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DRUG STUDY PROCEDURE

1. The HEL cells cultured in 12 or 24 well tissue culture plates with MEM containing 10% FCS, 1% NEAA, 1% pen-strep, 0.1% lactalbumin hydrolysate and 1 mM Na pyruvate. Cells are incubated at 35° in 5% CO_2.

2. When the cells are confluent (usually 5-7 days), drugs to be tested are diluted to the desired concentration in fresh tissue culture media (without Na pyruvate & lactalbumin hydrolysate).
   a) Fresh media is prepared and warmed.
   b) Plates are labelled. Four wells for each parameter for each day.
   c) Drugs are weighed, solubilized, and filter sterilized (stocks in DMSO cannot be filtered).
   d) Drugs are diluted to appropriate concentration in the fresh media. Prepare 10 mL (20 mL for 12-well plates) of media with drug for each parameter. Keep in incubator so media does not get cold and basic.

3. Fresh inoculum is obtained from the lungs of a rat heavily infected with P. carinii.
   a) A piece of lung weighing approximately 100 mg is ground in 6-15 ml in a Ten-Broek tissue homogenizer and spun at 400 X G for 5 minutes.
   b) The supernatant is decanted into a sterile tube and the P. carinii in supernatant are counted by placing 10 μL of supernatant onto a 1 cm² area etched onto a slide. The slide was air dried, fixed in MeOH and stained in Giemsa for 1 hour. The concentration desired is 15-20 trophs/1000X field (so final concentration on monolayers will be approximately 7 X 10^5 trophs/mL).

4. Media is suctioned from the wells of the plates to be used for the study.

5. Inoculum is added to the tubes of media containing drugs to be tested, 1:10 dilution (or the necessary dilution to achieve the desired final concentration).

6. This inoculated media containing drug dilutions is then immediately aliquoted into the wells, 0.5 mL/well (1.0 mL for 12-well plates).

7. The cultures are incubated at 35° in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2.

8. Cultures are sampled on days 1,3,5 and 7 post inoculation
   a) Draw the media into a sterile Pasteur pipette and rapidly expel it back into the well (repeat 3-5 times).
   b) 10 μL of supernatant from each well is put on a 1 cm^2 area etched in a microscope slide. Slides are dried, fixed, and stained with Giemsa.
   c) 300 μL of supernatant is collected from each well for ELISA (Combine samples from the 4 wells of the same parameter) into a microfuge tube. Samples are pelleted @ 8,000 rpm for 4 minutes, washed with 100 μL PBS-azide, pelleted as above, resuspended in 100 μL PBS-azide, and refrigerated until Day 8.

Spinner Flask Detailed Procedure
Cytodex 2,3

SILICONIZATION OF GLASSWARE

Cytodex sticks to everything!!! All glassware which comes into contact with beads must be siliconized. (SIGMACOTE cat. #SL-2) Siliconization must be done under the hood, because the fumes are toxic. Also, gloves must be worn because the liquid is caustic until dry.

To siliconize glassware, pour liquid from SIGMACOTE bottle into the glass container you wish to siliconize, make sure it coats all surfaces which come in contact with the beads. Pour excess fluid back into the SIGMACOTE container or into the next container to be siliconized, It doesn't take much fluid to be effective. SIGMACOTE should always be refrigerated.

HYDRATION OF BEADS

Rehydrate beads at a ratio of 3 mg beads per ml of final volume, (a 125 ml flask takes 375 mg beads.)

Use Ca and Mg free PBS to rehydrate. One recipe is below:

NaCl	7.65 g
Na_2HPO_4	0.724 g
KH_2PO_4	0.210 g

dissolve in 900 ml water. Adjust pH to 7.4 and refrigerate. Stable for 2 months.

Weigh out beads and put them into siliconized glass screw-cap tubes. Fill tube with at least 30 ml PBS and let stand at room temp for at least 3 hours. These tubes of beads can be done in batches of 10 or 20 because the autoclaved beads are stable in the refrigerator for 2 years.

Autoclave beads, allow to cool and refrigerate.

