Three-Dimensional Engineered High Fidelity Normal Human Lung Tissue-Like Assemblies (TLA) as Targets for Human Respiratory Virus Infections

Goodwin, T.J.[1], Deatly, A M.,[2] Suderman, M. T.[1] Lin, Y-H.[2], Chen, W.[2], Gupta, C. K.[2], Randolph, V. B.[2] and Udem, S. A.[2]

[1] Life Sciences Research Laboratories, NASA- Johnson Space Agency, Houston, Texas,
[2]Wyeth Vaccines, Pearl River, New York.

Unlike traditional two-dimensional (2D) cell cultures, three-dimensional (3D) tissue-like assemblies (TLA) (Goodwin et al, 1992, 1993, 2000 and Nickerson et al., 2001, 2002) offer high organ fidelity with the potential to emulate the infective dynamics of viruses and bacteria *in vivo*. Thus, utilizing NASA microgravity Rotating Wall Vessel (RWV) technology, *in vitro* human broncho-epithelial (HBE) TLAs were engineered to mimic *in vivo* tissue for study of human respiratory viruses. These 3D HBE TLAs were propagated from a human broncho-tracheal cell line with a mesenchymal component (HBTC) as the foundation matrix and either an adult human broncho-epithelial cell (BEAS-2B) or human neonatal epithelial cell (16HBE14o-) as the overlying element. Resulting TLAs share several characteristic features with *in vivo* human respiratory epithelium including tight junctions, desmosomes and cilia (SEM, TEM). The presence of epithelium and specific lung epithelium markers furthers the contention that these HBE cells differentiate into TLAs paralleling *in vivo* tissues. A time course of infection of these 3D HBE TLAs with human respiratory syncytial virus (hRSV) wild type A2 strain, indicates that virus replication and virus budding are supported and manifested by increasing virus titer and detection of membrane-bound F and G glycoproteins. Infected 3D HBE TLAs remain intact for up to 12 days compared to infected 2D cultures that are destroyed in 2-3 days. Infected cells show an increased vacuolation and cellular destruction (by transmission electron microscopy) by day 9; whereas, uninfected cells remain robust and morphologically intact. Therefore, the 3D HBE TLAs mimic aspects of human respiratory epithelium providing a unique opportunity to analyze, for the first time, simulated *in vivo* viral infection independent of host immune response.

Three-Dimensional Engineered High Fidelity Normal Human Lung Tissue-Like Assemblies (TLA) as Targets for Human Respiratory Syncytial Virus Infections

Thomas J. Goodwin PhD.

Miguel T. Suderman MDV, M.Sc.

CETNEP Laboratory, NASA/JSC, Houston, Texas ,
Universities Space Research Association, Houston,Texas,

Advanced Tissue Engineering Concepts

- **Development of Three-Dimensional Tissue Assembles (TLAs) for Research and Commercialization**

- **Development of Custom Design Media for Nutritional Requirements of TLAs**

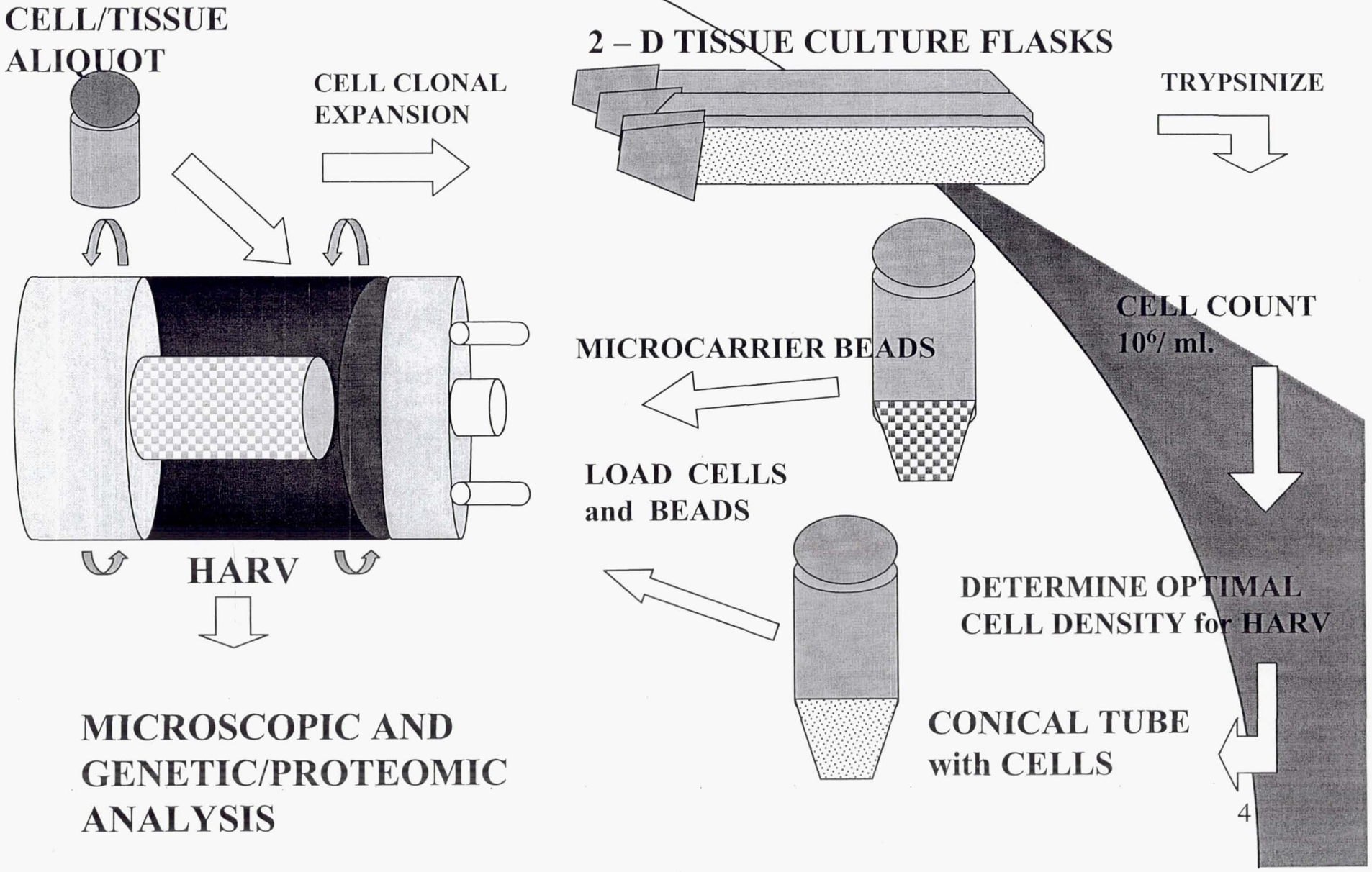
- **Development of a Process to Preferentially Stimulate Tissue Replication**

NORMAL HUMAN LUNG 3-D TISSUE CULTURE ASSEMBLIES

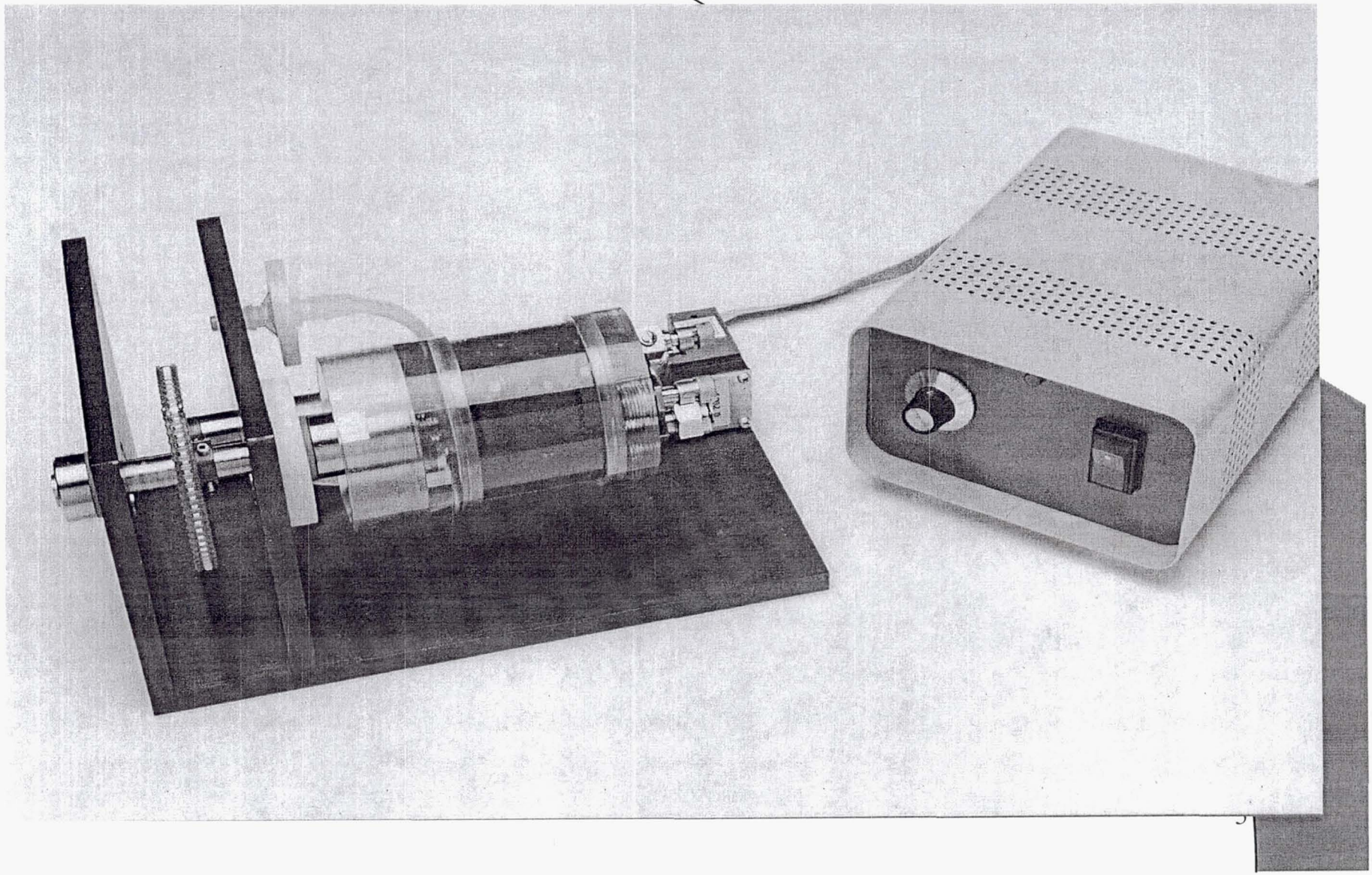
- PULMONARY TRACT TISSUE CELL TYPES:
- an ENDOTHELIAL-MESENCHYMAL:
  Human Bronchial Tracheal (HBTC)

- 2 EPITHELIAL CELLS:
  BRONCHIAL EPITHELIAL (BEAS-2B)
  16HBE14o- (PEDIATRIC CELL LINE)

ASSEMBLY of 3 – DIMENSIONAL TISSUE ASSEMBLY into HIGH ASPECT ROTATING WALL VESSEL (HARV)

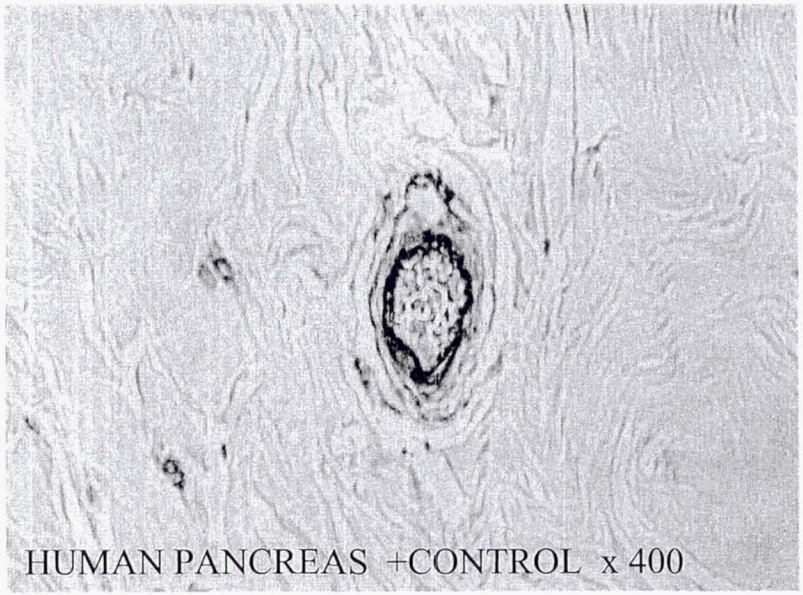


Rotating Wall Vessel

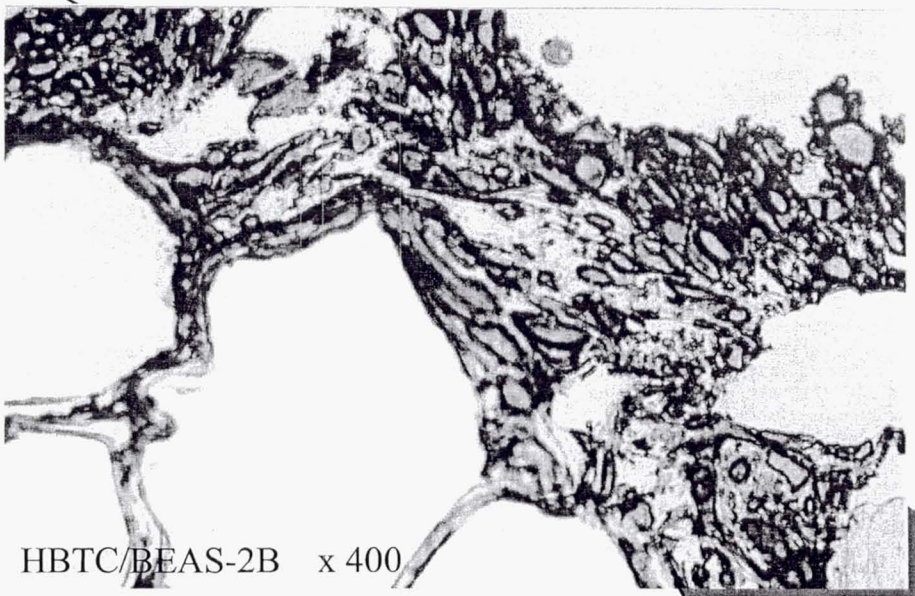


Immunohistochemistry of Human Bronchial Epithelium (HBE) Tissue Like Aggregate (TLA) of HBTC/16HBE14o- H&E 40x

