# APENDICE A

## TECNICA DE INCLUSION EN RESINA EPOXICA

1. Fragmentar el pulmón en porciones de 1mm$^3$, fijar en una solución de Karnovsky-Ito a temperatura ambiente por 30 minutos.
2. Lavar tres veces de cinco minutos con buffer de cacodilatos 0.1 M pH 7.2-7.4
3. Post-fijar con tetraóxido de osmio al 2% por 30 minutos.
4. Lavar tres veces de cinco minutos con buffer de Cacodilatos 0.1M pH 7.2-7.4
5. Contrastar en bloque con Nitrato de uranilo al 1% por 30 minutos.
6. Lavar tres veces de cinco minutos con buffer de cacodilatos 0.1M pH 7.2-7.4
7. Desidratar las muestras durante cinco minutos cada uno, con acetona al 30%, 50%, 70%, 90%, y tres veces con acetona al 100%.
8. Impregnación del tejido en una mezcla 1:1 de Acetona-Epon (Resinas epóxicas) durante toda la noche.
9. Pre inclusión a temperatura ambiente durante 60 minutos, colocando las muestras en moldes para inclusión con epon al 100%
10. Inclusión y polimerización a 60°C durante 24-48h

## FIJADOR KARNOVSKY-ITO

1. Se disuelven 16gr. de paraformaldehído en 400ml de agua desionizada.
2. Calentar cerca del punto de ebullición y agregar varias gotas de hidróxido de sodio para quitar la turbidez.
3. Agregar 32ml de glutaraldehído al 25%, 0.08gr de ácido pícrico y 400ml de buffer de cacodilatos 0.1M pH =7.2 - 7.4
4. Guardar en un frasco ámbar.

## TINCION DE CORTES SEMIFINOS CON AZUL DE TOLUIDINA

1. Obtener cortes (1μm) de pulmón procesado con resinas epóxicas
2. Colocar los cortes en un portaobjetos
3. Mediante el uso de una placa de calor pegar los cortes al portaobjeto.
4. Colocar una gota de Azul de toluidina ( 5gr de Borato de sodio, 100ml de agua desionizada, y azul de toluidina 1gr.) sobre los cortes por 10 segundos.
5. Lavar el portaobjeto con agua destilada hasta quitar el exceso de colorante
6. Pasar gentilmente una gasa limpia sobre los cortes para secar el exceso de agua.

## TECNICA DE CONTRASTACION DE CORTES FINOS

1. Obtener cortes finos (100nm)
2. Montar los cortes en rejillas de cobre
3. En una caja petri limpia colocar las rejillas con los cortes hacia abajo sobre gotas de acetato de uranilo (disolver 0.5g de acetato de uranilo en alcohol etílico al 50%)
4. Lavar las rejillas con agua ultrapura por un minuto y dejar secar
5. Colocar las rejillas con los cortes hacia abajo sobre gotas de citrato de plomo al 0.4% (0.04g de citrato de plomo en 10 ml de agua ultrapura) agregar tres gotas de hidróxido de sodio al 10% para ayudar a disolver)
6. Lavar con agua ultrapura por un minuto y dejar secar sobre papel filtro en una caja de petri cerrada.

## TABLA IV

## CULTIVO DE ORGANO: CARACTERISTICAS Y CONDICIONES EMPLEADAS PARA EL PULMON DE RATON CEPA CD1

| Ratones cepa CD1 | Incubación a 37°C (6-8 explantes de pulmón de 1mm³ con MEM y suplementos) | Concentración de PA1 | Total de placas de cultivo[b] | Tiempos de incubación[c] |
|---|---|---|---|---|
| 5 | Cuatro concentraciones de PA1 y controles sin P A1 de cada tiempo de cultivo | 0.0µg/ml | 4 | 1, 2, 4, 6, 8, 12 24h |
| | | 0.2µg/ml | 4 | |
| | | 2.0µg/ml | 4 | |
| | | 5.0µg/ml | 4 | |
| | | 25µg/ml | 4 | |

[a] Como ya se mencionó la Peroxisomicina A1 se disolvió primero en etanol absoluto y después en medio de cultivo.