SPINNER FLASK ASSEMBLY

Make sure spinner flask (SF) assembly is adequately siliconized. Water should not stick to inside surfaces. The flasks can be autoclaved 2 - 3 times without having to re-siliconize them.

When ready to use flasks, adjust the spin bar apparatus as far down in the flask as possible without touching the glass bubble on the bottom of the flask.

INITIATION OF CELL CULTURE

When you are ready to start a culture, suction PBS from on top of the beads. Replace PBS with MEM and swirl beads around a few times within a 5 min. period. Allow beads to settle and suction off the medium.

Replace medium with fresh complete MEM with 10% fetal calf serum and 1% drugs (and 1% NEAA for HEL cells). Swirl to suspend beads in medium and pipet medium into the side arm of a spinner flask (use a 10 ml siliconized glass pipet or pour beads into side arm carefully so as not to contaminate culture). If the tube has beads left in it, wash it with some more medium.

Add more fresh media to the flask until there is enough to cover the thin, flat bottom of the white spin bar.

Add cells at this time. Suggested amounts of cells are as follows:

(Cytodex 2) A-549 1 - 2 confluent flasks.
(Cytodex 2) WI-38 3 - 4 confluent flasks or 1 tube of frozen cells from the supplier.
(Cytodex 3) MINK 2 - 3 confluent flasks.
(Cytodex 3) HEL 1 - 2 confluent flasks.

Place flask on magnetic stirrer and stir for 1 minute every 60 minutes. Do this for 4 hours, then fill SF to full volume (usually 100 - 125 ml). Spin continuously. Let culture grow for 1 day. Change media as outlined below.

CHANGING MEDIUM- EVERY 3 DAYS

Take SF from incubator and sit it under the hood, close to the suction set-up. Let beads settle (about 5 minutes) then suction off half of the media. Replace media removed with fresh media. Monitor growth of cells by making wet mounts of beads and examining them under low power on the microscope.

INITIATING A P.C. CULTURE

When cells are confluent and ready to inoculate with Pneumocystis, change media as above (usually 5 - 7 days to become confluent).

Grind up a piece of lung of standard size for a 10 plate drug study (12.5ml inoculum contained 20-25 trophs per field for a 125 ml SF)

HARVESTING

Take SF out of incubator and put under hood. Let it sit for 10 minutes to be sure all of the beads have settled to the bottom. I usually make new media during this time.

Take the media off using a 10 ml pipet. Put media into centrifuge tubes. Take a 10 ml sample of media for counting, then spin hard 5 minutes at 10,000 RPM. Resuspend each tube in 1 ml saline and transfer to microfuge tubes. Spin for 2 minutes in the microfuge, remove the supernatant and resuspend pellet in 1 ml of saline. Put 10 ul on a slide for counting. On the first harvest especially, it is easier when counting if you freeze the tubes separately instead of combining them because of the volume of Pneumocystis which is collected when a good growth occurs. Add fresh media to 125 ml then 2nd harvest day 7 - 10.

It may be desirable to filter the media from the spinner flask through a 3 μm filter before centrifuging to remove host cells, but you may lose as much as half of the pneumocystis because the large clumps of organisms will also be removed by the filter.

SPINNER.PRO

VIABILITY STAIN

Preparation of stock solutions of Ethidium bromide and fluorescein diacetate.

Saline: Any convenient phosphate buffered saline (PBS)

We use 2.84g Na_2HPO_4 (anhydrous)
2.76g NaH_2PO_4 H_2O
8.5g NaCl
H_2O (distilled) to 1 liter pH 7.2

Stock EB solution: Make an 8X stock in PBS. Freeze aliquots in sealed tubes.
WARNING: Wear gloves- EB is a mutagen.
8X EB stock= 20 μl/ml or 2 mg/100ml PBS.

Stock FDA: Stock is made up in acetone. Wear gloves to keep skin esterases from FDA. Keep FDA in acetone in sealed tube in freezer 5 mg FDA in 1 ml acetone, shake to dissolve.

8X FDA working solution: Make fresh for each experiment 6 microliters in 10 ml PBS.

