value of >4000.

CytoFluor
0% leakage, y = <300
100% leakage, z = 3500 - 6000

Reporting of results
For the purposes of this validation scheme results should be calculated by

the above formula and not any other analysis programme. Incompatibilities between the test sample and the filter or o-ring of the filter insert should also be reported alongside numerical data, since the effect seen may be due to chemical interacting with the filter materials.

EXAMPLE CALCULATION

Test chemical results

Test chemical concentration (mg/ml)	% Fluorescein leakage
1	15
25	46
100	62
250	98

i) to calculate FL20 (mg/ml)

FL20= [(20-15)/46-15)] x (25 - 1) + 1

= (5/31) x 24 + 1

= 4.87 mg/ml

ii) to calculate FL50 (mg/ml)

FL50 = [(50-46)/(62-46)] x (100 - 25) + 25

= (4/6) x 75 + 25

= 44 mg/ml

iii) If the FL20 value is not reached by any concentration tested then the concentration (mg/ml) for the maximum percentage fluorescein leakage exhibited should be reported along with this FL value.

FLUORESCEIN LEAKAGE SUMMARY

CELL CULTURE	
MDCK Cells	ECACC - 84121903
Culture medium	DMEM : Ham's F12 (1 : 1)
Serum	10% FCS (heat-inactivated)

Other	benzylpenicillin, streptomycin, amphotericin B
Filter insert	Millicell-HA, 12mm, 0.45µm (Millipore)
Treatment of insert prior to seeding with MDCK cells	no
Cell seeding density	4 x 10^5 cells/ml (400 µl per insert)
Days to confluence	96 hours
Comments on growth of monolayer	refeed after 72 hours
TESTING PROCEDURE	
Rinsing procedure prior to treatment of monolayer with test chemical	none
TEST CHEMICAL: Amount and dose range tested	200 µl of test chemical in HBSS or light mineral oil 1 mg/ml or 1 mg/ml or 0.01 mg/ml 25 mg/ml or 25 mg/ml or 0.1 mg/ml 100 mg/ml or 50 mg/ml or 0.25 mg/ml 250 mg/ml or 75 mg/ml or 1.0 mg/ml neat or or 100 mg/ml or 10 mg/ml saturated solution N.B. use 750 mg/ml if testing a solid
	5 concentrations/chemical 3 inserts/concentration 1 chemical/plate
Exposure period to test chemical (minutes)	1/15
Temperature of plate at exposure	room temperature
REFERENCE CHEMICAL: Amount and dose range tested	100 mg/ml Brij 35
FLUORESCEIN LEAKAGE ASSAY	
Washing procedure before addition of fluorescein	2x with HBSS
Addition of fluorescein	inserts moved to 24-wp containing 400 µl HBSS; 400 µl 0.01% Na-fluorescein in HBSS added to insert

Incubation period with fluorescein (minutes)	30
Incubation temperature	room temperature
Determination of leakage	fluorescence intensity measured on Cytofluor 2300 fluorescent plate reader, excitation 485nm, emission 530nm
Calculation and expression of results	% fluorescein leakage vs. chemical concentration concentration of test chemical causing 20% fluorescein leakage (FL20) and 50% fluorescein leakage (FL50) calculated or read from graph

LABORATORY NUMBER: ASSAY: FLUORESCEIN LEAKAGE TEST

FINAL RESULTS (DEFINITIVE EXPERIMENTAL RUNS) - FL20 (mg/ml)

CHEMICAL NUMBER	DATE (dd/mm/yy)	ENDPOINT FL20 (mg/ml)	SOLVENT H or M	COMMENTS
	Mean +/- SEM			
	Mean +/- SEM			

	Mean +/- SEM			
	Mean +/- SEM			
	Mean +/- SEM			