[b] Cada placa de cultivo tiene 8 espacios útiles para colocar explantes de cultivo de órgano

[c] Las diferentes concentraciones de PA1 y controles correspondientes se cultivaron por triplicado para cada tiempo de incubación.

## METODO PARA DETERMINAR LA ACTIVIDAD DE LACTATO DESHIDROGENASA (LDH)

1. Centrifugar los medios de cultivo (14,000 rpm, 4°C 5-10 minutos) para obtener el sobrenadante.

2. Llevar a cabo la reacción enzimática directamente en la cubeta espectrofotométrica agregar 833µl de una solución de β-NADH (0.113mg/ml disuelto en el amortiguador de Tris-HCL, libre de inhibidores) mas 70 µl de sobrenadante del medio de cultivo, tapar la cubeta y agitar dos veces por inversión.

3. Anotar el valor de la absorbancia de "fondo" (background) a 340nm cada minuto hasta que la lectura se estabilice. Normalmente se determino la absorbancia durante 3 a 4 minutos.

4. Iniciar la reacción enzimática, agregar 167µl de piruvato de sodio (9.76 mM disuelto en amortiguador Tris-HCL) tapar la cubeta y agitar dos veces por inversión.

5.-Anotar la disminución en la absorbancia a 340nm cada minuto y monitorear hasta por 10 minutos, o hasta que ocurra un cambio lineal de la absorbancia de $\geq 0.2$

6. Para los cálculos de la actividad enzimática, se determina el cambio negativo en la absorbancia a partir de la adición del piruvato, se utiliza la absortividad molar del NADH ($6.22 cm^2/\mu mol$) y se aplica la fórmula correspondiente.

$$\mathbf{U/ml = \frac{\Delta\ Abs.(340nm)}{(6.22 cm^2/\mu mol)\ (1cm)\ (min)} \times \frac{1\ min}{10\ min} \times \frac{1.07\ ml}{0.07\ ml} *}$$

*ε =$6.22 cm^2/\mu mol$ de NADH

10 min = Tiempo de ensayo

1.07ml = Volumen total de ensayo en la cubeta

0.07ml = Volumen de la fuente de enzima en la cubeta

## CUANTIFICACION DE TNFα POR ELISA

1. En los pocillos de la placa de ELISA se colocaron 100µl de estándares de TNFα de 0 pg/ml, 35pg/ml, 140pg/ml, 560 pg/ml y 2240pg/ml por duplicado y 50µl de suero problema y controles. A todas las muestras de suero problema y controles se les agregó 50µl de buffer de lavado, se cubrió la placa con cinta adhesiva y se incubó a 37°C por 2 horas.

2.Transcurrido este tiempo se removió el líquido invirtiendo la placa y dando una fuerte sacudida. Cada uno de los pocillos se lavó cuatro veces con 200µl de buffer de lavado cada ves, la placa se colocó sobre papel secante para retirar el exceso de líquido.

3. Enseguida se agregó 100µl de conjugado HRP anti TNFα en cada pocillo, la placa se cubrió con cinta adhesiva y se incubó a 37°C por 1 hora.

4. Después de esta incubación, se removió el líquido y cada uno de los pocillo se lavó cuatro veces de la manera antes descrita, se secó el exceso de líquido.

5. Se agregó 100µl de solución de sustrato a cada pocillo, se incubo a temperatura ambiente en la oscuridad por 10 minutos.

6. Para detener la reacción se agregó 100µl de ácido sulfúrico 1M. El color azul de las placas cambió a un color amarillo, la intensidad del color es directamente proporcional a la concentración de TNFα.