Preparation of Final Concentration of EB/FDA.

For Promastigotes

50μl cells + 25μl EB (8X) + 25 μl FDA (8X).
Let sit 1 min 25°C or
5 min 0°C
50μl of above mix + 50μl
Protosle or Polyox
mix well, room temp
Read fluorescence by microscopy

For Amastigotes
(free or in macrophages)

50μl parasites + 25μl EB(8X) + 25μl FDA (8X)
Let sit i min 25°C or 5 min 0°C
50μl of above + 50 μl PBS
mix, room temp
read fluorescence by microscopy
*At this EB concentration dead amastigotes in living macrophages are not visible.

Protoslo: Cat # 885141, Carolina Biological Supply Co., Burlington, NC 27215. Use at 100% or 50% conc.

Polyox: Product WSR301, Polyethyleneoxide, Union Carbide Co., See J.Protozoology 24: 471-474.1977 for information on Polyox. Make 2% or 1% (w/r) in PBS. We use 1%.

Microscopy: FITC filters for FDA^+ cells BG12, KV418nm excitation
500 nm Dichroic
OG515nm Barrier

Rhodamine filters for EB^+ cells
546 nm excitation
560 nm dichroic
OG590 barrier

AO Fluorstar microscope; mercury vapor lamp.

FDA cells are easier to see if cells are mixed as soon as possible before observation. Some fluorescein leaks from cells leading to increase background fluorescence. This leads to perceived dimming of FDA^+ cells. FDA^+ cells will quench in UV beam. Esterases in samples release fluorescein from FDA and increase background fluorescence. Wash cell preparations in PBS before adding FDA.

Thin phase type hemocytometer can be used to count cells by FDA/EB. A little white light shining up through hemocytometer allows you to see lines in hemocytometer. (Use tissue paper to reduce white light.)

Supplied material: FDA	EB
Cat # 0615, Lot 836066	Cat E8751, Lot 90F3862
Polysciences, Warrington, PA.	Sigma Chem Co.

SCORING P.CARINNI IN TISSUE

5 - MORE THAN 100 PER FIELD AT 1000X

4 - BETWEEN 10-99 PER FIELD AT 1000X

3 - BETWEEN 1-10 PER FIELD AT 1000X

2 - BETWEEN 2-9 PER 10 FIELDS AT 1000X

1 - 1-10 IN 30 FIELDS AT 1000X

0 - 0 IN 30 FIELDS AT 1000X

PROTOCOL FOR TRANSTRACHEAL INOCULATION OF RATS

Rats used for evaluation of drugs for PC pneumonia have been from Harlan Sprague Dawley colony 202 in Indianapolis, IN. These rats are virus-free and PC-free when shipped from the supplier. To develop PC infection in the rats, we inoculate rats with infected rat lung.

Female Sprague-Dawley rats weighing between 120 to 140 grams are given dexamethasone (Butler) at 1.2 mg/L in drinking water for 4 days prior to transtracheal inoculation. We have shown that by 4 days there is 50% depletion of lymphocytes. Inoculum is prepared by grinding infected rat lung in saline, centrifuging , staining and counting as described for preparation of culture inoculum but adjusting numbers of trophozoites so that there are at least $2x\ 10^6$ per ml. If in examination of the Giemsa-stained preparations contamination is noted, the inoculum is discarded.

Animals are anesthetized with ketamine hydrochloride, 0.2 ml IM, To inoculate an animal a small midline incision is made, the trachea exposed by blunt dissection, and 0.2 ml of inoculum with 0.5 ml of air behind is injected directly into the trachea. The wound is closed with a clip.

Giemsa Stain

We use azure B Giemsa, the stain recommended for malarial parasites. Stain is made by Harleco and can be purchased from Baxter.

The alcoholic stock stain is diluted with phosphate buffered water (for most stain lots at pH 7.0 to 7.2) at 1:20 for staining time of 30 minutes or 1:40 for staining time of 45 to 60 minutes. The more dilute stain with longer stain time is best for materials used for photomicrographs as colors are more delicate.