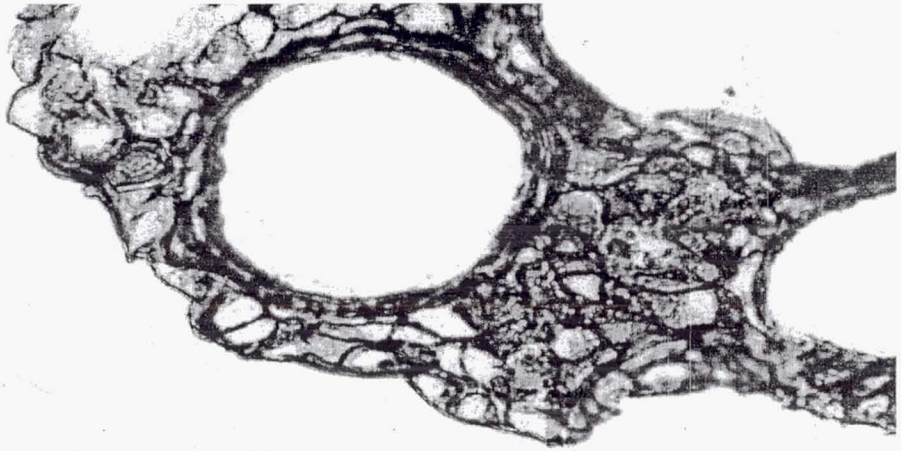
3D TLA Cellular Differentiation : ICAM-1 Antibody



HUMAN PANCREAS +CONTROL x 400



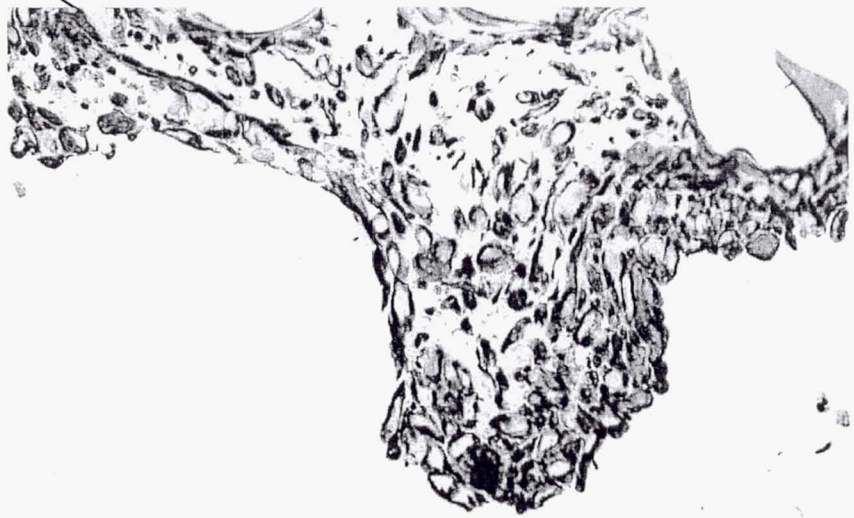
HBTC/BEAS-2B x 400



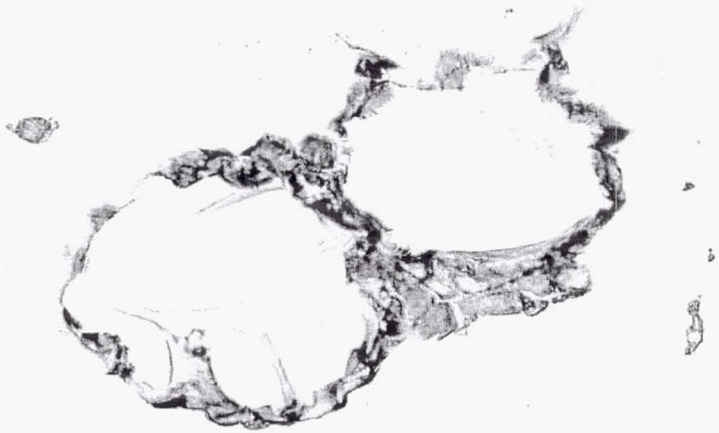
HBTC/16HBE14o- x 400

3D TLA Cellular Differentiation : Tubulin Antibody

HUMAN BREAST + CONTROL x 400

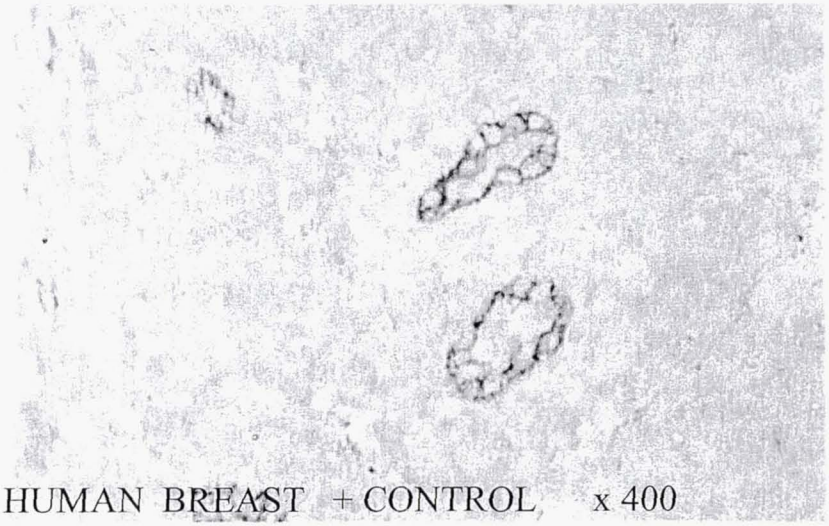


HBTC/BEAS- 2B x 400

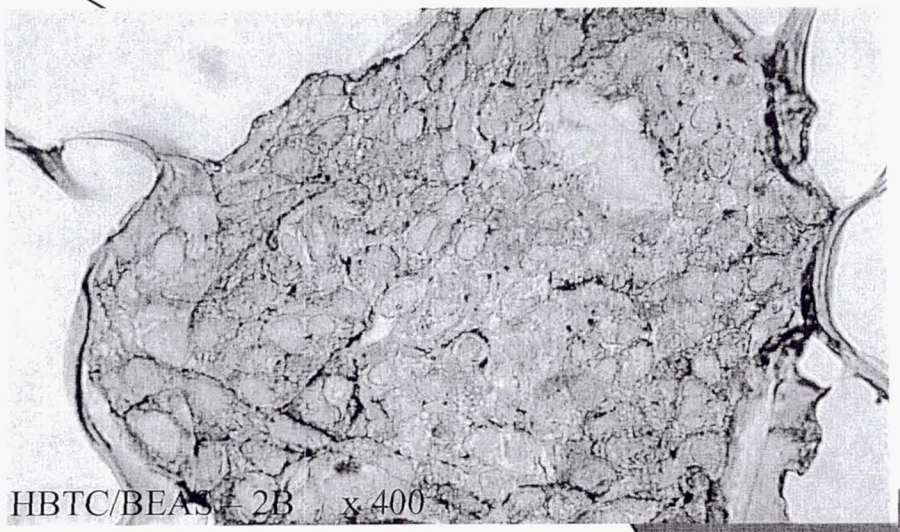


HBTC/16HBE14o- x 400

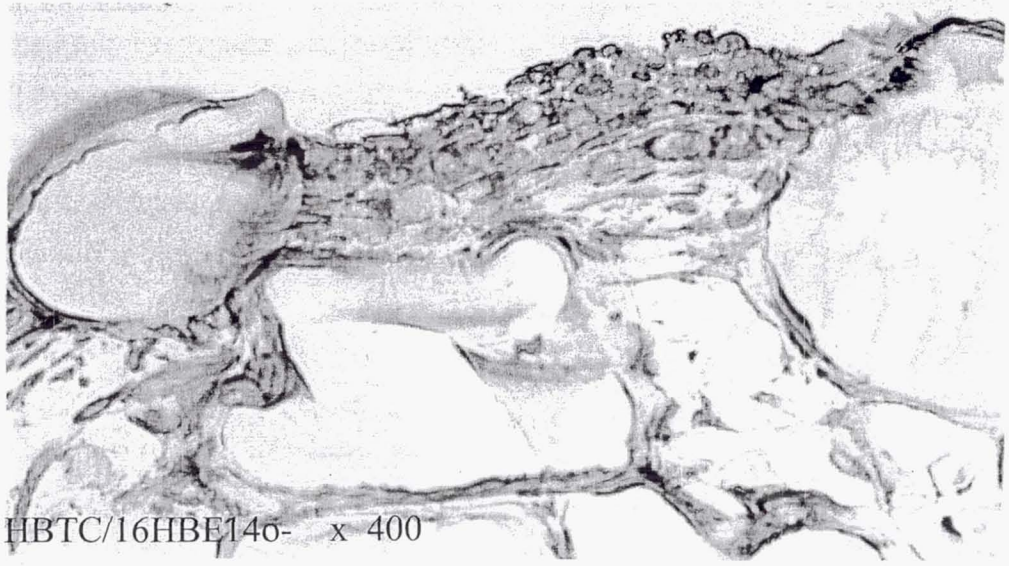
3D TLA Cellular Differentiation : ZO-1 Antibody



HUMAN BREAST + CONTROL x 400

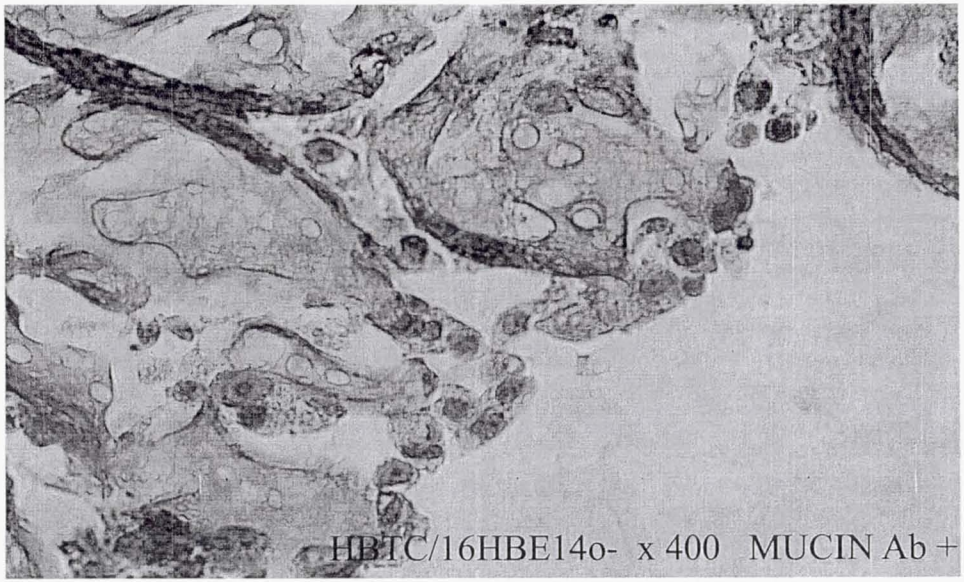


HBTC/BEAS-2B x 400

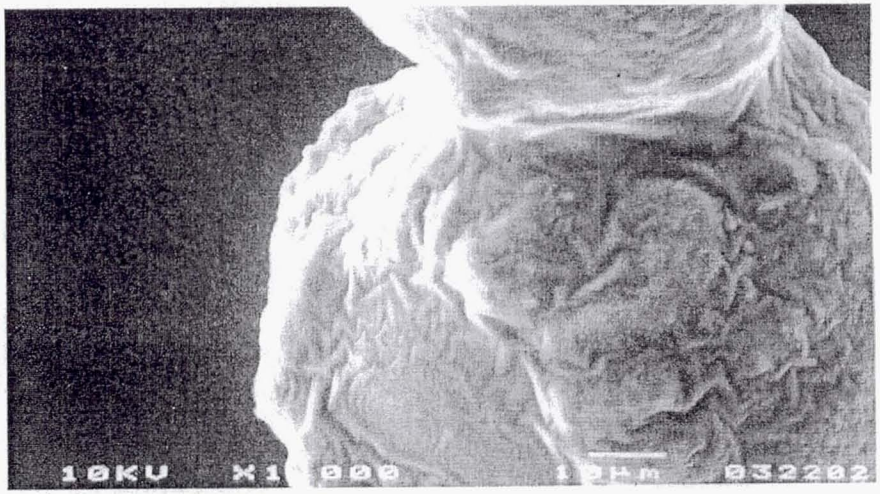


HBTC/16HBE14o- x 400

3D TLA Cellular Differentiation : Mucin Antibody



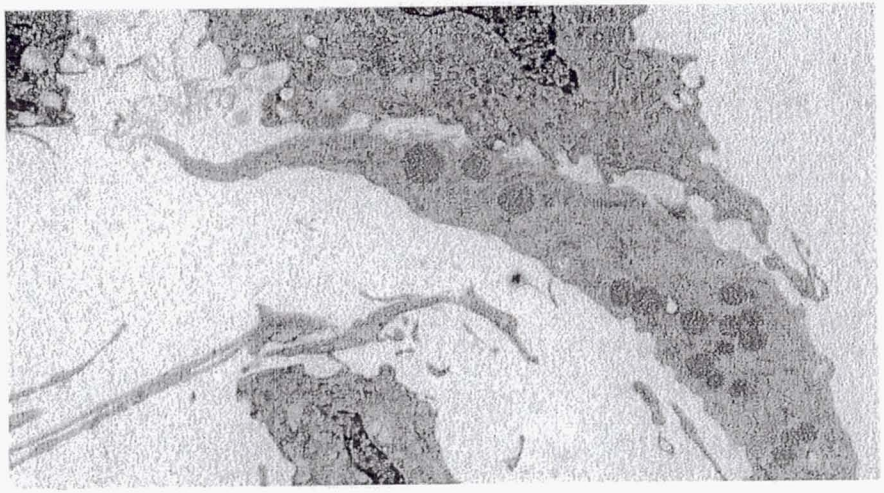
3-D Tissue Culture Assembly of Human Bronchial – Tracheal Epithelium Cells (HBTC):Monoculture



