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MODELOS EXPERIMENTALES DE FIBROSIS INTERSTICIAL PULMONAR DIFUSA

T E S I S

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P R E S E N T A

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INTRODUCCION.

La fibrosis pulmonar intersticial difusa (FPID), es el resultado final común de un conjunto heterogéneo de enfermedades agrupadas como neumopatías intersticiales crónicas, las cuales son habitualmente progresivas, irreversibles y letales y se caracterizan por presentar inflamación difusa y fibrosis en el parénquima pulmonar. A medida que el padecimiento avanza, los cambios morfológicos se acompañan de destrucción de la arquitectura pulmonar con pérdida progresiva de las unidades de intercambio gaseoso, hasta que el paciente muere (1).

A pesar de que se conocen múltiples entidades capaces de llevar a FPID, en cerca del 60% de los pacientes es imposible identificar el agente causal; a esta forma se le denomina fibrosis pulmonar idiopática y constituye un problema cada vez más frecuente en medicina (2). En este sentido, en los EEUU es responsable de más de 10,000 ingresos hospitalarios cada año y se calcula que tiene una prevalencia de 5 a 10 casos por 100,000 habitantes (3). En México no existen estadísticas confiables en relación a la morbimortalidad de este tipo de padecimientos, pero en el Instituto Nacional de Enfermedades Respiratorias se han atendido más de 400 pacientes nuevos en los últimos 5 años.

En términos generales, la FPID muestra rasgos clínicos y de laboratorio que permiten sospechar su diagnóstico (4). Los pacientes presentan disnea de esfuerzo progresiva que evoluciona hasta hacerse de reposo; en la radiografía de tórax aparecen imágenes reticulonodulares bilaterales con disminución de los campos pulmonares y las pruebas respiratorias muestran alteraciones predominantemente restrictivas con disminución de los volúmenes, capacidades y distensibilidad pulmonar con aumento de la presión de retracción elástica. En

ventilación e intercambio gaseoso se observa hipoxemia de reposo que se exacerba con el ejercicio e hipo o normocapnia.

El diagnóstico de certeza se obtiene mediante el estudio histológico realizado en muestras de biopsia. Básicamente se encuentran diferentes grados de inflamación intersticial, con presencia de células inflamatorias, habitualmente macrófagos, en los espacios alveolares, cuboidización del epitelio alveolar por reemplazo de neumocitos tipo I por neumocitos tipo II y/o células bronquiolares y fibrosis multifocal o difusa (5).

En las etapas terminales, la FPID se caracteriza por presentar el llamado "pulmón en panal", donde de observan espacios aéreos no funcionales de diferentes tamaños, separados entre sí por gruesas paredes de tejido fibroso y cantidades variables de músculo liso (6). Esto representa la forma más grave y avanzada de destrucción del parénquima y en estas condiciones el pronóstico del paciente es sombrío.

En los últimos 10 años se han realizado numerosos estudios en humanos y modelos experimentales con el objeto de esclarecer los mecanismos patogénicos involucrados en el desarrollo de la fibrosis pulmonar, pero los resultados han sido fragmentarios, contradictorios y poco concluyentes (7-15).

En general, los hallazgos obtenidos sugieren que existen 2 etapas durante el curso de la fibrosis. La primera potencialmente reversible, se caracteriza por la presencia de células inflamatorias en el intersticio y espacios alveolares junto con un profundo cambio en las poblaciones celulares del parénquima destacando una disminución de neumocitos tipo I y de células endoteliales con proliferación de neumocitos tipo II, células cebadas, células musculares lisas y fibroblastos (7).

La segunda etapa consiste en alteraciones moleculares de la matriz intersticial, con desarreglo progresivo en la ubicación, cantidad y tipos genéticos de colágena y destrucción - concomitante de las unidades alveolo-capilares. Estos fenómenos marcan el desarrollo de la lesión fibrosante y se consideran irreversibles (7).

Sin embargo, los mecanismos íntimos que ocurren en el microambiente pulmonar, responsables de la desorganización de - la arquitectura normal del parénquima siguen siendo desconocidos y hasta la fecha, por razones obvias, la secuencia de los eventos no ha podido ser estudiada en detalle en seres humanos. En términos generales, la mayoría de los conceptos actuales sobre este padecimiento, se basan en observaciones aisladas de puntos estáticos de su progresión en humanos. Es por ésto que se ha planteado la necesidad de contar con un modelo experimental de FPID, que permita el estudio dinámico de los fenómenos patogénicos.

Un buen modelo permitiría un mejor conocimiento de las - formas de presentación y de las relaciones estructura-función pulmonar y facilitaría los esfuerzos para definir la secuencia de su patogénesis y la búsqueda de medidas terapéuticas - racionales.

ALGUNAS CONSIDERACIONES SOBRE MODELOS EN GENERAL Y MODELOS DE FPID EN PARTICULAR

Los modelos son un ensayo de la realidad, una forma de aproximación al universo con el que se trata y se desea descubrir.

La premisa fundamental en el diseño de un modelo, es la exención de partes reales pero insignificantes del problema

en estudio, para concentrarse en las características relevantes del universo modelado. De esta manera, un modelo es una substracción conciente y a menudo radical que debe contener solamente aquellos elementos de la realidad que son indispensables para la resolución del problema.

Es por ésto que en la construcción de un modelo es necesario enfrentarse al problema de determinar explícitamente cuales atributos del fenómeno se desea investigar o que constituyen la clave de éste y en consecuencia incorporar dichos caracteres al modelo. Esto sin embargo, puede ser muy difícil ya que de un universo dado, pueden surgir varios arquetipos posibles.

En términos generales, el diseño de un modelo presenta dos problemas importantes. Uno de ellos, lo constituye paradójicamente, lo que es una de sus principales virtudes, la reducción selectiva que le es inherente. Como ésto implica necesariamente una simplificación, puede suceder que se confunda la precisión del modelo simplificado con la compleja realidad de la que ha sido extraído.

El otro problema se deriva de la exactitud de nuestro conocimiento de la realidad que se desea modelar. La realidad suele ser compleja y nuestra percepción de ella es subjetiva. Para conocer a la realidad hay que captar el fenómeno total de un problema determinado, lo que significa indagar y describir cómo se manifiesta el problema en dicho fenómeno. De esta manera, el éxito de un modelo radica finalmente en la precisión de nuestro conocimiento de la realidad del universo modelado.

Modelos Animales.

Un modelo animal se puede definir como un organismo vi-

viente con una enfermedad adquirida de manera natural o inducida experimentalmente que semeja estrechamente al padecimiento que ocurre en el humano.

Modelos Naturales de FPID.

Pirie y cols.(16), han descrito un modelo de FPID en bovinos que presenta rasgos clínicos y morfológicos similares a la enfermedad humana; sin embargo, este hallazgo no ha sido corroborado por otros autores.

Modelos Inducidos de FPID.

En 1968, Carrington (6) describió lo que a su juicio constituyen los criterios esenciales para un buen modelo de FPID. No obstante, este enfoque es incompleto ya que sólo abarca alteraciones morfológicas y funcionales y no considera aspectos clínicos y bioquímicos.

Con el objeto de desarrollar un modelo adecuado de este padecimiento, hemos seleccionado los preceptos que a la luz del conocimiento actual consideramos claves en la definición de dicho modelo.

Estos son:

1) Que sea un padecimiento crónico, progresivo y potencialmente letal.

2) Que produzca alteraciones morfológicas, difusas o multifocales, similares a las observadas en el humano. Esto es, inflamación intersticial e intraalveolar, proliferación epitelial, proliferación de fibroblastos y fibrosis.

3) Que exista un aumento en el contenido total de colágena pulmonar.

4) Que los animales presenten signos clínicos y funcionales de insuficiencia respiratoria.

En general estos criterios son difíciles de cumplir y una extensa revisión de la literatura que se anexa en la bibliografía del primer artículo, sugiere que a pesar de los numerosos modelos intentados, la mayoría de éstos no cumplen con estos requisitos.

En nuestro laboratorio intentamos previamente reproducir un modelo de FPID (17), utilizando varios de los agentes sugeridos en la literatura, sin preocuparnos del problema del agente etiológico dado que muchos casos humanos son idiopáticos y la enfermedad es muy similar independientemente de la causa que la provoca.