7. Se leyó la absorbancia a 450nm en un lector de placas de ELISA ajustando el 0 con el estándar de 0 pg/ml.

8. Al obtener las lecturas de los estándares se hizo una curva de concentración mediante un análisis de regresión lineal y se calculó la concentración de las muestras problemas. Se tomó en cuenta el factor de dilución 2 para la concentración final de los problemas.

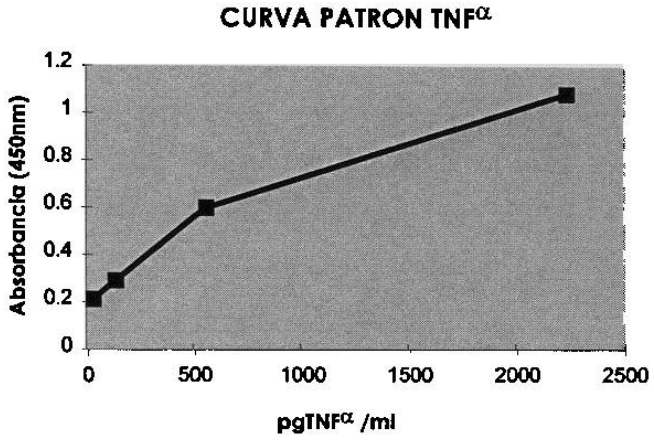


Figura 24. Curva patrón de TNFα cada punto representa los valores de los estándares utilizados y los puntos resultaron del análisis de cuatro determinaciones

## METODO DE LOWRY PARA DETERMINAR LA CONCENTRACION DE PROTEINA

Se llevo a cabo una precipitación de proteínas con ácido tricloracético de la siguiente manera:

1. Se colocó 0.5ml de la muestra (sobrenadante del homogenizado) en dilución adecuada mas 1 ml de Ácido tricloracético (TCA) al 10%, se reposaron los tubos por 10 minutos, posteriormente se centrifugaron a 2000rpm por 20-30 minutos, se decantó el sobrenadante de todos los tubos y se procedió a secar la pared de cada tubo.

2. A las muestras precipitadas en forma de pastilla se agregó 0.399ml de una solución que contenía: 0.033ml de tartrato de Na y K al 0.6%,
0.033ml de $CuSO_4$ al 0.3%,
0.033ml de $Na_2CO_3$ al 30%,
0.1ml de NaOH 0.8N y
0.2ml de agua desionizada.
El volumen final de cada tubo fue de 0.4ml.

3. Posteriormente se agregó 0.033ml de Folin Ciocalteau 2N y 0.167ml de agua desionizada.

4. Incubación a temperatura ambiente por 30 minutos.

5. Determinar la absorbancia a 750nm ajustando el cero con el blanco de reactivo.

6. Para los cálculos de concentración de proteína se utilizaron los valores de la curva patrón de BSA y se aplicó la fórmula correspondiente.

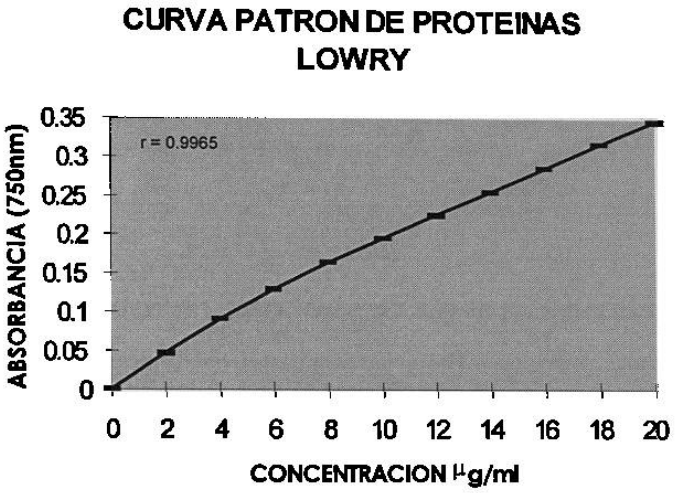


Figura 25. Curva patrón de proteína, cada punto representa el promedio de 10 curvas patrón con su desviación estándar y los puntos de cada curva resultaron del análisis de tres determinaciones.