A. HBTC:MONOCULTURE:MOCK:SEM 1000x

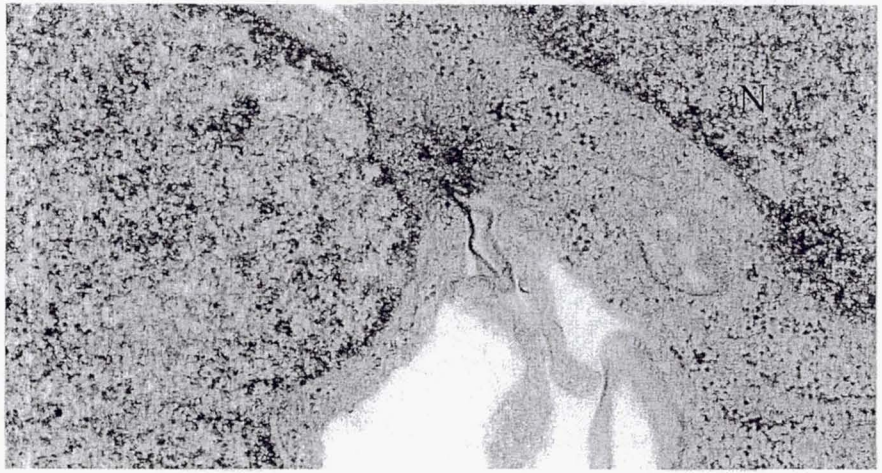
B. HBTC:MONOCULTURE:MOCK:TEM 25000x

C. HBTC:MONOCULTURE:MOCK:SEM 3500x

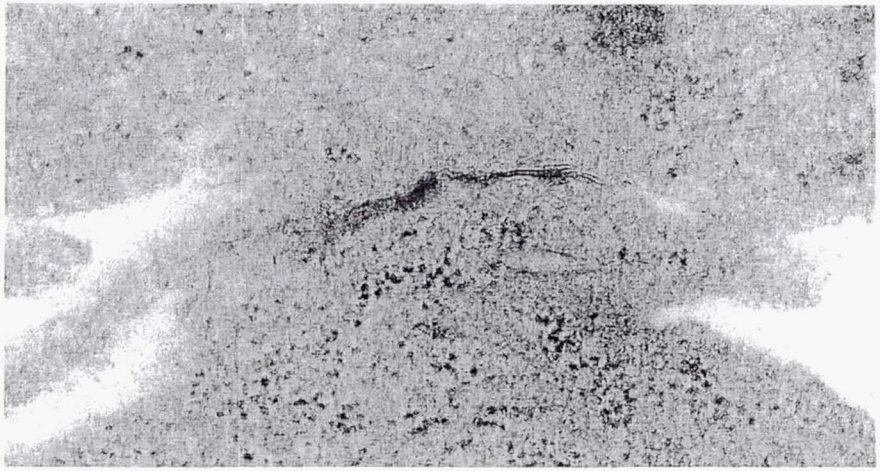


D. HBTC:MONOCULTURE:MOCK:TEM 50000x

3-D Tissue Culture Assembly of Human Bronchial Cell Line (BEAS-2B):Monoculture

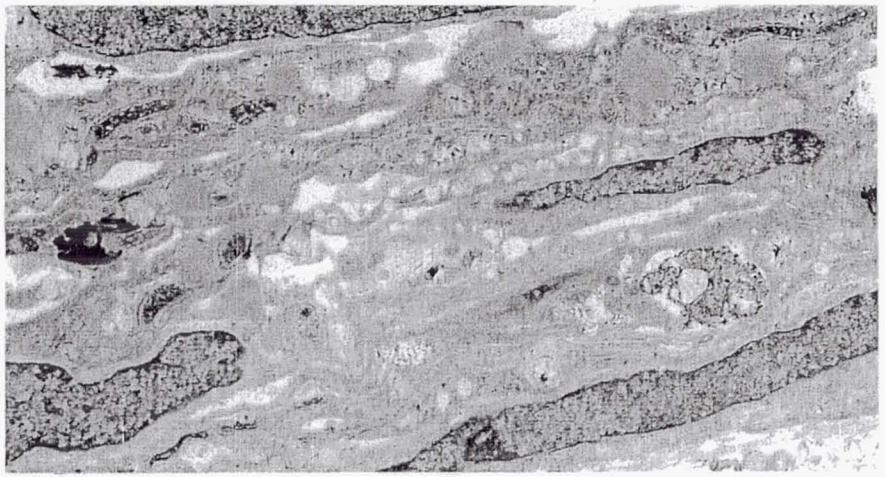


A. BEAS-2B : MONOCULTURE : MOCK : TEM 50000x



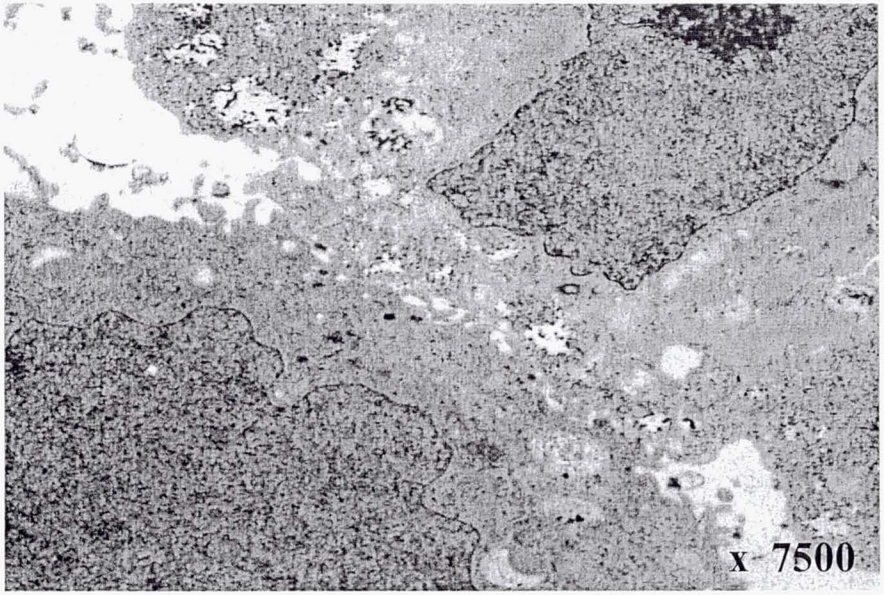
B. BEAS-2B : MONOCULTURE : MOCK : TEM 50000x

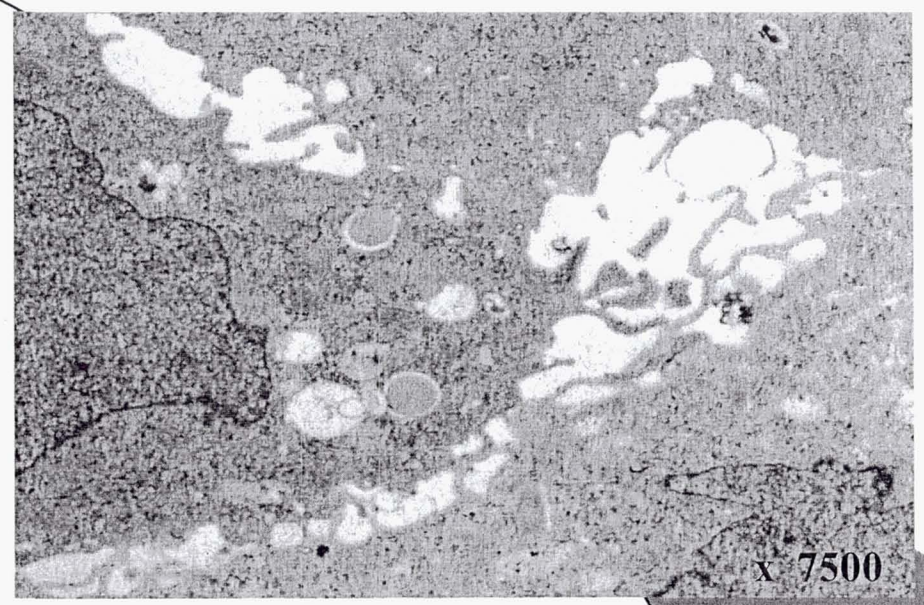
C. BEAS-2B : MONOCULTURE : MOCK : TEM 50000x

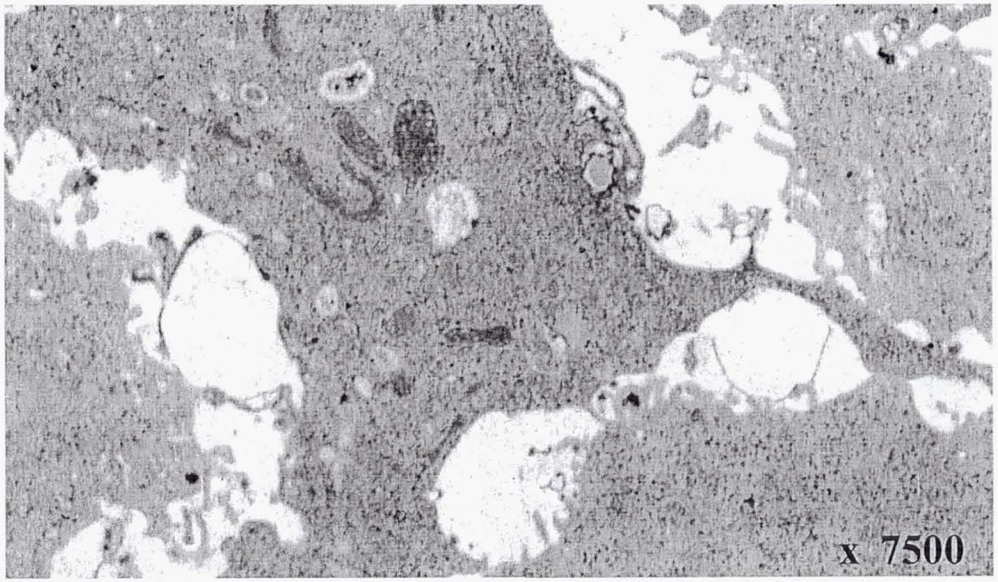


D. BEAS-2B : MONOCULTURE : MOCK : TEM 50000x

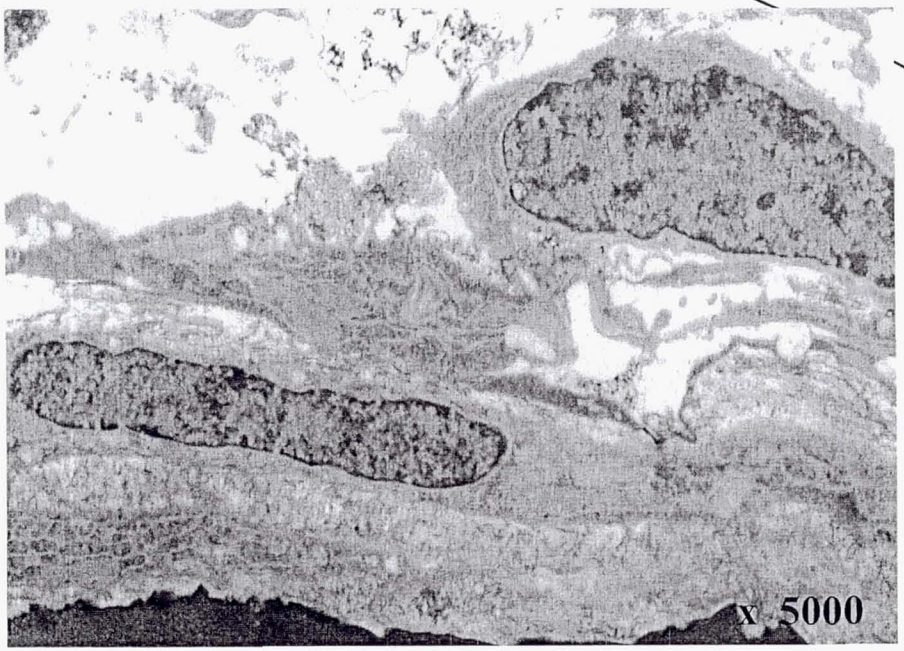
3-D Tissue Culture Assembly of Human Bronchial Cell Line (16HBE14o-):Monoculture

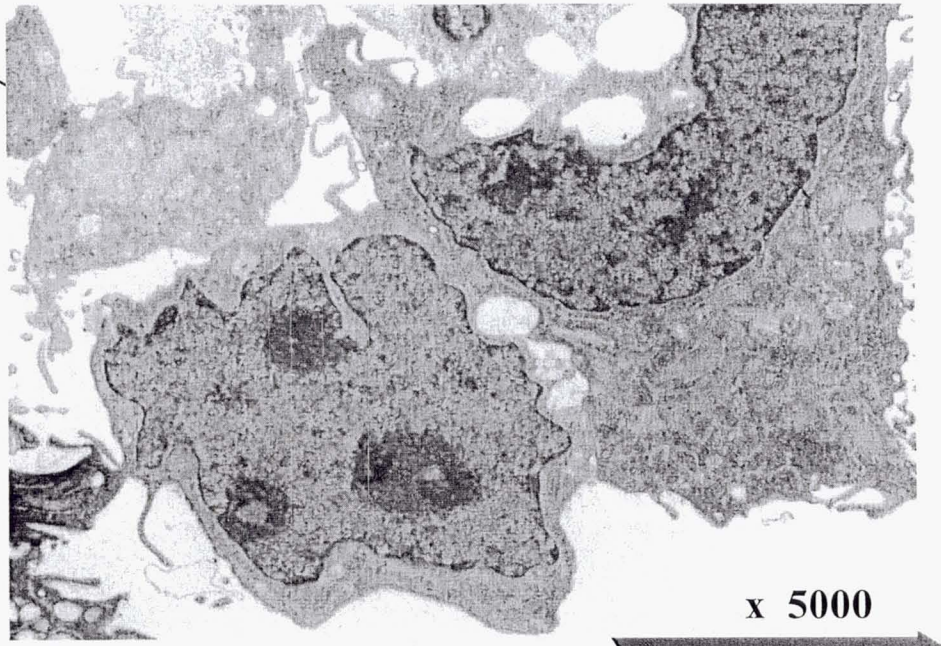


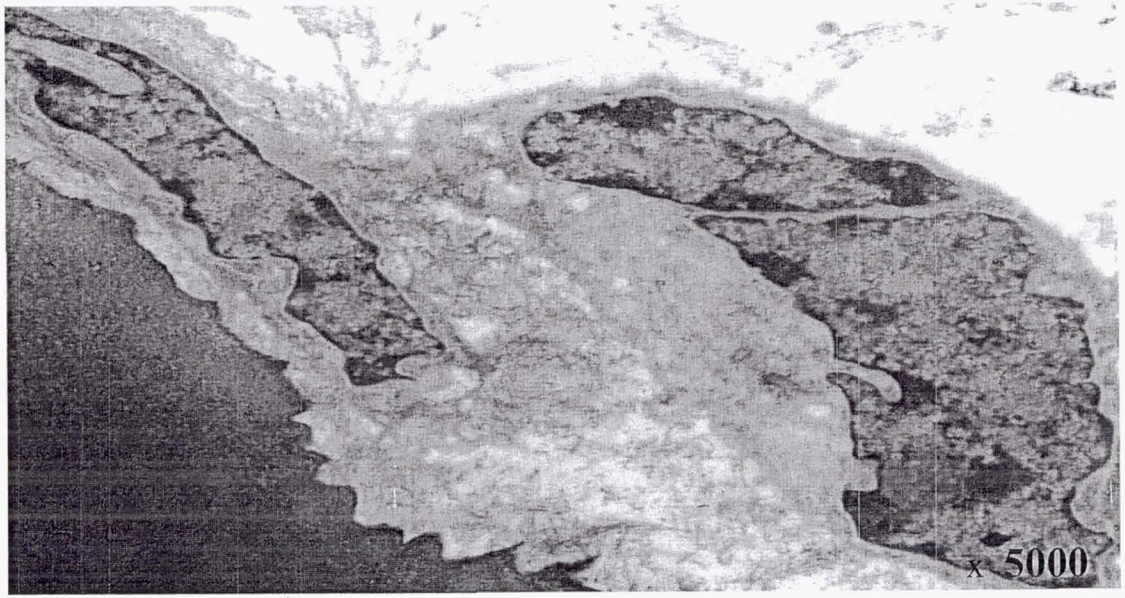




3D Co-Culture of HBTC/BEAS-2B







CHARACTERIZATION OF RESPIRATORY SYNCYTIAL VIRUS (RSV) - A2 STRAIN

- family PARAMXYOVIRIDAE, subfamily PNEUMOVIRINAE
- Virions can be irregular spherical (100-300 nm) or filamentous forms (60-100nm diameter, up to 10 um in length)
- Enveloped, Nonsegmented Negative - Strand RNA
- Host Cell Entry by Cell- surface Fusion
- Viral Replicative Cycle Restricted to Cytoplasm
- Progeny Virions Obtain Lipid Envelope by Budding from Plasma Membrane