Los modelos experimentales fueron dos tipos genéricos:

a) Acción de un solo agente nocivo.

b) Efecto de diversas combinaciones de dos agentes nocivos.

Las substancias tóxicas que se exploraron fueron bleomicina, ciclofosfamida, hidroxitolueno butilado, paraquat y oxígeno. En el caso de la combinación de dos agresores, uno de ellos fue siempre el oxígeno a concentraciones superiores a 70%. Los animales utilizados fueron ratón y rata y la substancia tóxica se administró por vía sistémica (intraperitoneal) o local (intratraqueal). Después de probar 30 modelos diferentes, comprobamos que la mayoría de ellos resultaron un fracaso, en términos de reproducir los criterios señalados.

No obstante, la combinación de paraquat y oxígeno resultó en extenso daño pulmonar con elevada mortalidad durante la primera semana. Los animales que sobrevivieron períodos más prolongados mostraron alteraciones morfológicas y bioquímicas

sugestivas de fibrosis incipiente y estos experimentos iniciales fueron la base de los trabajos que se presentan a continuación.

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A New Model of Diffuse Interstitial Pulmonary Fibrosis in the Rat[1]

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We have produced experimental diffuse interstitial pulmonary fibrosis in rats with a combination of low and repeated doses of paraquat plus continuous exposure to normobaric 74% O_2 in the breathing air for several weeks. Pulmonary fibrosis was evaluated histologically and biochemically, through the determination of total collagen content in the lung. Our procedure is characterized by low initial mortality, the development of extensive distortion of the pulmonary architecture, and the presence of severe and diffuse interstitial fibrosis. The model was compared with bleomycin-induced pulmonary fibrosis in the same rat strain, in which the process is focal and leaves most of the lung unaffected. We conclude that lung damage produced by the combination of low doses of paraquat plus normobaric 74% O_2 concentration in the breathing air is an adequate experimental model of diffuse interstitial pulmonary fibrosis as it occurs in many of the human cases of this condition.

INTRODUCTION

In recent years there has been renewed interest in diffuse interstitial pulmonary fibrosis (DIPF). A number of studies have been published in man (Campbell *et al.*, 1981; Crystal *et al.*, 1976, 1978; Dill *et al.*, 1975; Fraire *et al.*, 1975; Fulmer and Crystal, 1979; Fulmer *et al.*, 1979, 1980; Greenberg *et al.*, 1974; Heard, 1976; Hinson, 1970; Liebow, 1975; Scadding and Hinson, 1967; Turner-Warwick *et al.*, 1980) and several experimental models have been developed in various animal species, using agents such as oxygen (Adamson *et al.*, 1970b; Adamson and Bowden, 1974a; Balentine, 1966; Hestergerg and Last, 1981; Last *et al.*, 1979, 1981), bleomycin (Adamson and Bowden, 1974b; Aso *et al.*, 1976; Clark *et al.*, 1982; Collins *et al.*, 1981; Fasske and Morgenroth, 1983; Giri *et al.*, 1980; Hesterberg *et al.*, 1981; Laurent *et al.*, 1981; McCullough *et al.*, 1978; Phan *et al.*, 1980; Thrall *et al.*, 1979; Tryka *et al.*, 1983), paraquat (Butler, 1975; Greenberg *et al.*, 1978a; Greenberg *et al.*, 1978b; Omaye and Reddy, 1980; Smith *et al.*, 1974), butylated hydroxytoluene plus O_2 (Adamson *et al.*, 1977; Haschek *et al.*, 1981, 1982; Witschi *et al.*, 1980), radiation (Adamson *et al.*, 1970a; Law *et al.*, 1976; Pickrell *et al.*, 1978; Ward *et al.*, 1983), and several others (Haschek *et al.*, 1980; Hurych *et al.*, 1981; Kobrle *et al.*, 1982; McCall *et al.*, 1983; Niewohener and Hoidal, 1982; Saunier, 1982; Yamaguchi *et al.*, 1975, 1981).

In both human and experimental studies of DIPF a variety of morphologic and biochemical techniques have been used by the pathogenetic mechanisms responsible for the massive increase in interstitial connective tissue and the ultimate

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distortion of the normal lung architecture remain unknown. To contribute to our understanding of this complex problem we have developed a new experimental model of DIPF in the rat. In this paper we report details of the experimental procedure and a comparison of the lesions produced with those obtained in bleomycin-induced pulmonary fibrosis in the same animal strain.

MATERIALS AND METHODS

Animals. In all experiments male Wistar rats were used, weighing 170–230 g (9–10 weeks old).

Experimental model. Various combinations of paraquat (1,1'-dimethyl-4,4'bipyridylium) and exposure to normobaric O_2 at 74% concentration were explored (Table 1). Paraquat was always given as a 0.05% solution in saline, freshly prepared before each administration.

In order to achieve and preserve an atmosphere enriched in O_2 airtight chambers with a volume of 160 liters were built of sheets of transparent acrylic. Their size allowed the animals sufficient space to move around, and to eat and drink *ad libitum*. In experiments with continuous exposure to O_2 the animals were only removed from the chamber at the time of the ip injection of paraquat (2–5 min). Air and O_2 were combined in an O_2 mixer with a flow of 8–9 liters/min and 74% O_2 concentration; the gas mixture was humidified with a thermic nebulizer prior to delivery into the chamber. The O_2 concentration was monitored periodically with a gaseous mixture analyzer.

Bleomycin-induced pulmonary fibrosis. Twenty six male rats were treated according to Thrall *et al.* (1979) with a single intratracheal administration of 3.5–4.5 mg of bleomycin/rat. Eight of these animals received no further treatment, while the remaining 18 rats were exposed to continuous normobaric 74% O_2 in the breathing air for period of 5 days (6 rats), 7 days (6 rats), and 4 weeks (6 rats).

Preparation of lungs. Many rats were sacrificed when moribund and a few others were autopsied immediately after being found dead. Resisting animals (see Results) were sacrificed after completion of the 5-week period of exposure to O_2; in one experiment (T5) after completion of the 5-week exposure to O_2 the animals were placed for one more week in environmental air, and in another experiment (T7), for one more month, before they were sacrificed. The lungs were perfused intratracheally with buffered 10% formaldehyde, preserved *in situ* for 10 min, and then removed en block with the heart and placed in the same formaldehyde solution until further study.

Histological studies. The pulmonary tissue blocks were embedded in paraffin, cut at 4 μm, and stained with HE and the Verhoeff–Van Gieson stain for elastic fibers; other sections were cut at 10 μm and stained with the Picrosirius Red technique according to Junqueira *et al.* (1979). When the latter preparations are observed under polarized light they reveal a very complete picture of collagens types I and III in pleura, vascular, and parenchymal areas; in addition, collagen type II in bronchial cartilage is also stained. Care was taken to block the lung tissue as to include the maximal amount of surface in the sections. Thus, in practically all histology slides examined a complete section of an entire lung was present, including pleura, hilar structures, and all intervening pulmonary parenchyma. In those few instances in which the sampling was less than complete multiple sections of the lung were examined.

TABLE I
Variations in Dose and Schedule of Paraquat Administration and in Time of Exposure to Normobaric 75% O_2

	Number of animals	Dose of paraquat (mg/kg body wt)	Number of ip injections	Interval between injections	Maximum time of exposure to 75% O_2
Acute experiment	12	5.0	5	48 hr	2–9 days
Chronic experiments					
T_1	8	3.5	5	1 week	5 weeks
T_2	8	4.0	5	1 week	2–5 weeks
T_3	7	4.5	5	1 week	5 weeks
T_4	13	4.5	1	—	2–5 weeks
T_{5a}	9	4.0, 5.0, 6.0	3	1 week	3 weeks
T_{5b}	9	4.0, 7.5, 7.5	3	1 week	3 weeks
T_6	12	4.0	10	72 hr	3 weeks
T_7	8	3.5	10	72 hr	5 weeks
T_8	8	2.5	20	72 hr	9–10 week

Collagen measurement. All the left lung and the remaining fragments of the right lung (after removal of a thin block for histologic study) were dried to constant weight, ground, and 20- to 50-mg aliquots were hydrolyzed with 6 *N* HCl for 20 hr at 105°C, filtered, dried, resuspended in distilled H_2O, and the hydroxyproline content was measured in triplicates by the technique of Rojkind and González (1974).