# APENDICE B

Los resultados obtenidos en este trabajo de tesis doctoral se presentaron en dos congresos internacionales (se anexan copias de los resúmenes).

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# ABSTRACTS

## PART II

### Abstracts 3618-6467
### Tutorials T1-T18

## Experimental Biology 98®
## San Francisco, CA
## April 18-22, 1998

An Annual Meeting of Professional Research Scientists

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Volume 12, Number 5, March 20, 1998

4565

**IMAGING OF LIPOPOLYSACCHARIDE (LPS) RELEASE AND OUTER MEMBRANE DISRUPTION IN *Pseudomonas aeruginosa* BY X-RAY MICROSCOPY.**

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LPS is an important factor in pathogenesis of inflammation characteristic for the chronic *P.aeruginosa* infection in Cystic fibrosis (CF) patients. *P.aeruginosa* is believed to survive in the CF lung as microcolonies covered by biofilms; in addition, the *P.aeruginosa* strains isolated from CF patients are also highly mucoid. These factors suggest that *P.aeruginosa* surface is masked and should not be inducing such inflammation. We used a bench-top source of laser (nsec) plasma to generate X-rays to image live cells (in 0.9% phosphate buffered saline). A 5μl cell suspension was placed on a photoresist, covered with a (100nm) SiN window, sealed and then positioned in vacuum close to the X-ray source. The resist was then scanned by atomic force microscope to generate an image of differential X-ray absorption. By this technique we have captured images (exposure <10 nanosec) of live cells of *Pseudomonas aeruginosa*. LPS release and membrane disruption was induced by adding gentamicin at 25μg/ml for 15 minutes. X-ray micrographs of gentamicin treated cells show formation of distinct bead-like structures on cell surface, cell envelope dissociation and presence of granular content (0-7μm scale) between cell and envelope. We propose that use of some antibiotics, may lead to continuous release of LPS from partially damaged bacteria to result in sustained *in vivo* induction of inflammatory mediators from neutrophils and macrophages leading to severe lung damage. This work was supported by a grant from Mr.John Brittingham to Orlando Regional Healthcare Foundation.

4566

**UPREGULATION OF MURINE ICAM-1 IS INDEPENDENT OF TNF-a AND IL-1b IN A549 CELLS EXPOSED TO LIPOPOLYSACCHARIDE (LPS)**

Cathy Fakler, Bei Wu, Marilyn McMicken, Stephen Welty. Baylor Coll. of Med.

ICAM-1 is increased in mouse lungs exposed to hyperoxia or LPS. The goals of this study were to determine the mechanisms of ICAM-1 regulation in lung cells (A549) exposed to LPS. ICAM-1 mRNA is induced at 1.2 and 4h of exposure. To determine whether TNF-a or IL-1b mediate ICAM-1 induction the cytokines were measured after LPS exposure. TNF-a and IL-1b levels were not detectable. To determine whether levels below the limits of detection could induce ICAM-1, cells were incubated with ab to the cytokines. There was no inhibition ICAM-1 induction. To determine DNA sequences of the murine ICAM-1 gene involved in the regulation of ICAM-1, we sequenced the 5' flanking region of murine ICAM-1 from -2404 to the start site, and made serial deletion constructs. We reported that LPS induced reporter gene expressions in A549 cells transfected with sequences -355 and -309, but not with -280, suggesting that the DNA between -309 and -280 is important for induction. To determine the sequence in this region to which protein binds we conducted mobility shift assays in which we used as a probe the sequence from -305 to -281, which contains an AP-1 like site, and observed protein binding. Mutating the AP-1-like site TGACTCC to CAGTTCC abolished binding. However, the same mutation in -355 and -309 did not abolish induction. In conclusion, LPS induction of ICAM-1 is independent of TNF-a and IL-1b in A549 cells, and protein binding to an AP-1-like site in the ICAM-1 promoter does not appear to elicit ICAM-1 induction. Further studies to identify mechanisms for ICAM-1 expression are crucial to molecular interventions in sepsis. Supported by NIH HD27823.