(Fields Virology, 4th Edition, 2001)

CHARACTERIZATION of RESPIRATORY SYNCYTIAL VIRUS: EPIDEMIOLOGY

- Single highest causative factor in hospitalization of infants and young children worldwide

- Bronchiolitis or pneumonia most frequent between the ages of 6 weeks to 9 months, lower respiratory tract involvement between 2 and 7 months

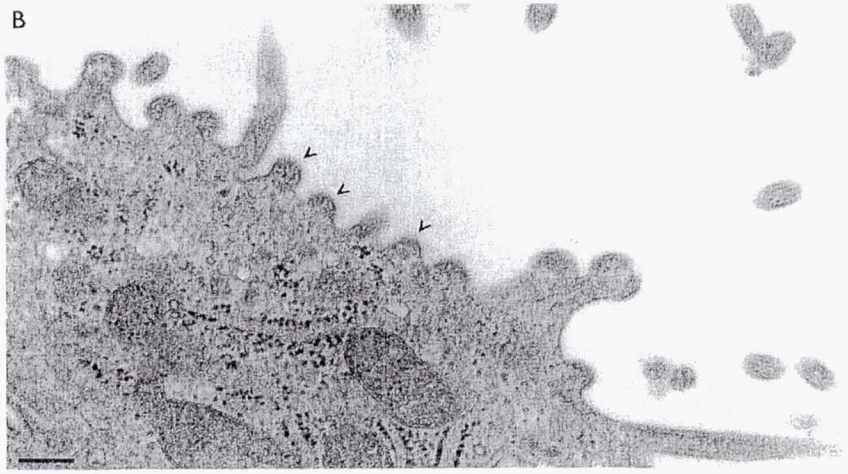
- Increased incidence in the elderly and immuno-suppressed individuals

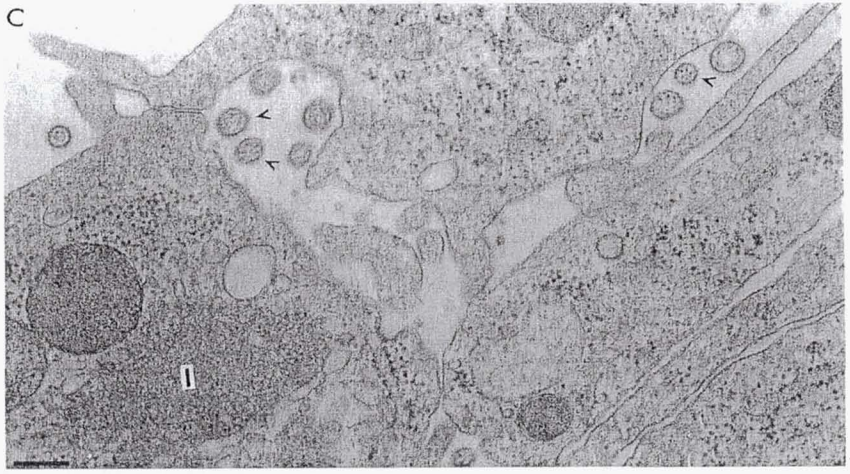
- Infection and Re-infection frequent

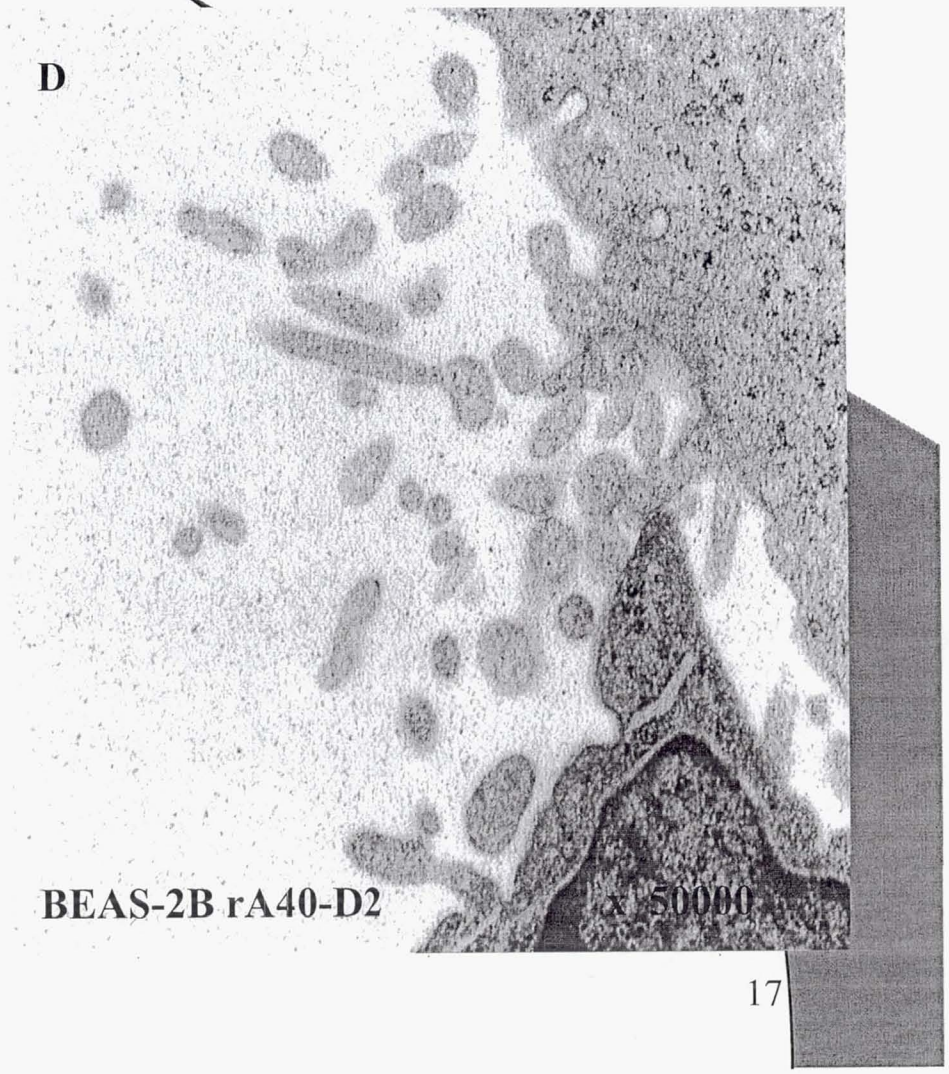
Comparison of Published 2-D TEM's to Current Observations: (Fig. B, C) RSV in Vero C1008 cells

(Roberts et al., 1995)

Fig. D current study



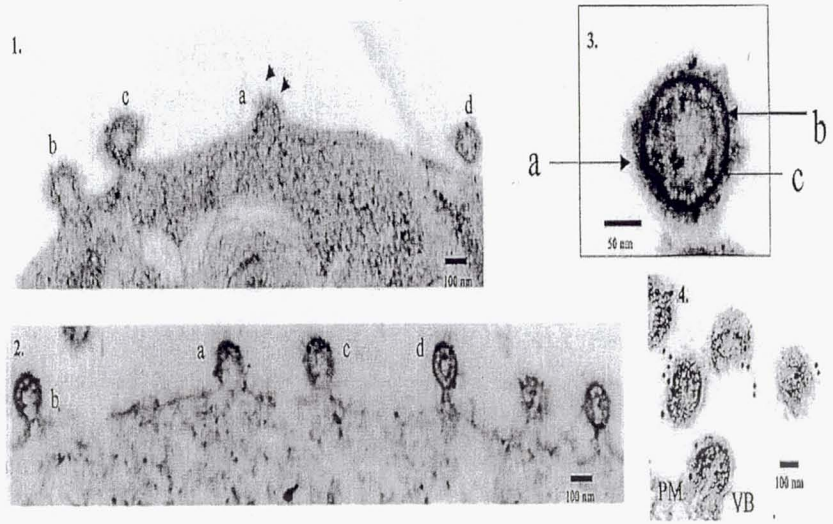


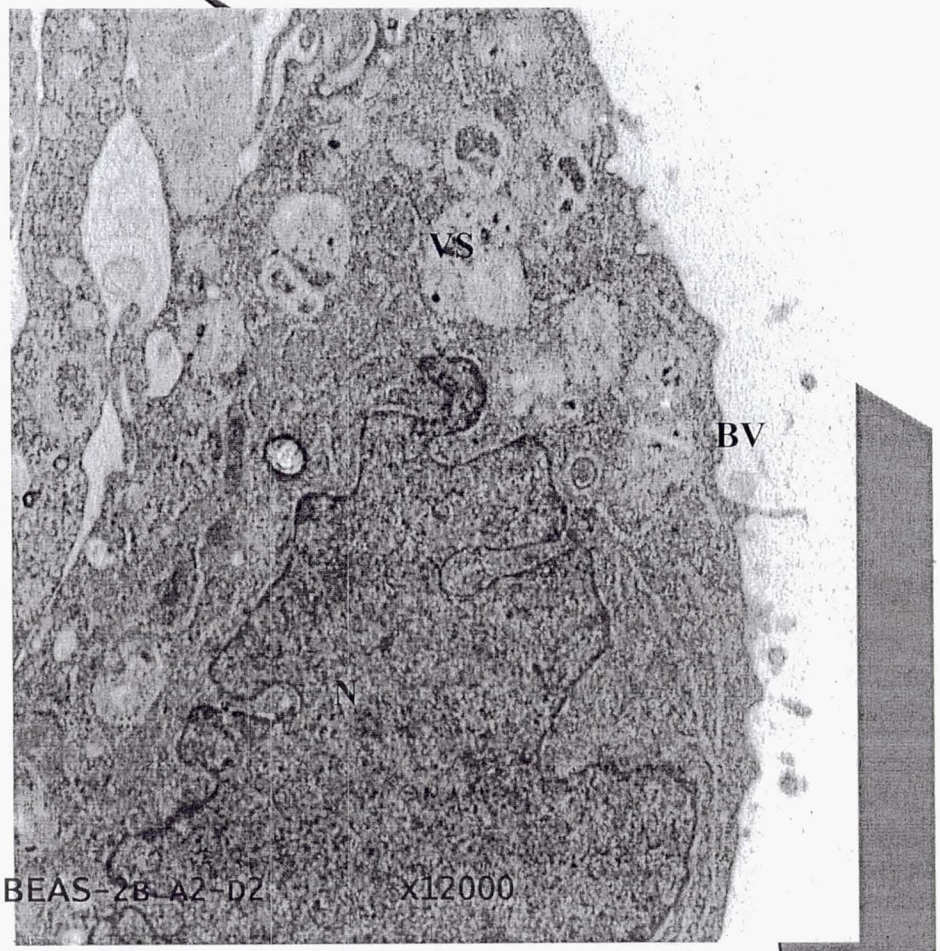


Comparison of Published 2-D TEM's of RSV.

A.

RSV budding from Vero C1008 cells. Brown et al.,2002





2-D cell culture RSV TEM's of Viral Inclusion Bodies (IB) Garcia et al., 1993 compared to current 3-D Cell Model

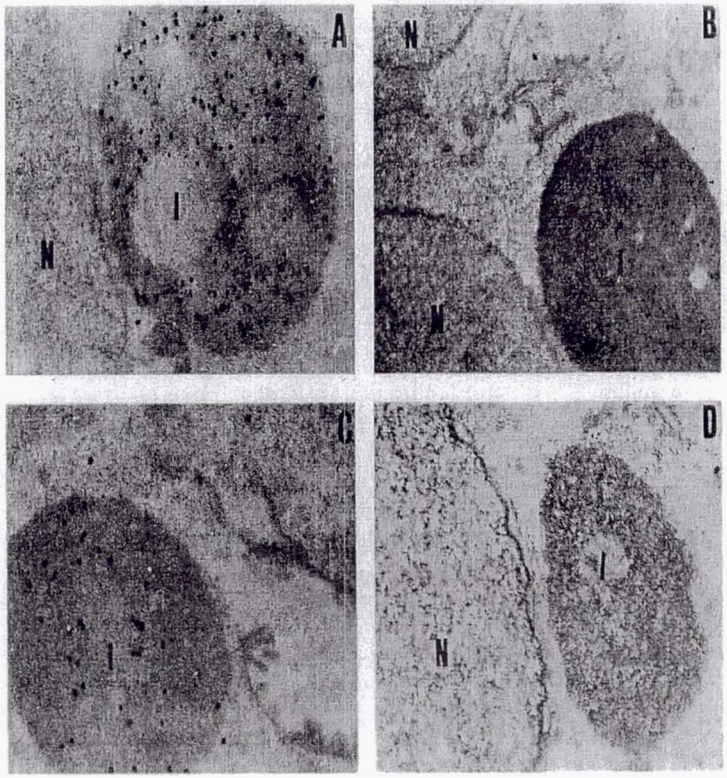
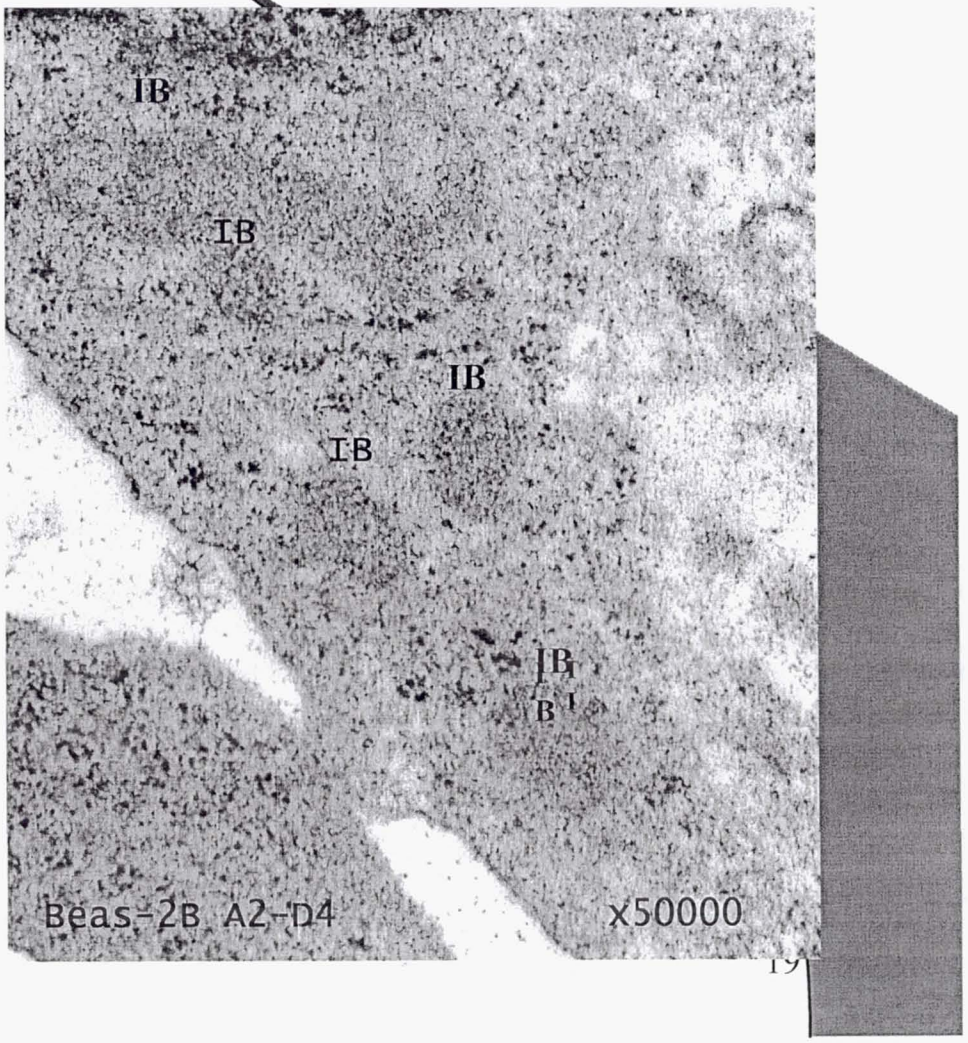
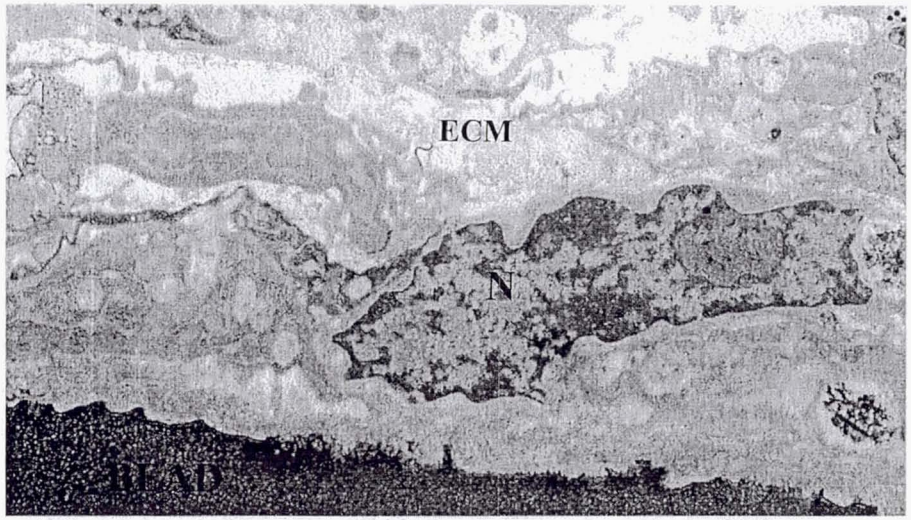