RESULTS

Clinical observations. The entire group of rats subjected to the various combinations of paraquat ± O_2 (Table I) was clearly separable into two subgroups according to their general response (or absence of it) to treatment, regardless of the dose and/or frequency of administration of paraquat or O_2: the *susceptible* animals showed, during the first 24 to 48 hr of treatment, various signs and symptoms of severe and progressive respiratory insufficiency, such as bradypnea, intercostal pull, nasal twitching, and cyanosis. These rats uniformly lost weight during the experiment (10–30% of their initial body weight) and their mortality was 100%, usually early within the first 10 days in the experiments with the higher doses of paraquat (Table II). Occasionally these animals showed intense psychomotor depression or excitation with physical stimuli, both probably conditioned by cerebral hypoxia. On the other hand, in some of the groups there were some *resistant* rats which failed to show any one of the signs and symptoms described above; they gained weight at the expected normal rate and appeared quite normal at the time of sacrifice.

Animals treated with intratracheal bleomycin failed to reveal any clinical changes. They increased in weight at the normal rate, there were no signs of respiratory distress, and there was no mortality.

Histopathologic features. Susceptible animals in the various groups treated with paraquat ± O_2 revealed generalized and diffuse pulmonary lesions, equally severe in subpleural, hilar, and intervening lung parenchyma. In the acute experiments deaths occurred after 3 and 8 days following the initiation of treatment; these animals showed irregular but clearly noticeable dilatation of distal air spaces

TABLE II
Time of Survival and Lung Collagen Content of Animals Given Paraquat and Exposed to Normobaric 74% O_2

	Time of survival	Number of surviving animals	Collagen content (mg/lung)
Acute experiment	3 days	9/12	13.5 ± 1.5
	8 days	3/12	*28.0 ± 0.9
Chronic experiments			
T_1	5 weeks	6/8	10.2 ± 3.2
T_2	5 weeks	4/8	13.6 ± 4.7
T_3	5 weeks	4/7	15.6 ± 6.8
T_{4a}	9–12 days	7/13	20.2 ± 9.9
T_{4b}	5 weeks	6/13	18.0 ± 10.10
T_{5a}	9–18 days	5/19	*18.1 ± 3.2
T_{5b}	25 days	4/9	16.0 ± 4.2
T_6	9–21 days	12/12	*17.8 ± 2.9
T_{7a}	15–20 days	4/8	*22.9 ± 6.9
T_{7b}	30 days + 1 month	4/8	*28.6 ± 7.4
T_{8a}	3 weeks	3/8	13.2 ± 1.8
T_{8b}	9–10 weeks	5/8	*17.1 ± 3.4
Normal animals	—	20	11.1 ± 2.6

* The difference from the normal is statistically significant at $P < 0.01$.

with collapse of the intervening alveoli and profuse intralveolar hemorrhage. In the chronic experiments deaths occurred as early as 9 days and as late as 10 weeks after the initiation of treatment; indeed, in three groups (T4, T7, and T8) it was possible to consider the animals as falling into two different subgroups, early and late, in relation to their survival time. Because of the rather uniform character of the histologic changes observed, which seemed to depend more on the time of survival than on the different doses and administration schedules of paraquat, and in order to avoid repetitions, the microscopic lesions in the lungs are briefly described in only two groups of animals: early and late survivors.

Early survivors. The lungs appeared grossly irregular, with subpleural hemorrhages and focal areas of increased consistency. Under the microscope there were three major alterations: (a) pronounced, widespread, and irregular dilatation of the distal air spaces, seen in many cases to be lined at one end by bronchial epithelium; (b) collapse of the intervening alveoli, with edema and infiltration by mononuclear cells, present both within the alveolar septa and in the alveolar lumen; some animals revealed more intralveolar cellular accumulation than others (Fig. 1); (c) with the Picrosirius Red stain it was appreciated that the pulmonary collagen network was distorted but did not appear increased.

In addition, two other features were prominent in the histological picture of early survivors: there was perivascular and peribronchial edema, sometimes quite pronounced, with few mononuclear cells infiltrating the surrounding loose connective tissue. The larger intrapulmonary bronchi were not dilated but, on the contrary, their normal aspect contrasted with the generalized dilatation of the distal air spaces.

Late survivors. Pulmonary changes in animals surviving longer than 3 weeks consisted of the same irregular dilatation of the distal air spaces and collapse of the intervening alveoli described in early survivors, but in addition there was further condensation of the collapsed parenchyma, increased interstitial infiltra-

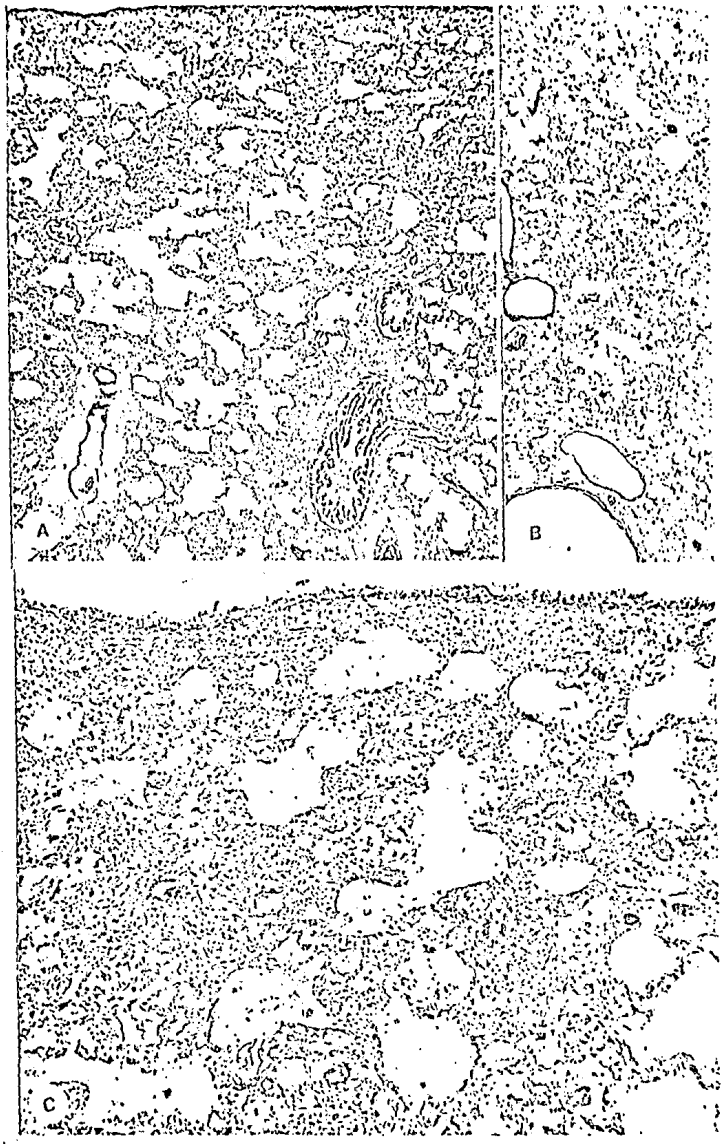

FIG. 1. Histologic aspect of the lung in early survivors of treatment with paraquat + O_2 as described in the text. (A) Low-power micrograph to illustrate the diffuse nature of pulmonary damage, the dilatation of the terminal air ducts and collapse of the intervening alveoli. ×85. (B) A section of normal rat lung photographed at low power for comparison. ×85. (C) higher magnification of the same slide as in (A) to demonstrate the interstitial infiltration by mononuclear cells and the collapsed alveoli. ×213.

tion of mononuclear cells, presence of many elongated, spindle-shaped, fibroblast-like cells in both the lumen and thickened septa of collapsed alveoli (Fig. 2), and the Picrosirius Red stain revealed a clear and definite increase in the amount of interstitial collagen fibers (Fig. 3). In addition, larger pulmonary bronchi also appeared dilated and there was peribronchial fibrosis.