4567

**KETAMINE SUPERINDUCES HEME OXYGENASE-1(HO-1) IN MURINE PERITONEAL MACROPHAGES AFTER EXPOSURE TO ENDOTOXIN (LPS)**

Keith Hunter, Augustine MK Choi*, Melville Wyche, (SPON:MCHOI). Departments of Anesthesiology, Howard University, Washington, DC 20060 and Pulmonary and Critical Care Medicine*, Johns Hopkins University, Baltimore, MD 21224

Ketamine is a sympathomimetic anesthetic agent that has been recognized for its protective role in endotoxic shock and in LPS-induced lung injury. Antioxidant enzymes have been shown to provide protection against the deleterious effects of LPS. Here we chose to examine whether one such antioxidant enzyme and stress response gene heme oxygenase-1 (HO-1) may play a role in the ketamine-induced protective effect against LPS. Murine peritoneal macrophages (RAW 264.7 cells) were exposed to LPS (1 μg/ml) in the presence or absence of ketamine and the steady state levels of HO-1 mRNA were determined by Northern blot analysis. LPS treatment induced HO-1 mRNA levels in RAW 264.7 cells in a time and dose dependent manner. When cells were pretreated with ketamine (10 μg/ml) for 1 h prior to exposure to LPS (1 μg/ml), we observed that ketamine caused a superinduction of HO-1 mRNA. Ketamine alone also induced HO-1 mRNA levels in RAW 264.7 cells. Our data suggest that the stress response gene HO-1 may play a role in ketamine-induced protection against LPS.

Supported by: Foundation for Anesthesia Education and Research

4568

**CD14 and interleukin-8 are localized primarily to alveolar macrophages in a rabbit model of acute bacterial pneumonia**

C.W. Frevert, O. Kajikawa, K. Wynant, T.R. Martin Seattle VAMC and the University of Washington, Seattle, WA 98108

Neutrophil (PMN) recruitment is an important component of early host defenses in bacterial pneumonia. Evidence suggests that membrane CD14 mediates the recognition of gram negative cell wall products by macrophages, resulting in the production of a variety of inflammatory mediators including the PMN chemotactic factor, IL-8. Even though *in vitro* data has shown that many cells are capable of making this chemokine, it is unknown which cells are responsible for IL-8 production *in vivo*. **Goals**: To study the tissue distribution of CD14 and IL-8 in the lungs of normal rabbits and rabbits treated with intratracheal (IT) *Escherichia coli*. **Methods**: Rabbits were treated with intratracheal *E. coli* ($1 \times 10^7$ cfu) and then studied 4 hr later. CD14 and IL-8, were localized in tissue sections using immunohistochemistry with polyclonal anti-rabbit CD14 antibody (5 μg/ml) and an affinity purified polyclonal anti-rabbit IL-8 antibody (5 μg/ml). **Results**: Using the anti-CD14 antibody, we observed that CD14 staining localized primarily to AM in the lungs of normal and *E. coli* treated rabbits. When slides were stained using the anti-IL-8 antibody, IL-8 staining was seen only in the lungs of rabbits instilled with *E. coli* and this staining was localized primarily to AM in these lungs. Thus, within the sensitivity of immunohistochemistry, CD14 and interleukin-8 are localized primarily to alveolar macrophages in a rabbit model of *E. coli* pneumonia. Supported by NIH GM37696 and American Heart Association of Washington

4569

**PEROXISOMICINE A-1 INDUCES TNFα SECRETION AND PMN INFILTRATION IN THE LUNG MICRDVASCULAR BED OF THE ALBINO MICE (CD-1).**

R. Ballesteros, S. Flores, A. Piñeyro and J. Sepúlveda). Dept. de Histología, Microbiología y Farmacología, Facultad de Medicina, U.A.N.L., Monterrey, Nuevo León, México, 64460.