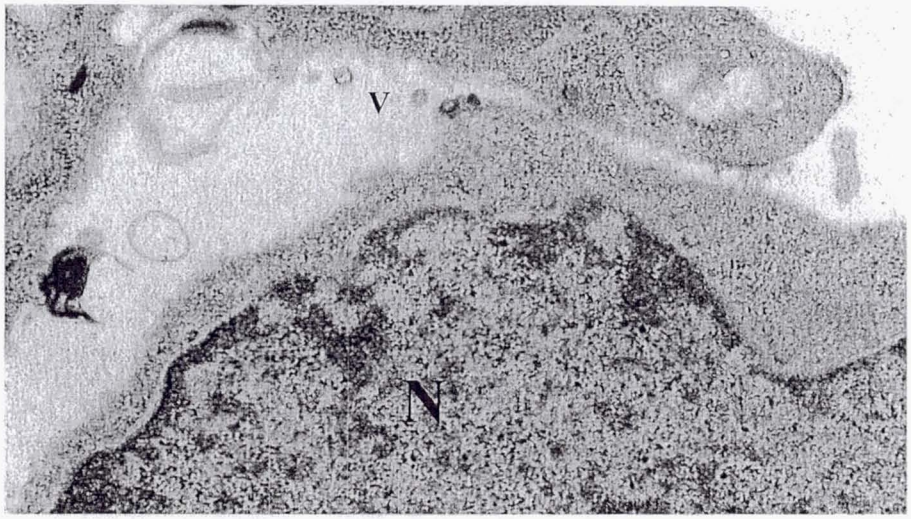
FIG. 1. Immunoelectron microscopy of RS virus-infected cells. Hep-2 cells were infected with the RS virus Long strain (m.o.i. 1–2 PFU/cell). Twenty-four hours later, the cells were fixed for 1 hr at 4° with 2% formaldehyde and 0.2% glutaraldehyde in sodium cacodylate buffer (pH 6.9). Monolayers were detached from the plates, dehydrated, and included in Lowicryl resin K4M before being processed for immunogold labeling as described in Vivo *et al.* (*18*). The samples were incubated with MAbs directed against P (67P, panel A), NP (42N, panel B), 22K (37M2, panel C), and M (66M, panel D) proteins. After washing, the samples were incubated with a sheep anti-mouse Ig, conjugated to 30-nm gold particles. Magnifications: (A) ×32,000, (B) ×19,000, (C) ×41,800, and (D) ×53,200. N, cell nuclei; I, cytoplasmic inclusions.