Animals treated with bleomycin alone, or with bleomycin + O_2, revealed small multifocal areas of perivascular, peribronchial, and subpleural inflammation and fibrosis. The univolved lung tissue, which occupied most of the histologic sections, was completely normal (Fig. 4). The Picrosirius Red stain revealed increased collagen fibers in the focal areas of fibrosis.

Lung collagen content. Total lung collagen in 20 normal rats of the same age and sex as those used for the experiments described had an average content of 11.1 mg ± 2.6 mg/lung. In the acute experiments total lung collagen was not increased after 3 days but it appeared to be definitely higher than normal after 8 days; nevertheless, this figure is based only on three animals and should be considered as preliminary (Table II). In the more chronic experiments a trend was observed for late survivors to show significantly higher total lung collagen content than normal rats, although again the relatively small number of animals in each group and, in some instances, the large standard deviations found, prevent any futher analysis of the data.

In bleomycin-induced pulmonary fibrosis there was significant increase in total collagen content in the lungs only after 13 weeks; in all the other animals the differences with the normal rats were not significant (Table III).

DISCUSSION

An adequate experimental model of human DIPF should fulfill the following minimal requirements: (a) be a chronic, progressive, and potentially lethal disease; (b) develop clinical, radiologic, and functional data of pulmonary insufficiency; (c) resemble the histologic and ultrastructural features of the human disease, which at different times are described as alveolar septal fibrosis, alveolar "desquamation," cuboidalization of alveolar lining cells, interstitial inflammation, smooth muscle proliferation, narrowed (or normal, or dilated) airways, etc. (Carrington, 1968; Crystal *et al.*, 1976; Dill *et al.*, 1975; Fraire *et al.*, 1973; Greenberg *et al.*, 1974; Heard, 1976; Scadding and Hinson, 1967); these various pathologic changes may not all occur simultaneously in every human case of DIPF, but those present in a given case should have at least a multifocal, and preferably a generalized or diffuse distribution throughout the pulmonary parenchyma; (d) reveal an absolute increase in the total amount of lung collagen.

In recent years several experimental models of human DIPF have been reported. They should be examined against the criteria proposed above, in order to establish their adequacy as models for the better understanding of the human disease (Carrington, 1968). We believe that many of the recently proposed experimental models of DIPF either fail to fulfill the criteria mentioned above or are yet to be critically examined from that point of view. One of the major difficulties is that human DIPF is far from being a neat, tight and closely defined clinicopathological entity (Liebow, 1975). On the other hand, although many of the experimental models proposed may not be applicable to human DIPF, they may still contribute valuable insights into some of the mechanisms probably involved in one or more aspects of the human disease.

FIG. 2. Histologic aspect of the lung in late survivors of treatment with paraquat + O_2, as described in the text. (A) More advanced dilatation of air ducts and alveolar collapse, with thickening of the remaining septa. ×104. (B) Higher magnification illustrating infiltration by mononuclear cells and alveolar desquamation. ×266.

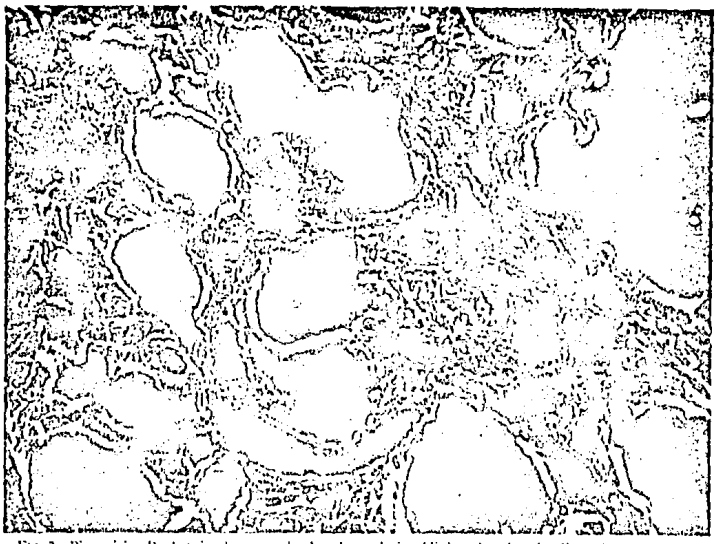

FIG. 3. Picrosirius Red stain photographed under polarized light, showing the distortion and increase in the pulmonary collagen network. ×223.

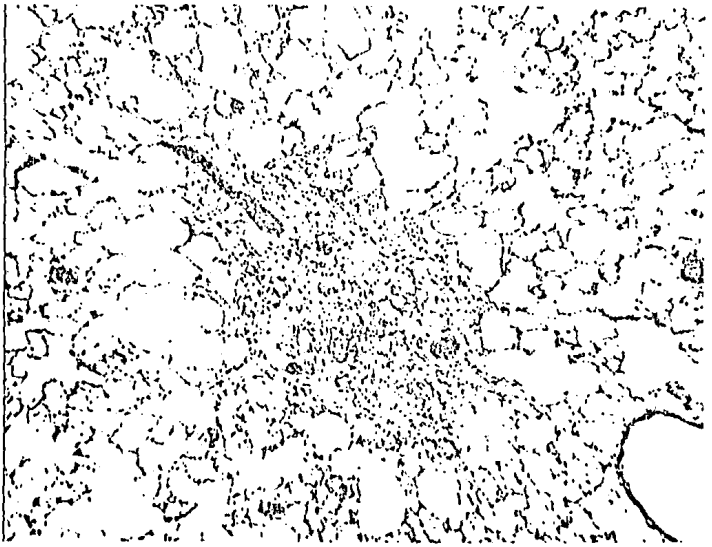

FIG. 4. A small area of focal fibrosis in the lung of an animal treated with bleomycin as described in the text. ×121.25.

TABLE III
Time of Survival and Lung Collagen Content of Animals Treated with Bleomycin and O_2

	Time of survival	Number of surviving animals	Collagen content (mg/lung)
Bleomycin alone	7 weeks	2/2	34.4 ± 16.0
	13 weeks	6/6	26.2 ± 10.0
Bleomycin + O_2	5 days	2/6	18.0 ± 5.5
	7 days	2/6	11.3 ± 1.4
	4 weeks	2/6	16.2 ± 4.5

Of the various experimental models of DIPF mentioned in the literature only one seems to approximate the histologic and biochemical features of the human condition, namely the butylated hydroxytoluene + 70% O_2 model in BALB/c mice of Haschek, Witschi *et al.* (1981, 1982, 1983). These authors reasoned that the destruction of pneumocytes type I would expose the interstitial cells in the alveolar septa, which could then be experimentally stimulated to produce fibrous tissue. They accomplished the first part of their scheme with the butylated hydroxytoluene, which has been described to be highly toxic for pneumocytes type I (Adamson *et al.*, 1977), and the second part was brought about by exposure to 70% O_2, although X radiation seems to work equally well (Haschek *et al.*, 1980). The model is probably species and strain specific, because we have tried to reproduce it both in CA mice and in Wistar rats without any success (Selman, M., Montaño, M., and Pérez-Tamayo, R., unpublished observations).

Preliminary experiments with the development of acute pulmonary lesions using paraquat in Wistar rats led us to try the combination of different doses of this toxic plus exposure to normobaric 74% O_2 in the breathing air for various periods, following the idea of the double pulmonary injury of Haschek *et al.* (1981), as well as the repeated observation that O_2 enhances the pulmonary damage produced by paraquat (Fisher *et al.*, 1973; Keeling *et al.*, 1981). Our results indicate that in Wistar strain rats relatively low doses of paraquat (2.5–3.5 mg/kg body wt) repeated every 72 hr plus continuous exposure to normobaric 74% O_2 for 5 to 10 weeks results in relatively low mortality during the early part of the experiment and in the development of widespread histologic pulmonary lesions. These include considerable distortion of the normal lung architecture, interstitial mononuclear cell infiltration, alveolar collapse, and interstitial fibrosis. Neither paraquat nor the continuous exposure to O_2, used in the same experimental doses but in isolation, caused any pulmonary change. In other experiments (Selman *et al.*, 1985) we have demonstrated that the enhancing effect of O_2 on the pulmonary damage produced by paraquat is relatively short lived, so that multiple doses of paraquat should not be separated by more than 72 hr.