Accidental ingestion of ripe fruits from genus *Karwinskia* plants causes flaccid paralisis in intoxicated humans. After a literature review we found that death in intoxicated humans is caused by lung disfunction such as:respiratory failure, pneumonia, and cardio-respiratory arrest. In a previous study we reported that T514 or Peroxisomicine A-1, one of the toxic substances present in the fruit of *Karwinskia*, causes lung damage charcterized by polimorfonuclear (PMN) infiltration and breakage of the alveolo-capillary membrane. This lesion is similar to the one described for the Adult Respiratory Distress Syndrome (ARDS). To further charcaterize the lung damage, we studied the cell pattern in the lung of intoxicated mice and the presence of tumor necrosis factor alfa (TNFα) both in circulating blood and in lung homogenates, since it is well established that TNFα is one of the main mediators involved in the production of ARDS. Mice were injected intraperitoneally with the following treatments: 1) peroxisomicine dissolved in carthamus oil, 2) peroxisomicine in salt solution, 3) peroxisomicine in haemaccel, 4) lipopolysacharide (LPS, positive control), 5) carthamus oil, 6) salt solution, 7) haemaccel and 8) negative control without any treatment.

Each half hour after treatments lung and blood samples were collected, beginning at 0.5 h and up to 5 h. Part of the lung was embedded in epoxy resin for light and electron microscopy evaluation, an the remainning was homogenized for TNFα quantification by ELISA. TNFα was quantified in blood also. Peroxisomicine induced lung damage characterized by infiltration of PMN. Macrophages and lymphocytes were also present in increased amounts compared to negative controls. TNFα was present in the lung and serum of mice treated with peroxisomicine, although higher concentrations were found when peroxisomicine was diluted in aquose solutions. LPS causes the most severe damage recorded and the highest induction of TNFα both in blood and lung homogenate. Carthamus oil induced a slight PMN infiltration in the lung and TNFα increase both in lung and serum.

4570

**IL-8 RECRUITS NEUTROPHILS TO THE LUNGS IN RESPONSE TO AEROSOLIZED ENDOTOXIN**

B.T. Peterson, E.J. Miller and P. McWaters, University of Texas Health Center, Tyler, TX 75710 and CSIRO Div. of Animal Health, Paskville, Victoria, Australia.

To determine the mechanism of neutrophil (PMN) movement through the lungs in response to aerosolized *E.Coli* endotoxin (LPS), we anesthetized 6 sheep with halothane, ventilated them with 25 mg LPS or saline (n=2), collected lung lymph and plasma every 30 minutes, and performed 2 single-cycle lung lavages 3 hours later. The PMN concentration in the epithelial lining fluid (ELF) was significantly greater in the sheep that received LPS vs. the control sheep (542±188 (se) vs. 3±2 $10^6$cells/ml ELF, $p<0.01$). The IL-8 concentration also increased above control values (1818 ± 443 vs 15 ± 4 ng/ml ELF) and there was a strong correlation between the PMN and IL-8 concentrations among the 12 lavages ($r = 0.96$, $p<0.001$). The lung lymph contained no PMNs and negligible IL-8 concentrations. However, in 4 additional sheep anesthetized with pentobarbital, the fraction of PMNs in the lung lymph increased to 44 ± 8% but the IL-8 concentration increased to only 53 ± 28 ng/ml lymph, a value considerably less than that in the ELF (2019 ± 542 ng/ml ELF) or the plasma (172 ± 41 ng/ml). Therefore, we conclude that IL-8 released into the air spaces in response to LPS recruits neutrophils into the lungs, but it does not explain the appearance of PMNs in the lung lymph. (Supported by NIH grant R29-HL55622)

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# Peroxisomicine A-1 induces TNFα secretion and pmn infiltration in lung microvascular bed of the albino mice (CD-1)