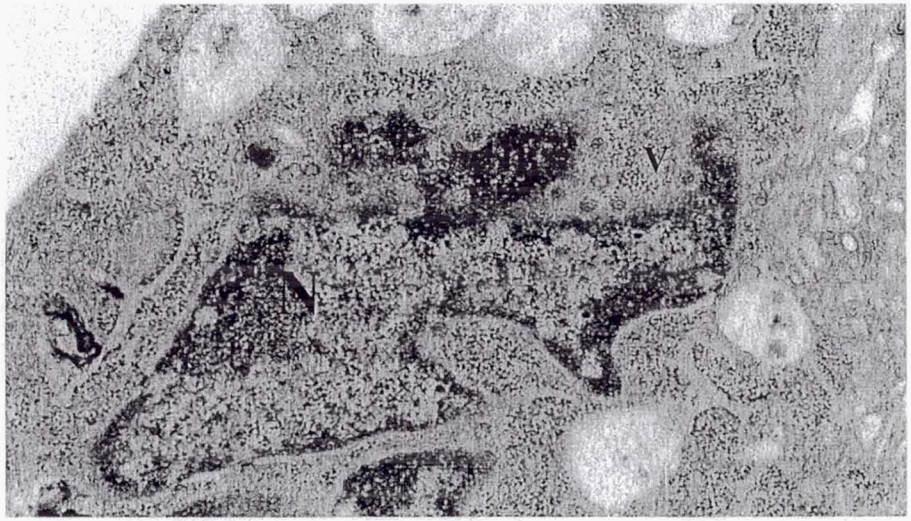
A2 1 Hr. post-inoculation



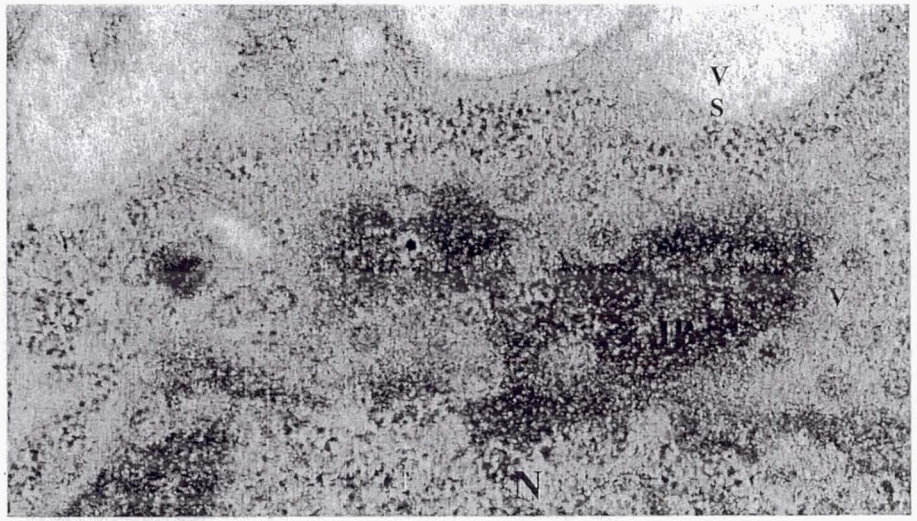
A:BEAS-2B:MONOCULTURE:A2-1 Hr:TEM 7500x



B:BEAS-2B:MONOCULTURE:A2-1 Hr: 25000x

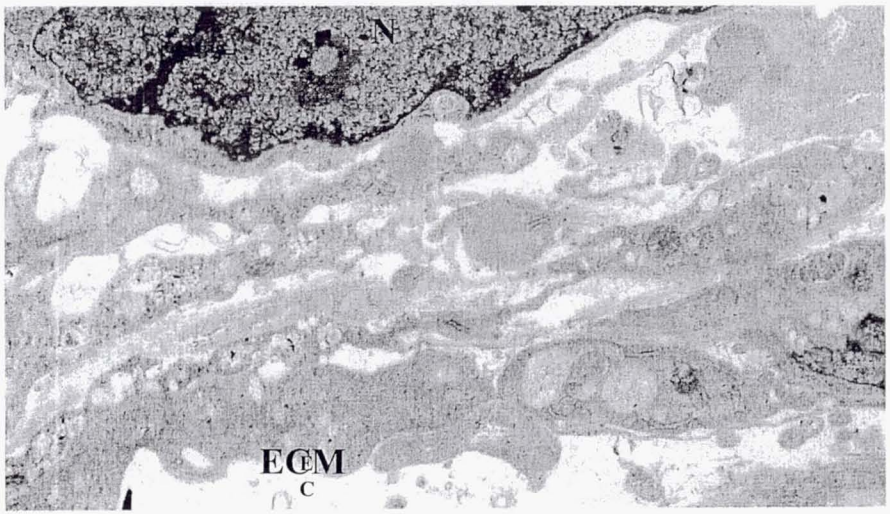


C:BEAS-2B:MONOCULTURE:A2- 1Hr:TEM 25000x

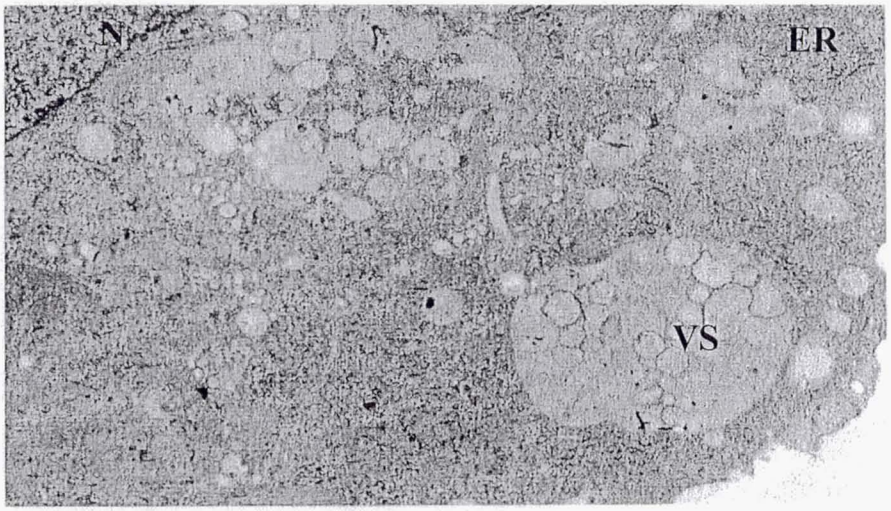


D:BEAS-2B:MONOCULTURE:A2-1Hr:TEM 50000X

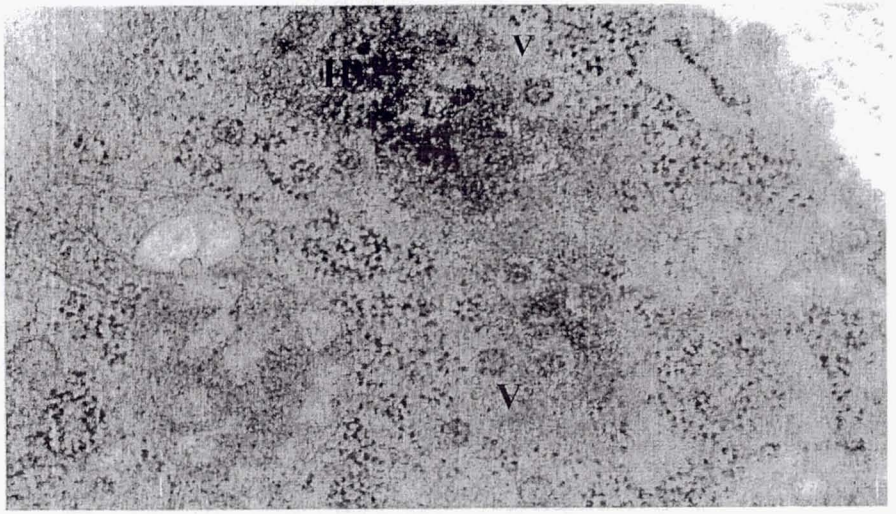
RSV A2- Day 4 post-inoculation



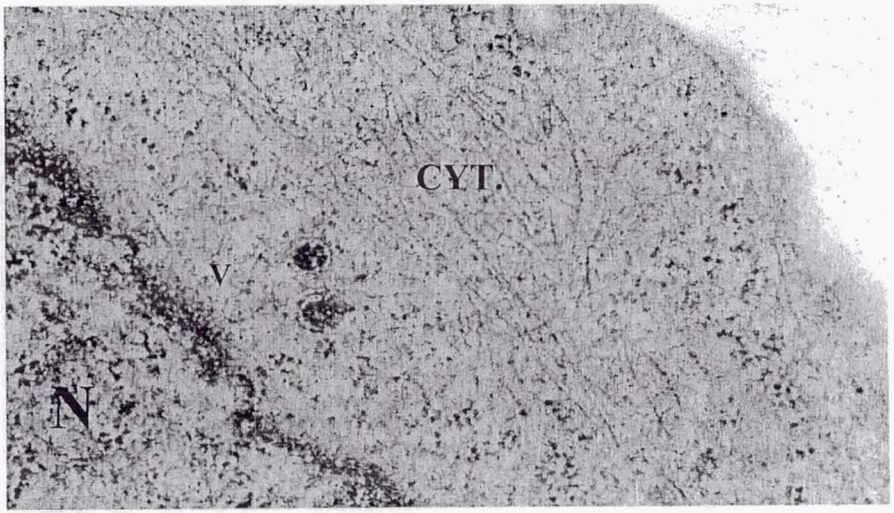
A.BEAS-2B:MONOCULTURE:MOCK:TEM 7500x



B.BEAS-2B:MONOCULTURE:A2-D4:TEM 12000x

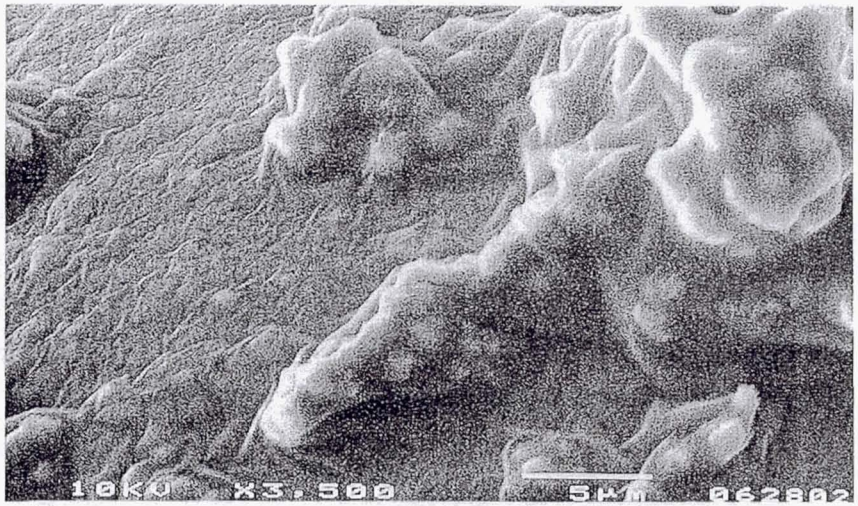


C:BEAS-2B:MONOCULTURE:A2-D4:TEM 50000x

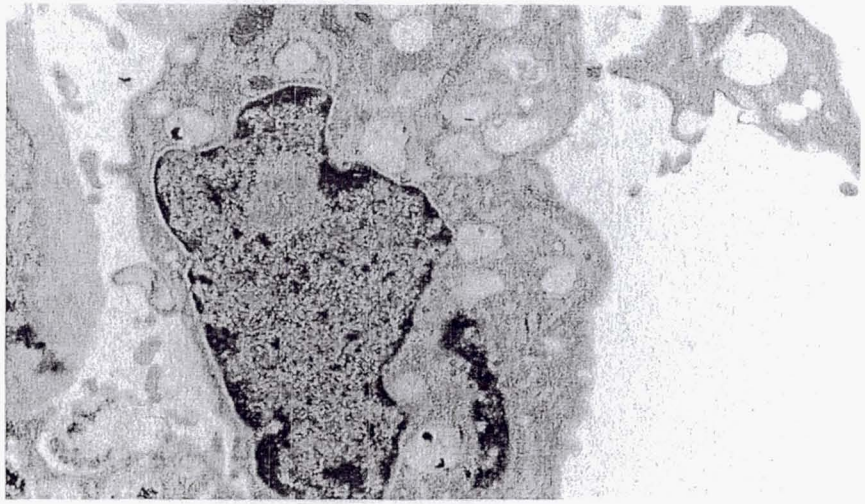


D.BEAS-2B:MONOCULTURE:A2-D4:TEM 50000x

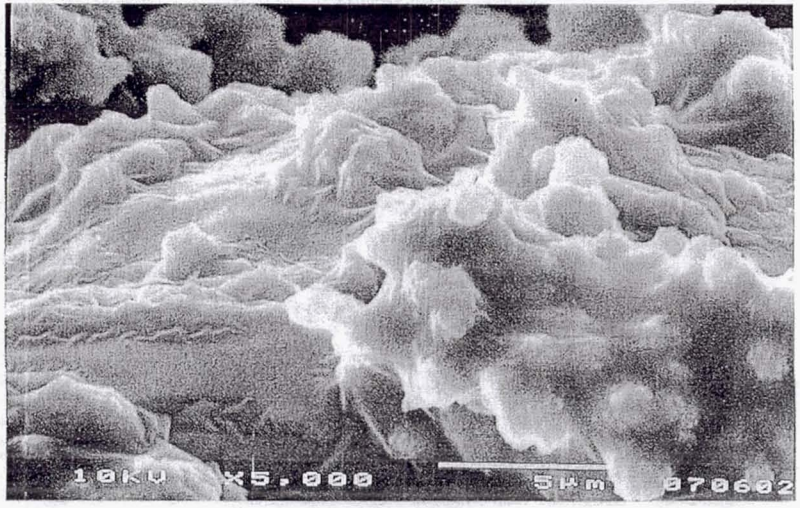
RSV A2- Day 9 post-inoculation



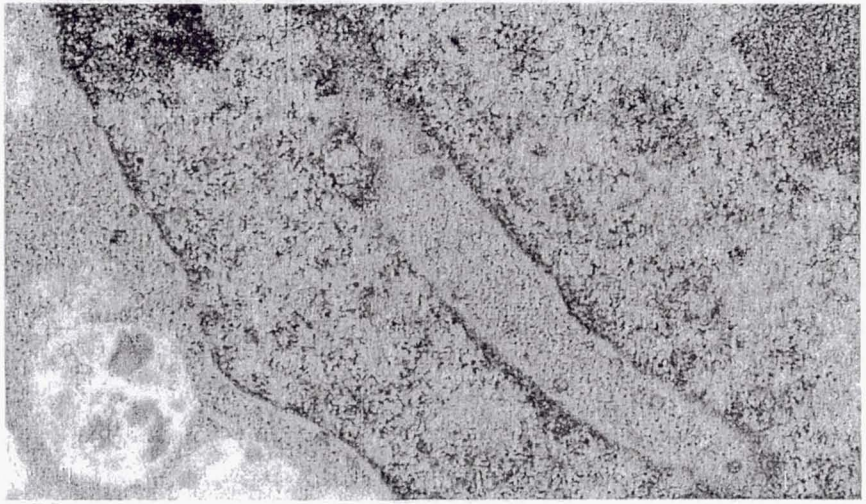
A.HBTC:MONOCULTURE:A2-D9:SEM 3500X



B:BEAS-2B:MONOCULTURE:A2-D9:TEM 12000x

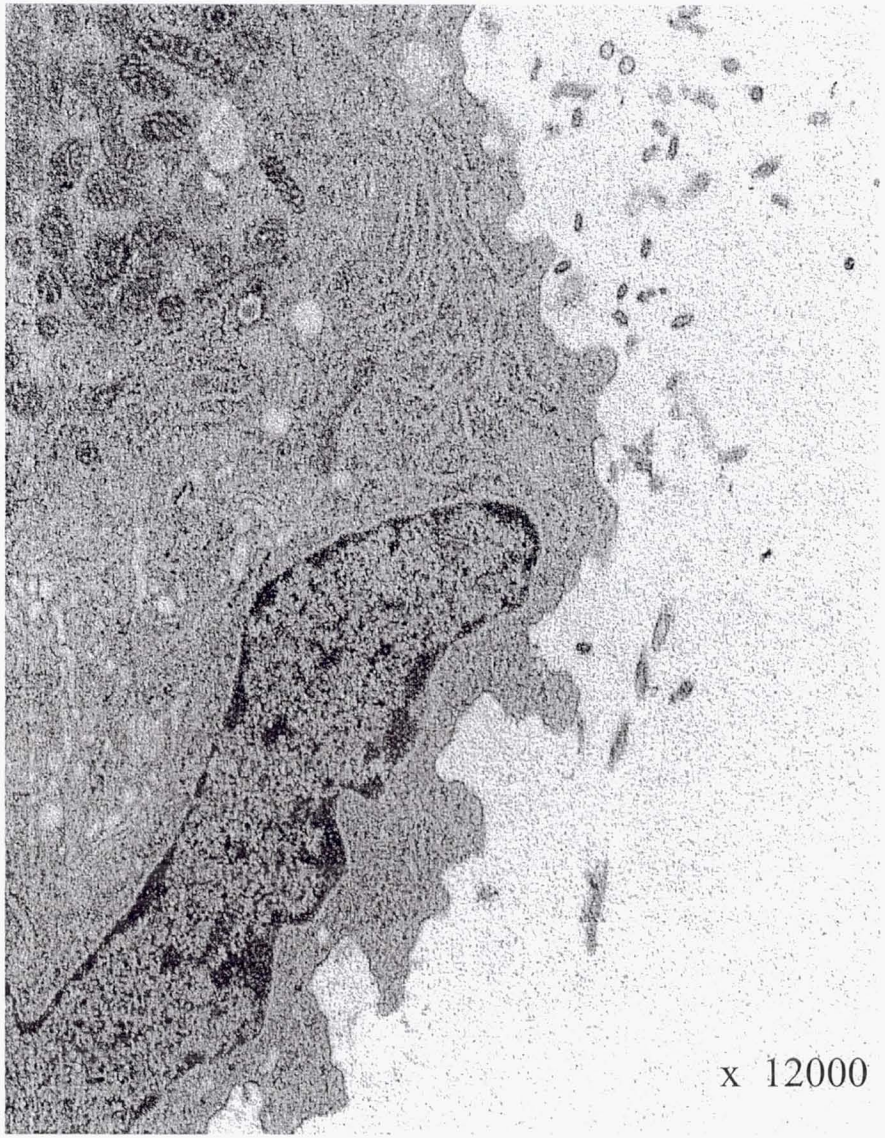


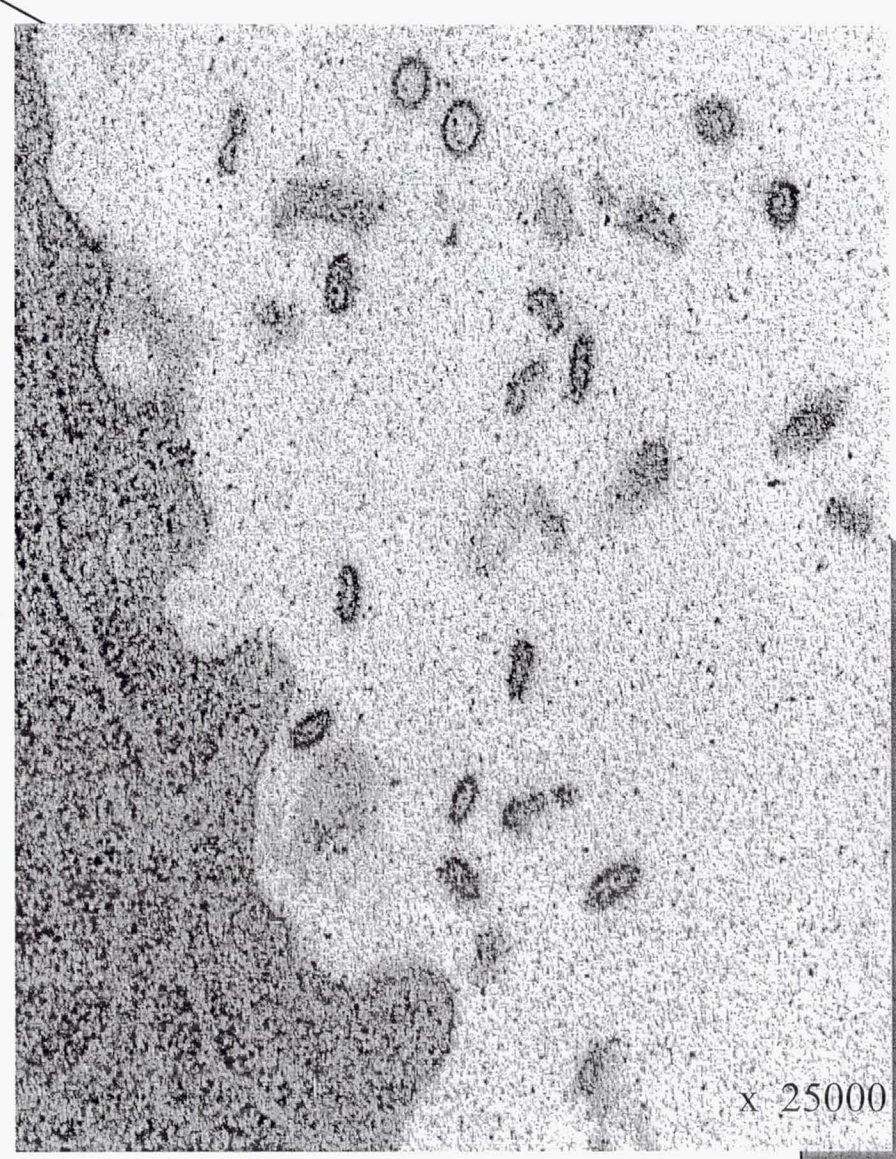
C.HBTC:MONOCULTURE:A2-D9:SEM 5000x



D.BEAS-2B:MONOCULTURE:A2-D9:TEM 25000x

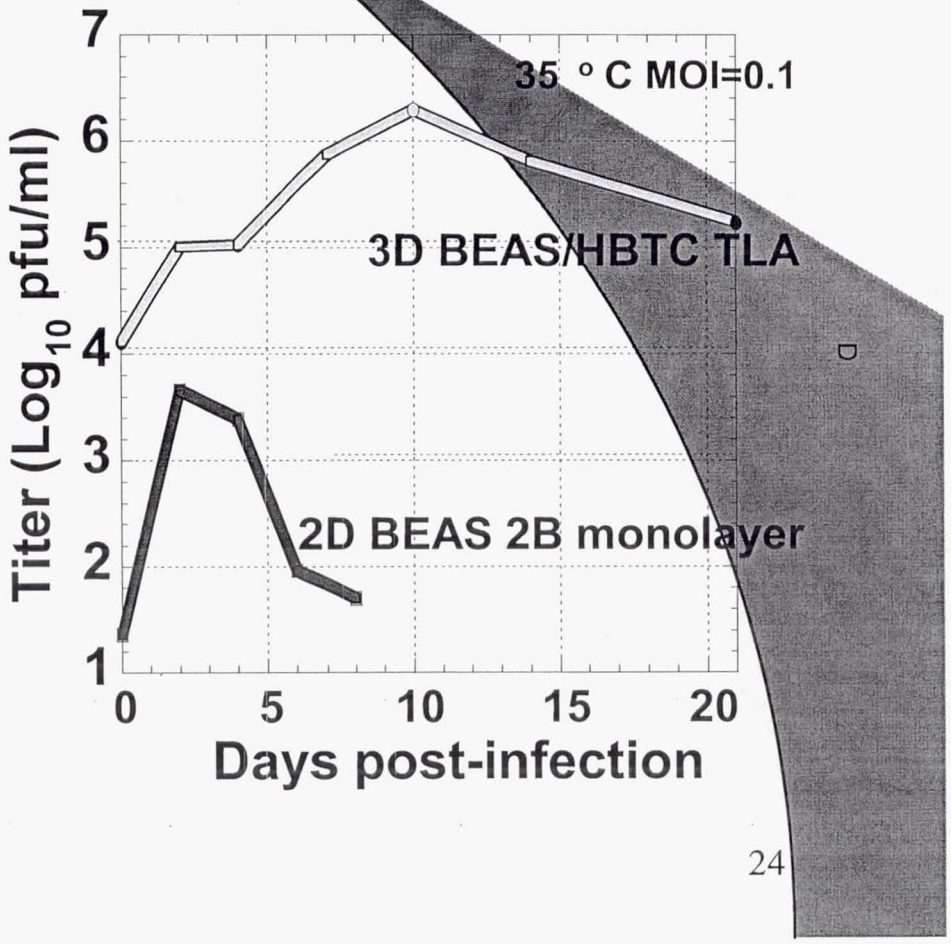
HBTC/BEAS –2B Co-Culture: RSV A2 Day 12 post-inoculation