Intratracheal administration of bleomycin to male rats of the Wistar strain resulted in the development of focal fibrosis, with most of the pulmonary parenchyma remaining unaffected. Although this peculiar resistance of Wistar rats to bleomycin has been previously reported (Costa *et al.*, 1980) it remains unexplained. When Wistar rats are the only strain available (as in our case), the combination of low doses of paraquat and normobaric high O_2 concentrations in the breathing air results in a better experimental model of human DIPF than the intratracheal administration of bleomycin.

The pulmonary lesions produced by the combination of paraquat + normobaric

high O_2 concentration are diffuse and involve with equal severity the peribronchial, subpleural, and intervening lung parenchyma. The salient feature is the dilatation of the distal air spaces with collapse of the surrounding alveoli; terminal bronchioles are usually not dilated and may even appear partially collapsed. There is considerable peribronchial and perivascular edema. Mononuclear cells infiltrate the loose connective tissue surrounding bronchi and blood vessels, as well as the collapsed parenchyma. In animals surviving 5 weeks or more, there is proliferation of spindle cells and deposit of a fine web of neoformed collagen fibers. The destruction of useful exchange surface in the lung is so extensive that many of the rats appeared to survive only because of the increased O_2 concentration they were breathing within the plastic chamber. It was not unusual for animals that later were shown to have advanced pulmonary lesions, to develop clinically obvious respiratory insufficiency when they were finally removed from the plastic chamber, at the end of the period of exposure.

The similarity of experimental DIPF produced in the rat by our combined procedure with the lesions described by Churg *et al.* (1983) in patients dying after developing acute respiratory distress and treatment for prolonged periods on mechanical ventilation with high positive end-expiratory pressure and O_2 concentration is striking. These authors suggest that the process is similar to bronchopulmonary dysplasia as seen in the newborn and propose that it is caused by the combination of high respiratory pressure and high oxygen concentration in the inspired air.

Regardless of the dose of paraquat and the length of exposure to O_2, in every experimental variation of our model of human DIPF there were always some animals that failed to develop clinical signs or symptoms of paraquat intoxication or of respiratory insufficiency; the lungs of those animals were histologically and biochemically normal. Although we have no explanation for this phenomenon, we believe that such "resistance" to paraquat + O_2 is not a permanent feature of those animals, since upon repeat of the same, or even less aggressive schedules of pulmonary damage, many of them succumbed with lesions identical to those present in the initially "susceptible" group (Selman, M., Montaño, M., and Pérez-Tamayo, R., unpublished observations).

According to Keeling *et al.* (1981), and Smith *et al.* (1974) the primary target of paraquat is the alveolar epithelium, which is rapidly destroyed. Both pneumocytes types I and II reveal severe degenerative changes as early as 4 hr after the administration of paraquat, and 2 or 3 days later they appear desquamated in the alveolar lumen, which also shows edema. The alveolar walls reveal congestion and mild acute inflammatory reaction; eosinophilic hyaline members are common. These changes occur after the administration of paraquat doses 4 to 20 times higher than the ones used in our study; with the lower doses of paraquat no changes are visible in the lung under the light microscope (Smith *et al.*, 1974). Yet, we believe that the alveolar epithelium is damaged and that as a consequence the increased O_2 concentration that the animal is breathing in our model of human DIPF can cause further harmful disturbances which affect the stability of the distal air spaces and bring about the dilatation of the respiratory ducts and the collapse of the surrounding alveoli.

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EXPERIMENTAL AND MOLECULAR PATHOLOGY 43, 000–000 (1985)

The Duration of the Pulmonary Paraquat Toxicity—Enhancement Effect of O_2 in the Rat[1]

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The duration of the pulmonary paraquat toxicity-enhancement effect of O_2 has been examined in Wistar rats. In one experiment, various groups of normal animals were given a single dose (5 mg/kg body wt) of paraquat and after different periods were exposed to continuous breathing of normobaric 74% O_2 in airtight chambers until dead or up to 10 days. In a reverse experiment, a large number of rats were first exposed for 6 days to continuous breathing of normobaric 74% O_2 and were then separated into various groups which received a single dose of paraquat (5 mg/kg body wt) after various periods of breathing normal air, ranging from 0 to 96 hr. The extent of pulmonary damage in both experiments was evaluated by histologic examination and by biochemical determination of total collagen content of the lungs. It was found that the duration of the pulmonary damage induced by paraquat that is enhanced by continuous breathing of high O_2 concentration lasts 24 to 48 hr. It was also observed that 12 to 24 hr after paraquat administration and continuous breathing of high O_2 concentration pulmonary lesions are severe and extensive, and in animals surviving 6 or more days there was also incipient interstitial fibrosis. The reverse sequence of treatment (O_2 + paraquat) resulted in no mortality and no pulmonary lesions. Additional controls treated with each of the pulmonary toxins alone also revealed no lung changes.

INTRODUCTION

Several authors have noted (Fisher *et al.*, 1973; Smith and Heath, 1975; Douze and van Heijst, 1977; Heath and Smith, 1977; Kehrer *et al.*, 1979; Keeling *et al.*, 1981) that the toxic effect of paraquat (1,1'-dimethyl-4,4'-bipiridylium) on the rat lung (Clark *et al.*, 1966; Smith and Heath, 1967; Vijeyaratnam and Corrin, 1971; Smith *et al.*, 1974; Smith and Heath, 1974; Kimbrough, 1974; Smith and Heath, 1976) is markedly enhanced by exposure to high O_2 concentration in the breathing air. Since paraquat has been shown to damage preferentially pneumocytes type I and II (Heath and Smith, 1977; Keeling *et al.*, 1981; Clark *et al.*, 1966; Heath and Smith, 1967; Vijeyaratnam and Corrin, 1971; Smith *et al.*, 1974; Smith and Heath, 1974; Kimbrough, 1974; Smith and Heath, 1976; Sykes *et al.*, 1977; Greenberg *et al.*, 1978) and oxygen poisoning results primarily in capillary endothelial cell lesions (Schaffner *et al.*, 1967; Kistler *et al.*, 1967; Weibel 1971; Gould *et al.*, 1972; Sevitt, 1974; Chvapil and Peng, 1975), it would seem logical that the combination of both agents would result in the addition of their respective effects. This, however, does not seem to be the actual experimental result, since Keeling *et al.* (1981) have found that both paraquat and 85% O_2 predominantly damage pneumocytes type II.

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During the development of an experimental model of human diffuse interstitial pulmonary fibrosis in the rat, various combinations of paraquat plus exposure to normobaric 74% O_2 concentration in the breathing air were tested in our laboratory (Selman *et al.*, 1984). Low but repeated doses of paraquat given simultaneously with continuous breathing of high O_2 concentration for periods of 5 weeks or longer yielded many animals with severe distortion of the pulmonary architecture and interstitial fibrosis. Other groups of rats treated with the same doses of paraquat but exposed only to atmospheric air, or given no paraquat and exposed to the same high O_2 concentration for equal periods, revealed no pulmonary lesions. Finally, when the same low doses of paraquat were given and the exposure to high O_2 concentration was delayed for 1 week after the last paraquat injection, again no pulmonary lesions developed. It was then considered of interest to systematically examine the duration of the paraquat effect that is enhanced by breathing high O_2 concentrations, in order to further characterize its nature. In this paper we report the results of such study, which show that the paraquat lung damage enhanced by breathing high O_2 concentration lasts 24 to 48 hours. In addition, we also show that the reverse combination, namely prolonged continuous exposure to high O_2 concentration in the breathing air, followed after various periods by administration of the same low doses of paraquat, results in no pulmonary damage at all.

METHODS

Animals. Male rats of Wistar strain, weighing 170–230 g (9–10 weeks old) were used in all the experiments.

Paraquat administration. Paraquat dichloride was dissolved in saline at 0.05% concentration freshly before each injection, and given intraperitoneally once at the dose of 5 mg/kg of body wt.