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Accidental ingestion of ripe fruits from genus *Karwinskia* plants causes flaccid paralisis in intoxicated humans. After a literature review we found that death in intoxicated humans is caused by lung disfunction such as:respiratory failure, pneumonia, and cardio-respiratory arrest. In a previous study we reported that T514 or Peroxisomicine A-1, one of the toxic substances present in the fruit of *Karwinskia*, causes lung damage charcterized by polimorfonuclear (PMN) infiltration and breakage of the alveolo-capillary membrane. This lesion is similar to the one described for the Adult Respiratory Distress Syndrome (ARDS) [2].

To further characterize the lung damage, we studied the cell pattern in the lung of intoxicated mice and the presence of tumor necrosis factor alfa (TNFα) both in circulating blood and in lung homogenates, since it is well established that TNFα is one of the main mediators involved in the production of ARDS. Mice were injected intraperitoneally with the follwing treatments: 1) peroxisomicine dissolved in carthamus oil, 2) peroxisomicine in salt solution, 3) peroxisomicine in haemaccel, 4) lipopolysacharide (LPS, positive control), 5) carthamus oil, 6) salt solution, 7) haemaccel and 8) negative control without any treatment.

Each half hour after treatments lung and blood samples were collected, beginning at 0.5 h and up to 5 h. Part of the lung was embedded in epoxy resin for light Fig 1and electron microscopy evaluation, and the remainning was homogenized for TNFα quantification by ELISA.

Peroxisomicine induced lung damage characterized by infiltration of PMN Fig 2. Macrophages and lymphocytes were also present in increased amounts compared to negative controls. TNFα was present in the lung and serum of mice treated with peroxisomicine, although higher concentrations were found when peroxisomicine was diluted in aquose solutions. LPS causes the most severe damage recorded and the highest induction of TNFα both in blood and lung homogenate[3]. Carthamus oil induced a slight PMN infiltration in the lung and TNFα increase both in lung and serum.

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3.-Denis M., A Mouse model of the lung injury Induced by Microbial Products. Am. J. Respir. Cell Mol. Biol. Vol.10. pp. 658-664, 1994.