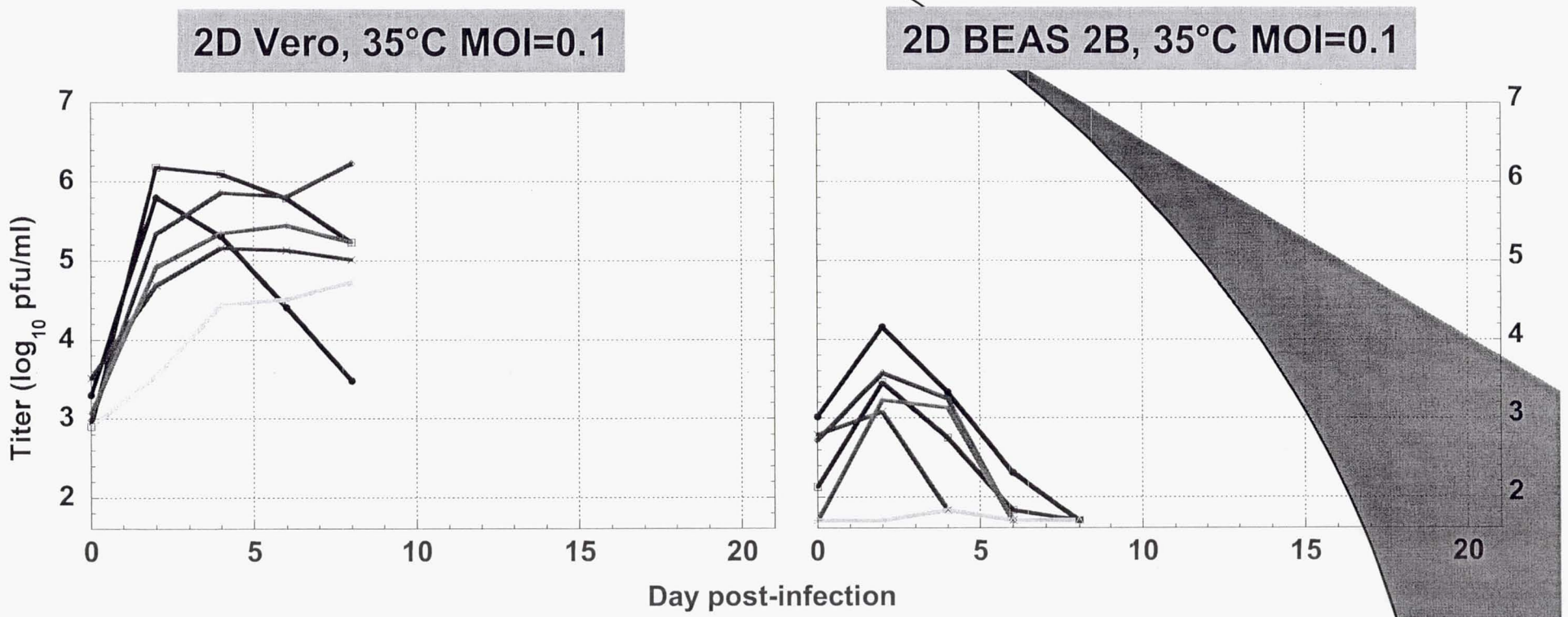


Replication of Wild Type RSV in HBE Cells

- A2 replicates more efficiently in 3D HBE TLAs relative to traditional 2D monolayer cultures of HBE cells.
  - **The peak titer is 6. 3 $\log_{10}$ pfu/mL in 3D HBE TLAs and 3.7 $\log_{10}$ pfu/mL in 2D HBE cells.**
  - **Peak replication is on day 10 in 3D HBE TLAs and on day 2 in 2D HBE cells.**
  - **A2 replicates efficiently up to day 21 in 3D HBE TLAs and rapidly declines after day 4 in 2D HBE cells.**

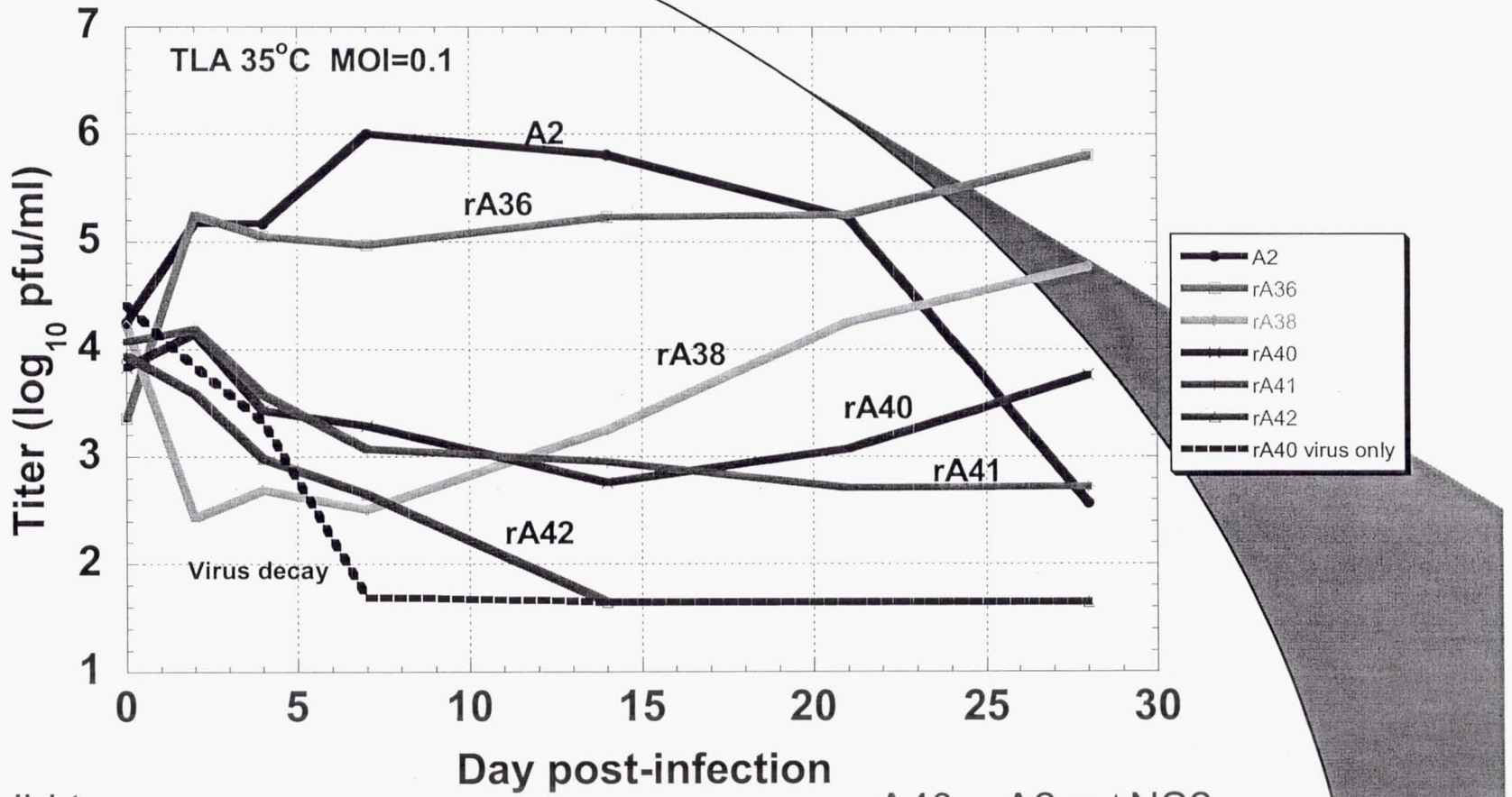


Replication Efficiencies of Most Attenuated RSV Strains Are Not Distinct from Wild Type A2 in Traditional Monolayers



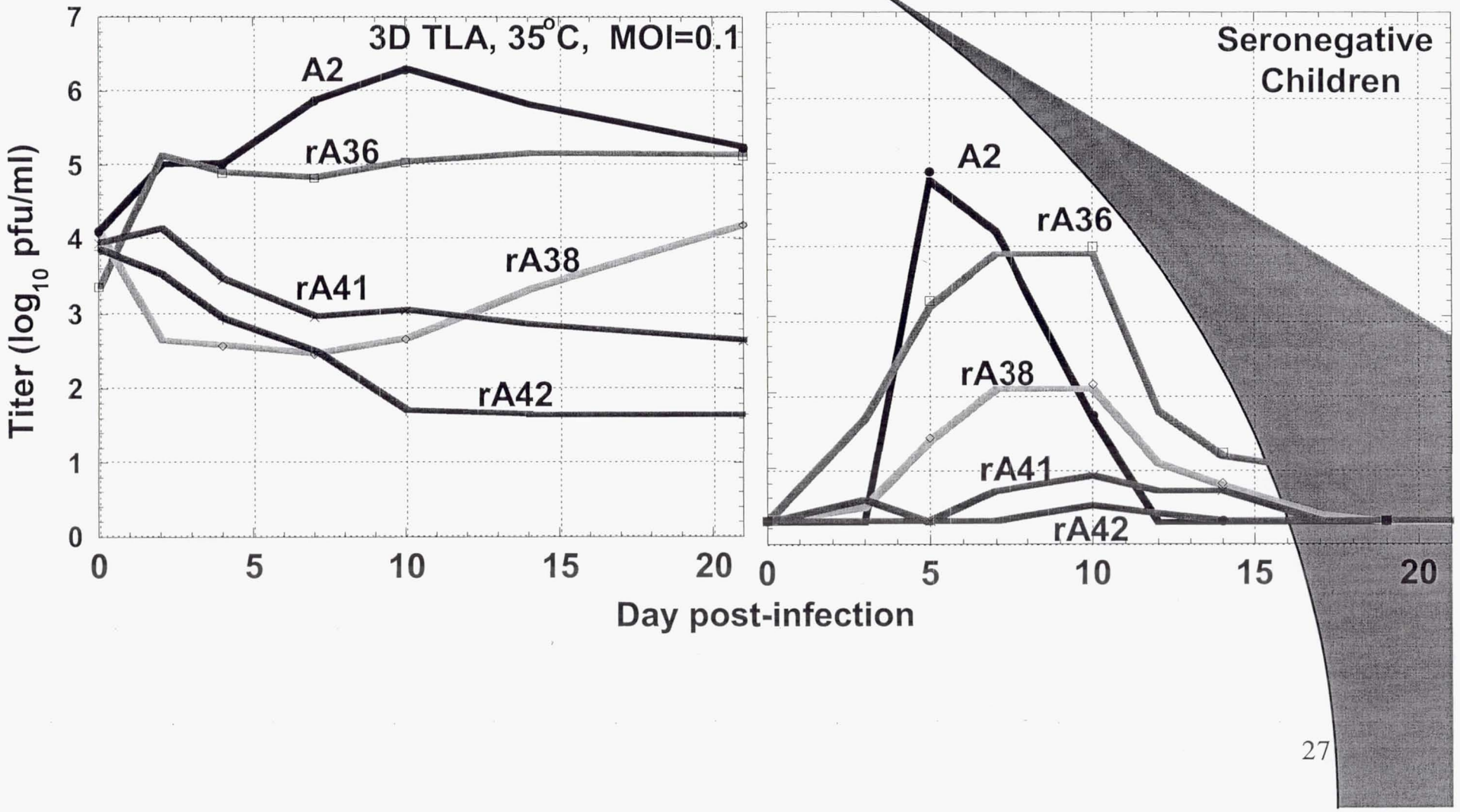
A2	wild type
rA36	rA2cp248/404ΔSH
rA38	rA2cp248/404/1030ΔSH
rA40	rA2cpΔNS2
rA41	rA248/404ΔNS2
rA42	rA2cp530/1009ΔNS2

Replication efficiencies of attenuated and wild type RSV strains are differentiated in 3D HBE TLAs

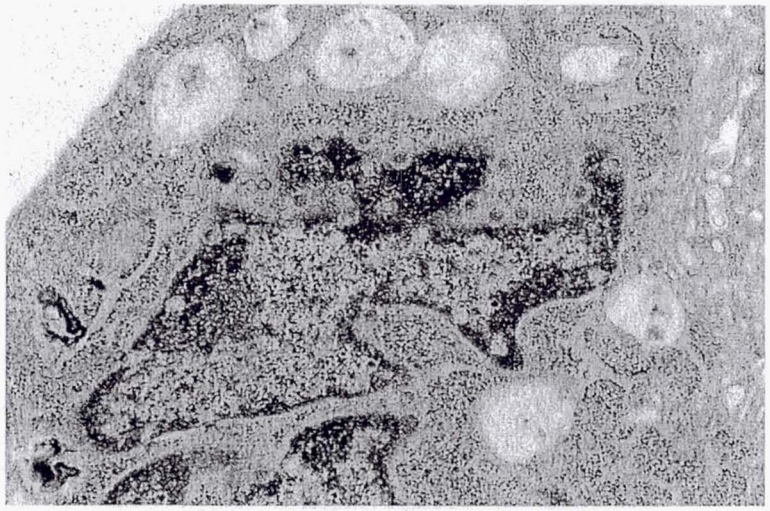


A2	wild type
rA36	rA2cp248/404ΔSH
rA38	rA2cp248/404/1030ΔSH
rA40	rA2cpΔNS2
rA41	rA248/404ΔNS2
rA42	rA2cp530/1009ΔNS2

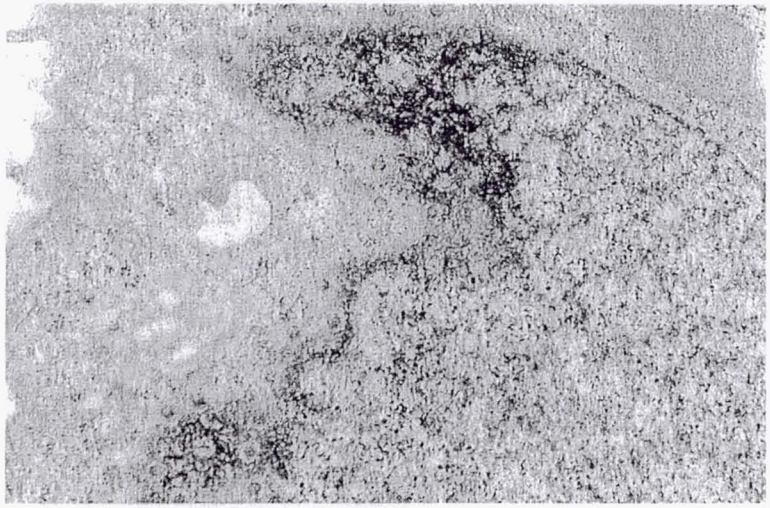
Relative replication efficiencies of attenuated RSV strains in 3D HBE TLAs parallel relative attenuation levels observed in human subjects