Exposure to O_2. Continuous exposure of animals to normobaric 74% O_2 concentrations in the breathing air was carried out in airtight chambers constructed with transparent sheets of acrylic, with a capacity of 160 liters. The chambers are quite spatious and the animals can freely move around and feed *ad libitum*. Air and O_2 were combined in an O_2 mixer with a flow of 8–9 liters/min and a 74% O_2 concentration. The gas mixture was humidified with a thermic nebulizer prior to delivery into the chamber. The O_2 concentration was monitored periodically with a gaseous mixture analyzer.

Experimental design. Two major experiments were performed:

(1) Five groups of 6 to 23 animals each were given a single intraperitoneal injection of paraquat (the dose was mentioned above) and were then placed in the airtight chamber after 0, 6, 12, 24, and 48 hr of breathing atmospheric air, respectively. Two other groups of 6 animals each were treated respectively with only one of the pulmonary toxic agents, namely paraquat or high O_2 concentration. All animals exposed to high O_2 concentration were kept in the airtight chamber until dead or for 10 days.

(2) Twenty five animals were first exposed to continuous breathing of 74% O_2 concentration for 6 days, and after that they were separated into 5 groups of 5 animals, each one receiving a single dose of paraquat (5 mg/kg of body wt) 0, 24, 48, 72, and 96 hr after breathing again normal air. They were all sacrificed 10 days after release from the airtight chamber.

Preparation of lungs. Animals were sacrificed when moribund or at the end of

10 days. Autopsies were performed on all dead animals, including those found dead in the morning. Unfortunately, because of poor preservation, it was not possible to prepare histologic sections and to perform biochemical studies on the lungs of all animals found dead. The lungs of all sacrificed and autopsied rats were perfused intratracheally with buffered 10% formaldehyde, preserved *in situ* for 10 min, and then removed en block with the heart and placed in the same formaldehyde solution until further study.

Histological studies. Blocks of lung tissue were embedded in paraffin, cut at 4 μm, and stained with HE and the Verhoeff–Van Gieson stain for elastic fibers; other sections were cut at 10 μm and stained with the Picrosirius Red technique according to Junqueira *et al.* (1979). When the latter preparations are observed under polarized light they reveal a very complete picture of collagens type I and III in pleura, vascular and parenchymal areas; in addition, collagen type II in bronchial cartilage is also stained. Care was taken to block the lung tissue as to include the maximal amount of surface in the section. Thus, in practically all histology slides examined a section of an entire lung was present, including pleura, hilar structures, and intervening pulmonary parenchyma. In those few instances in which the sampling was less than complete multiple sections were examined.

Collagen measurement. All the left lung and the remaining fragments of the right lung (after removal of a thin block for histologic study) were dried to constant weight, ground, and 20 to 50-mg aliquots were hydrolyzed with 6 *N* HCl for 20 hr at 105°C, filtered, dried, resuspended in distilled H_2O, and the hydroxyproline content was measured in triplicates by the technique of Rojkind and González (1974).

RESULTS

Mortality. Mortality of the animals included in the 7 groups of the first experiment appears in Table 1. It should be noted that in group 1 13 animals died within the first 36 hr and only 7 survived for 7 to 10 days, whereas in groups 5, 6, and 7 there was no mortality. Groups 2, 3, and 4 displayed intermediate mortality rates.

TABLE 1

Mortality of the Various Groups of Rats Exposed First to Paraquat and after Various Periods to Continuous Breathing of High O_2 Concentration for 10 Days

Group No.	Hours after paraquat	Time of survival	Mortality (dead/total exposed)
1	0	36 hr	13/20
		7–10 days	7/20
2	6	2–3 days	10/20
		10 days	10/20
3	12	2–6 days	14/23
		10 days	9/23
4	24	3–5 days	10/20
		10 days	10/20
5	48	10 days	0/6
6	Paraquat alone	10 days	0/6
7	High O_2 alone	10 days	0/6

None of the animals included in the five different groups of the second experiment died during the 10 days of observation after release from the airtight chamber, so they were all sacrificed at the end of that period.

Histological changes in the lungs. Histologic changes in the lungs of the animals in the first experiment were quite variable and ranged from minimal congestion to diffuse and dramatic distortion of the architecture of the respiratory parenchyma, massive hemorrhage, alveolar cell desquamation, inflamatory infiltration, and some degree of interstitial fibrosis. Congestion, edema, and hemorrhage were more frequent and severe in the animals of group 1, which had the shortest average survival, and were minimal or absent in groups 5, 6, and 7. In addition to the changes mentioned, interstitial inflammation was also present in both groups 1 and 2, but it was focal and primarily peribronchial and perivascular (Fig. 1). The infiltrating cells were predominantly mononuclear (lymphocytes, plasma cells, and macrophages) with few or no polymorphonuclear leukocytes present. In groups 2 and 3, to the changes described an extensive revision of the normal structure of the lung was added, characterized by wide dilatation of the respiratory bronchioles (both air ducts and atria) with collapse of the intervening alveoli (Fig. 2), desquamation of alveolar cells, extension of the perivascular and peribronchial inflammatory infiltrate to the alveolar walls and even incipient interstitial fibrosis, as evidenced by the presence of spindle fibroblast-like cells and apparent collagen increase in many fields of collapsé alveoli (Fig. 3).

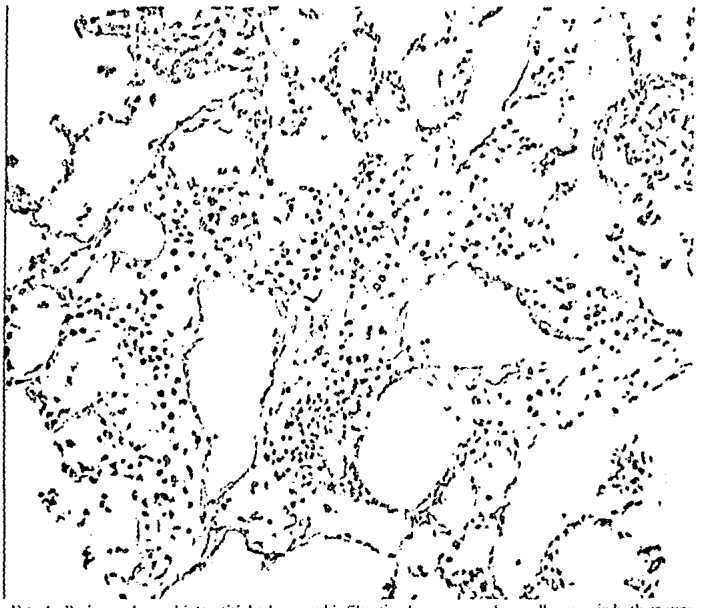

FIG. 1. Perivascular and interstitial edema and infiltration by mononuclear cells, seen in both groups 1 and 2, as described in the text.

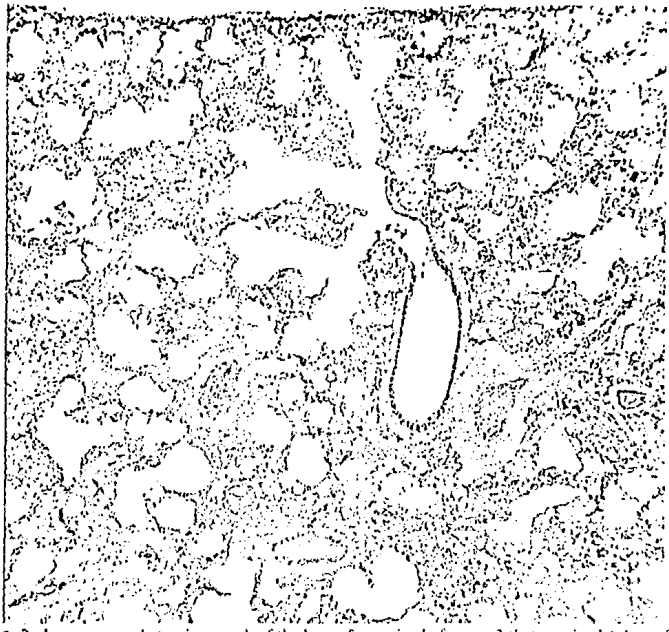

FIG. 2. Low-power photomicrograph of the lung of an animal of group 3 that survived 6 days after paraquat administration and continuous exposure to high O_2 concentration. The widespread dilatation of bronchioles and air ducts with collapse of the intervening alveoli are apparent.