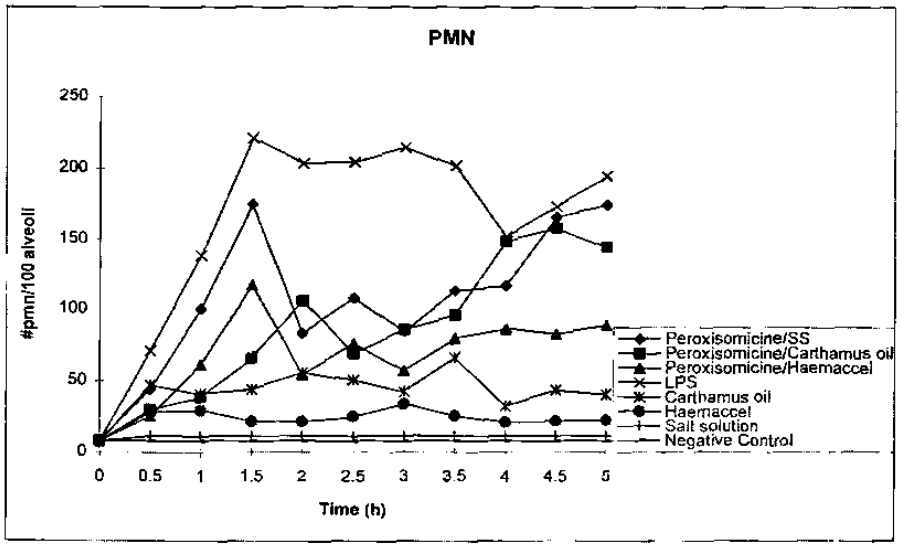


**Fig. 1.** PMN in mouse lung treated with peroxisomicine A-1 and controls.

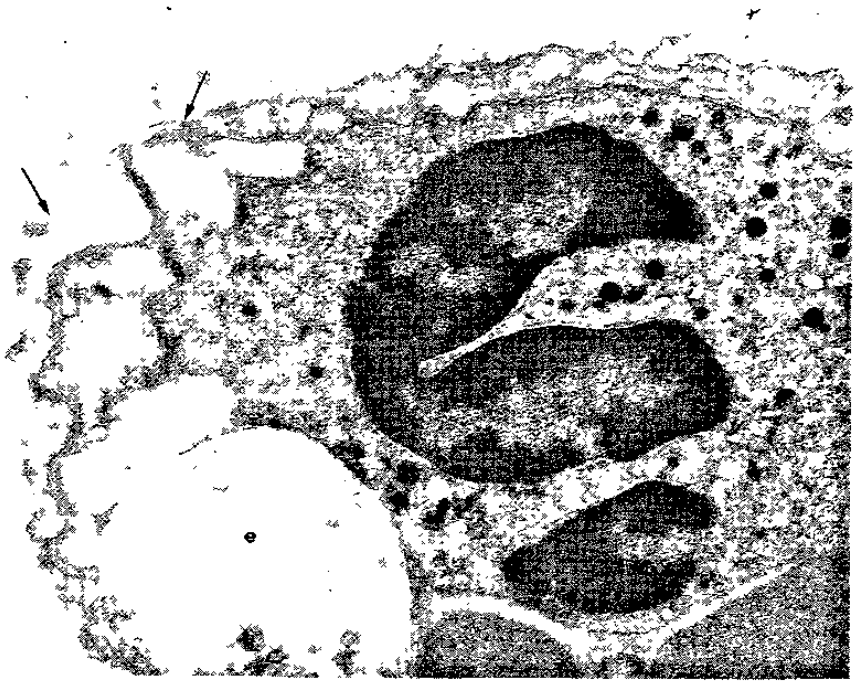


**Fig. 2.** PMN in alveolar capillary adherent to the endothelial cell (e). Edema and destruction of the alveolar-capillary barrier is present (arrow) 7000X.

**RESUMEN AUTOBIOGRAFICO**

**Raquel Guadalupe Ballesteros Elizondo**

**Candidato para el grado de**

**Doctor en Ciencias con especialidad en Morfología**

**Titulo de Tesis:** **FACTOR DE NECROSIS TUMORALα Y GRANULOCITOS NEUTROFILOS EN LA PRODUCCION DEL SINDROME DE INSUFICIENCIA RESPIRATORIA DEL ADULTO CAUSADO POR PEROXISOMICINA A-1**

**Área de Estudio: Morfología**

**Biografía:**

**Datos personales:** Nacida en Monclova, Coahuila, el 29 de mayo de 1965, hija de Sr. Andrés Ballesteros de la Cruz y Sra. Romelia Elizondo Elizondo.

**Escolaridad:** Egresada de la Facultad de Medicina de la Universidad Autónoma de Nuevo León, con el título profesional de Químico Clínico Biólogo en Noviembre de 1988.

**Experiencia Profesional:** Químico del Laboratorio de Análisis Clínico del Hospital San José de Monterrey de 1988-1992, Personal Profesional no Docente de tiempo completo del Departamento de Histología de la Facultad de Medicina de la Universidad Autónoma de Nuevo León desde 1989, Maestro de teoría y práctica de la materia de Histología desde 1994, Coordinador del laboratorio de Análisis de Imágenes e Histoquímica desde 1996, 15 trabajos de investigación presentados en congresos nacionales e Internacionales, Estudiante distinguido del Doctorado en Ciencias con especialidad en Morfología en 1998, dos artículos publicados en revistas con arbitraje internacional, Miembro de la sociedad de Química, Miembro de la sociedad de Microscopía electrónica, Candidato a Investigador del Sistema Nacional de Investigadores desde 1998.