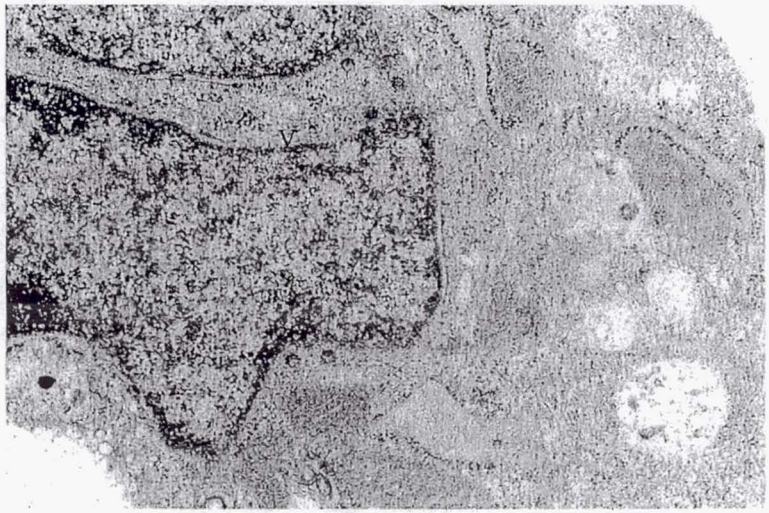
RSV Strains 1 Hr. post-inoculation



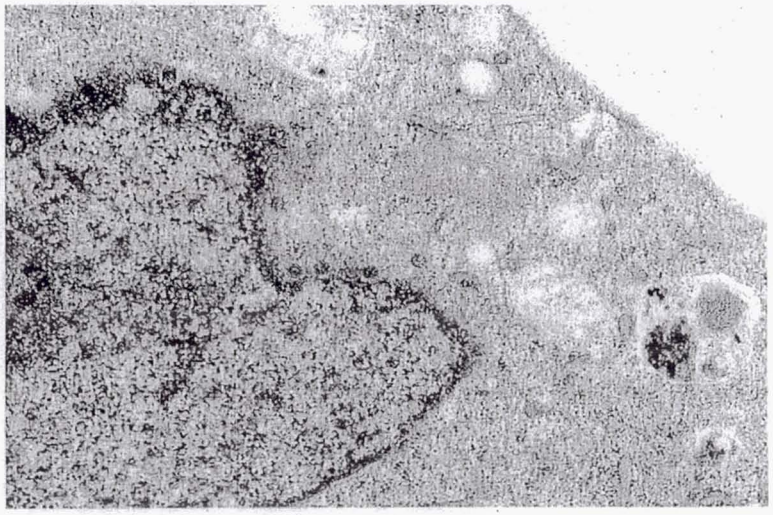
A:BEAS-2B:MONOCULTURE:A2-1Hr: 25000x



B:BEAS-2B:MONOCULTURE:248-1Hr: 25000x

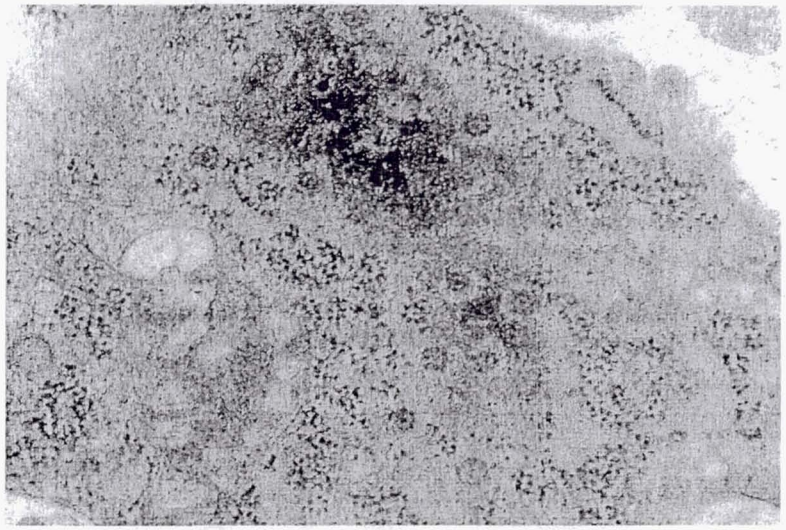


C:BEAS-2B:MONOCULTURE:rA40-1Hr. 25000x

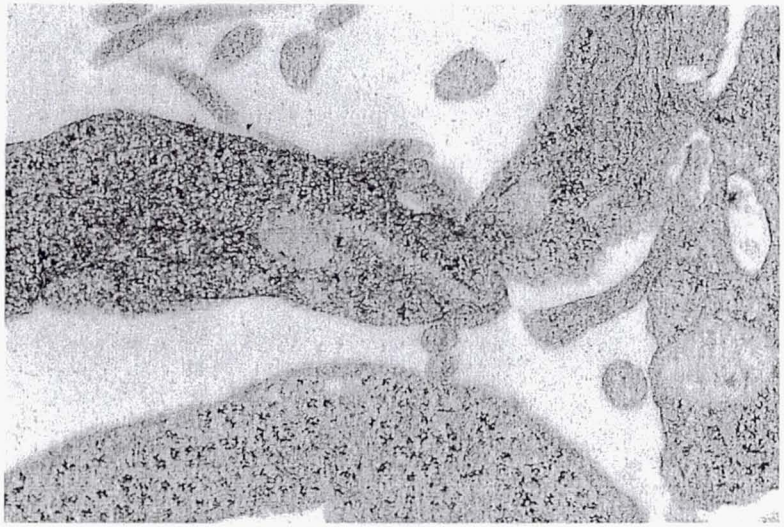


D:BEAS-2B:MONOCULTURE:rA40-1HR: 25000x

RSV strains 4 days post-inoculation

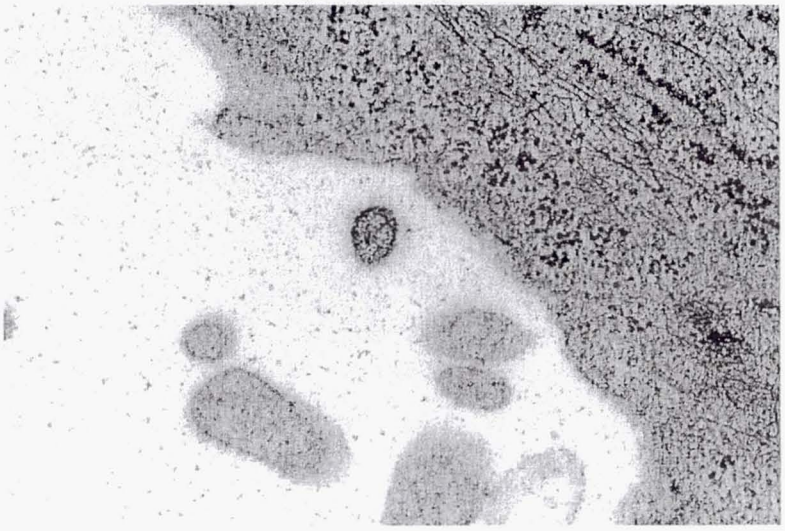


A:BEAS-2B:MONOCULTURE:A2-D4 25000x



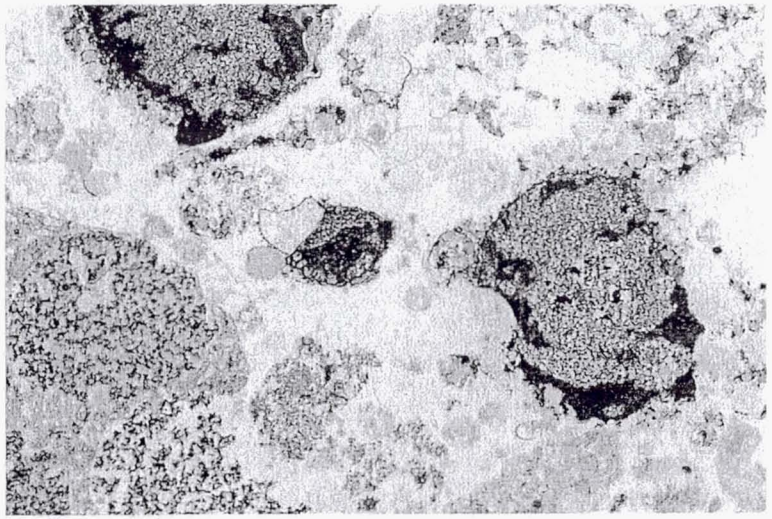
B:BEAS-2B:MONOCULTURE:A2-D4 25000x

C:BEAS-2B:MONOCULTURE:248-D4 25000x



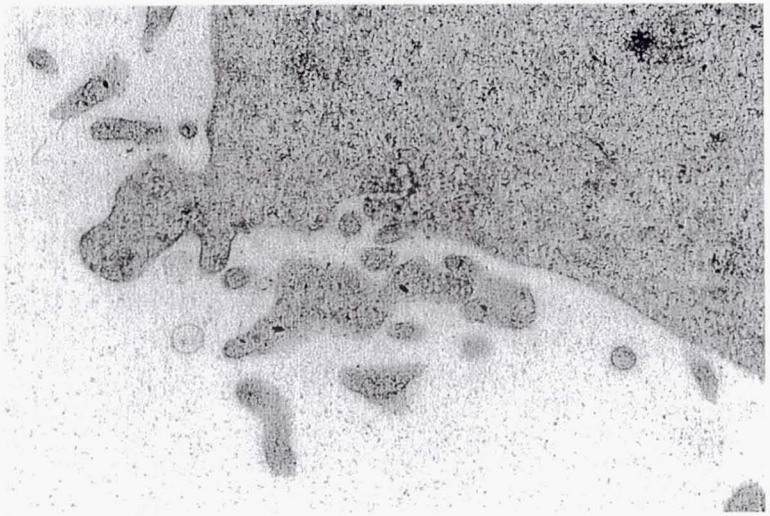
D:BEAS-2B:MONOCULTURE:rA40-D4 50000x

RSV Strains 9 days post-inoculation



A.BEAS-2B:MONOCULTURE:A2-D9:TEM 7500x

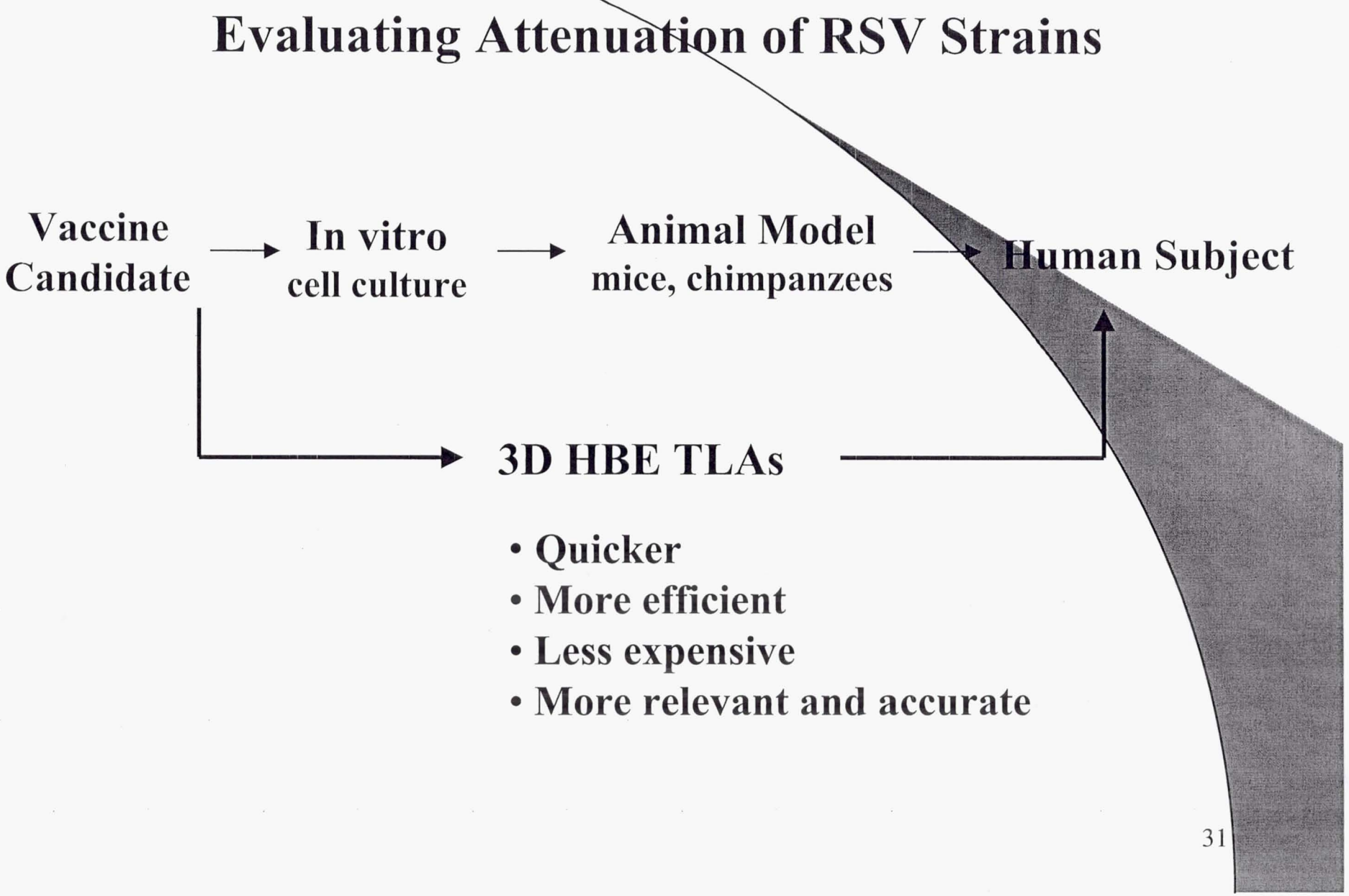
B.BEAS-2B:MONOCULTURE:248-D9:TEM 25000x



A:BEAS-2B:MONOCULTURE:rA40-9D:TEM 25000x

D.BEAS-2B:monoculture:rA40-D9:TEM 25000x

Evaluating Attenuation of RSV Strains



Conclusions from Current Research

- Three-Dimensional tissue models serve as successful targets for viral pathogens, pharmacology, and toxicology studies
- Complex tissues allow analyses of genomics and proteomics for vaccine development and cellular physiological responses
- Tissue Engineering Models may be used to augment animal test models.

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