In groups 6 and 7, which were controls treated with each pulmonary aggressor alone, the lungs were completely normal. The same thing occurred with the lungs of all the animals included in the second experiment, in which exposure to high O_2 concentration in the breathing air was followed at different periods by a single dose of paraquat.

Total lung collagen. Table II gives the time of survival and average total lung collagen (mg of collagen/lung) for each group of animals in the first experiment. No statistically significant differences were apparent at a P level below 0.025, and that only in the animals surviving for 10 days in groups 2 and 4. Similar results were obtained in all the animals included in the second experiment.

DISCUSSION

Our results indicate that the duration of the pulmonary paraquat toxicity-enhancement effect of O_2 in Wistar rats lasts approximately 24 to 48 hr. During this period, exposure to normobaric 74% O_2 concentration results in early death and the development of severe pulmonary lesions, consisting of intense congestion, massive hemorrhage, perivascular and peribronchial or generalized edema, and mold inflammatory infiltrate with mononuclear cells. When the animals survive a few more days, in addition there is dramatic dilatation of the air ducts with

FIG. 3. Incipient interstitial pulmonary fibrosis in one rat of group 4 which survived 10 days. There is irregular thickening of alveolar septa with infiltration by round and spindle cells, desquamation of alveolar cells, and fine fibrillary material.

collapse of the intervening alveolia and incipient fibrosis, which is suggested both morphologically and biochemically. Two days after paraquat administration continuous exposure to the same high O_2 concentration in the breathing air for 10 days has no effect at all on the survival and on the normal histology and collagen content of the lungs. On the other hand, the reverse experiment, namely first continuous exposure of animals to high O_2 concentration for a prolonged period and then administration of a single dose of paraquat, after different periods of breathing normal air, produced no mortality and no pulmonary histologic or biochemical changes in the following 10 days.

The absence of enhancement of the pulmonary O_2 toxicity by paraquat contradicts the opinion of Fisher *et al.* (1973), while the opposite observation, namely that it is the paraquat lesion that is enhanced by high O_2 in the breathing air, supports the conclusion of Keeling *et al.* (1981). These authors based their suggestion on the ultrastructural finding of damaged alveolar epithelium type II and intact capillary endothelial cells. Nevertheless, their animals did not survive 24 hr and their final decision was made on a semiquantitative appraisal of degree of cell damage, comparing type II pneumocytes with capillary endothelial cells. With a different experimental set up, such as the one described in this paper, the results could be at variance with those described.

The microscopic study of the pulmonary parenchyma damaged by the combi-

TABLE II
Average Total Lung Collagen in Rats Exposed First to Paraquat and after Various Periods to Continuous Breathing of High O_2 Concentration for 10 Days

Group No.	Hours after paraquat	Time of survival	Number of animals studied	Average total lung collagen (mg/lung)
1	0	36 hr	6	13.0 ± 1.1
		7–10 days	7	21.9 ± 6.6
2	6	2–3 days	6	13.2 ± 2.4
		10 days	6	16.5 ± 4.3
3	12	2–6 days	9	14.7 ± 3.7
		10 days	6	14.8 ± 5.2
4	24	3–5 days	6	13.4 ± 1.8
		10 days	6	16.2 ± 2.9
5	48	10 days	6	14.4 ± 3.4
6	Paraquat alone	10 days	6	11.9 ± 1.0
7	High O_2 alone	10 days	6	11.7 ± 1.1
Controls	—	—	10	10.9 ± 2.2

nation of paraquat + O_2 gives no clue as to the nature of the lesions induced by paraquat. With higher doses (4 to 20 times higher) paraquat is toxic for the alveolar epithelium (Keeling *et al.*, 1981; Clark *et al.*, 1966; Smith and Heath, 1967; Vijeyaratnam and Corrin, 1971; Smith *et al.*, 1974; Kimbrough, 1974; Smith and Heath, 1976; Sykes *et al.*, 1977; Greenberg *et al.*, 1978) which shows detectable lesions as early as 4 hr after paraquat injection, and becomes sloughed and desquamated in 48 to 72 hr (Smith and Heath, 1975, 1976). With the same high doses of paraquat, a few days later an intralveolar fibrosis develops which eventually produces pulmonary insufficiency and kills the experimental animal (Heath and Smith, 1977; Keeling *et al.*, 1981; Greenberg *et al.*, 1978). Similar lesions have been described in human cases of intoxication with paraquat (Mathew *et al.*, 1968; Toner *et al.*, 1970; Editorial, 1971; Malone *et al.*, 1971; Copland *et al.*, Thurlbeck and Thurlbeck, 1976). The ultrastructural study of the lungs of the animals used in our first experiment, which is currently underway, should perhaps reveal the damage produced by low doses of paraquat.

Regardless of what is eventually found at the ultrastructural level, it is very interesting that the sum of a single low dose of paraquat plus continuous exposure to high O_2 concentration in the breathing air, which by themselves produce no pulmonary lesions visible with the light microscope, should result in such high mortality and such dramatic distortion of the normal architecture of the lung. It is well established that O_2 is toxic for the alveolar cells of human and animal lungs (Schaffner *et al.*, 1967; Kistler *et al.*, 1967; Weibel, 1971; Gould *et al.*, 1972; Sevitt, 1974; Chvapil and Peng, 1975; Deneke and Fanburg, 1980), but very high O_2 concentrations and prolonged exposures are needed before pulmonary changes become apparent, and then they are of a different type than those described in this paper and elsewhere (Selman *et al.*, 1985).

The factors contributing to the structural stability of the distal respiratory units include the presence of adequate amounts of surfactant, healthy alveolar cells (pneumocytes types I and II), normal capillary perfusion, and an adequate mix-

ture of gases in the breathing air. By altering one or more of these factors, and perhaps by interfering with some other as yet unknown element(s) necessary to preserve the normal reciprocal relations of the various compartments of the distal air spaces in the normal lung, the changes herein described are produced.

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Conclusiones y Perspectivas

La FPID se caracteriza por la acumulación excesiva de colágena en la matriz extracelular, con la consecuente destrucción de la arquitectura tisular.

Como la mayoría de los estudios que se realizan en humanos sólo abarcan momentos puntuales y estáticos de la enfermedad, existe la necesidad de contar con un modelo experimental que semeje estrechamente al padecimiento y que permita analizar dinámicamente los fenómenos que ocurren en el pulmón.

Nuestros estudios demuestran que la administración prolongada de paraquat a dosis bajas, sumada a la exposición a concentraciones elevadas de O_2, ya sea simultáneamente a la inyección del herbicida, o diferida algunas horas, induce en ratas una enfermedad pulmonar muy semejante a la observada en la FPID humana; las lesiones son difusas y comprometen desde el vértice hasta la base y del hilio a la pleura, provocando en los animales grave insuficiencia respiratoria. Después de algunas semanas, se observa proliferación de fibroblastos, con aumento de colágena que se puede detectar morfológica y bioquímicamente. El modelo ha demostrado ser reproducible, aún cuando su eficiencia podría ser optimizada, dado que independientemente de las dosis utilizadas, alrededor del 50% de los animales fallecen en los primeros 10 días.

Como se ha mencionado previamente, este modelo será utilizado para estudiar de manera secuencial los eventos celulares y las alteraciones de la matriz intersticial que ocurren durante el desarrollo de la FPID.

En este contexto, y como un acercamiento preliminar, hemos examinado recientemente los cambios ultraestructurales pulmonares que ocurren en el modelo agudo. En este experimen

to, los animales se distribuyeron en tres grupos: El grupo problema recibió una dosis única de 5 mg/kg de paraquat y 12 horas después se colocó en la cámara de O_2 a una concentración constante de 75% y se fueron sacrificando a las 12, 24, 72 horas, así como 7 días después. Un grupo control recibió solamente O_2 por 7 días, al cabo de los cuales se sacrificó. El otro grupo recibió solamente paraquat, a la dosis mencionada y los animales se sacrificaron a las 4 y 12 horas y a los 7 días. Los pulmones se fijaron in situ por vía intratraqueal y por perfusión circulatoria con glutaraldehido al 2.5% en buffer de cacodilatos y se obtuvieron múltiples bloques de cada uno, los que se incluyeron por las técnicas habituales. Los cortes se examinaron en un microscopio Hitachi HU-IIA.

Tanto el paraquat como el oxígeno solos, produjeron una lesión moderada en las células endoteliales de los capilares pulmonares, caracterizada por la separación de la membrana basal y la presencia de áreas focales de tumefacción (Fig.1). La combinación de los dos agresores produjo lesiones endoteliales más intensas, con microtrombos y edema intersticial (Fig. 2). Posteriormente aumentan el número de fibroblastos y de células inflamatorias en el espesor de los tabiques alveolares (Fig. 3). A los 7 días, se apreció un aumento aparente de la colágena intersticial (Fig. 4).

Este mismo estudio se realizará en el modelo crónico, intentando identificar y separar en el tiempo, si es posible, las etapas de lesión inicial, inflamación intersticial, proliferación de fibroblastos y fibrosis.

Por otro lado, estamos muy interesados en analizar los cambios en el metabolismo de la colágena que llevan finalmente al desarrollo de la fibrosis; ésto es, alteraciones en la biosíntesis y/o degradación de esta proteína.

En nuestro laboratorio, hemos analizado recientemente este problema en un grupo de pacientes y a continuación se expone la metodología y los resultados.

Se estudiaron 11 pacientes con fibrosis pulmonar idiopática (FPI). En el momento de la biopsia, el tiempo promedio de evolución del padecimiento era de 12.4 ± 3.1 meses. Todos los pacientes llenaron los criterios clínicos y de laboratorio aceptados para hacer el diagnóstico de FPI (1,2) y en el estudio morfológico del tejido pulmonar se encontró evidencia de fibrosis intersticial difusa e inflamación septal e intraalveolar. En todos los casos predominaron las lesiones fibróticas sobre las inflamatorias. En ninguna de las muestras examinadas hubo vasculitis, granulomas ni presencia de material inorgánico a la luz polarizada. En el estudio bacteriológico no se cultivaron bacterias, mycobacterias ni hongos.

El grupo control, estuvo constituído por 6 pacientes a los que se les practicó resección de tejido pulmonar para extirpar algún tipo de tumoración, pero que no presentaban datos clínicos, radiológicos o fisiológicos de enfermedad intersticial fibrosante del pulmón. En ninguna de las muestras seleccionadas para el estudio bioquímico se encontraron alteraciones en microscopía de luz.

Metabolismo de la colágena.

Todos los análisis bioquímicos se realizaron en alícuotas del mismo tejido pulmonar utilizado para el estudio morfológico.

a) Cuantificación de la colágena.

Fragmentos de tejido pulmonar de 100-150 mgs de peso

húmedo fueron secados a peso constante e hidrolizados con HCl 6N por 24 horas a 100°C. Posteriormente, fueron filtrados, secados, resuspendidos en agua bidestilada y se midió el contenido de hidroxiprolina a través de un método colorimétrico - (3). La concentración de colágena en las alícuotas se calculó, multiplicando la cantidad de hidroxiprolina por 7.41, suponiendo que este residuo constituye alrededor del 13% del total de aminoácidos de la cadena alfa (4).

a) Síntesis de la colágena.

Las muestras pulmonares fueron divididas en porciones que pesaban entre 100-200 mgs de peso húmedo. Estas alícuotas se incubaron en 3 ml de medio de cultivo Dulbecco modificado por Eagle que contenía 10% de suero fetal de ternera, 50 μg/ml de ácido ascórbico, 70 μg/ml de sulfato ferroso, 200 U/ml de penicilina y 200 μg/ml de estreptomicina. Los cultivos se - equilibraron con una mezcla gaseosa de 95% de O_2 y 5% de CO_2 y se incubaron a 37°C en un baño metabólico de agitación constante. Después de 1 hora, el medio fue reemplazado con 3 ml - de medio fresco al que se agregó 30 μCi de prolina tritiada, manteniéndose el explante tisular en cultivo durante 4 horas. Al término del período de incubación, las muestras de tejido se homogenizaron y las proteínas marcadas se precipitaron con ácido tricloro acético (TCA) al 10% y 3 lavados sucesivos con TCA al 5%. Para medir la síntesis de colágena (hidroxiprolina [H^3]) y la síntesis de proteínas totales (incorporación de - prolina [H^3], el material precipitado con TCA se hidrolizó - por 24 horas en HCl 6N a 100°C, se filtró, evaporó y resuspendió en 2 ml de agua bidestilada. Ambos residuos fueron separados por el método de Rojkind y cols. (3). Los resultados se expresaron como porcentaje de síntesis después de corregirlos por el bajo contenido de prolina en proteínas no colagénicas (5).

c) Actividad colagenolítica.

Para este análisis, se utilizó el método de Ryan y Woessner modificado (6). Muestras de tejido de 450-500 mg (peso húmedo) fueron homogeneizadas y divididas en 6 alícuotas. Tres de ellas se incubaron en un baño metabólico por 24 horas a 33°C en presencia de Cl_2Ca 0.005M, NaCl 0.15M y Tris buffer 0.04M, pH 7.4; las 3 restantes se incubaron en idénticas condiciones pero con EDTA (inhibidor de colagenasa). Con el objeto de que los fragmentos obtenidos fueran más pequeños que una cadena alfa de la proteína, los homogeneizados se centrifugaron a 4°C y el sobrenadante se filtró a través de una membrana con límite de exclusión de 100.000 daltones. La digestión enzimática se detectó por la liberación de polipéptidos que contenían hidroxiprolina soluble, la cual se midió por el método de Rojkind y González (3). La actividad colagenolítica se expresó en /ug de colágena degradada por mg de colágena incubada en 24 horas.

RESULTADOS.

Concentración de la colágena.

La medición de hidroxiprolina es el método más aceptado para conocer la cantidad de colágena de un tejido, ya que este iminoácido es casi exclusivo de la colágena y constituye alrededor del 13% de los residuos de esta proteína, mientras que se encuentra en porcentajes insignificantes en unas pocas proteínas no colagénicas (7).

En las tablas 1 y 2 se muestran los resultados obtenidos sobre la concentración de la colágena. Como habíamos demostrado previamente (8), hubo un aumento significativo de la cantidad de esta proteína en el tejido pulmonar de los pacientes con FPI en relación a los controles, con una media de 327 ± 76 vs.

185± µg/mg de tejido seco ($p < 0.001$).

Síntesis de colágena en los cultivos de explante pulmonar.

Los tejidos pulmonares de ambos grupos, incorporaron activamente prolina [H^3] a colágena recién sintetizada. Los resultados obtenidos se muestran en las tablas 1 y 2. Como puede observarse la colágena representó del 1.2 al 3.45% de todas las proteínas sintetizadas por las muestras de biopsia - de los pacientes con FPI y del 1.45 al 2.75% en los controles. En el análisis estadístico no hubo diferencias significativas entre ambos grupos (2.2±0.8% vs. 2.08±0.5%).

Actividad colagenolítica.

La degradación endógena de colágena, medida a través de la solubilización de fragmentos polipeptídicos más pequeños que una cadena alfa, estuvo notablemente disminuída en los homogeneizados de pulmón de los pacientes con FPI. Los valores mostraron una media de 1.719±1.03 vs. 5.416±0.925 µg de colágena degradada/mg de colágena incubada/24 horas ($p < 0.001$) - (tablas 1 y 2). Aunque en todos los pacientes se observó un incremento en la concentración de colágena y disminución de la actividad colagenolítica, no hubo relación aparente entre estos dos parámetros.

Como puede observarse, el hallazgo más importante en este trabajo, fue la disminución de la degradación enzimática de esta proteína, lo que no había sido descrito previamente en FPID, aunque estudios realizados en hígados cirróticos de humano y animales experimentales han demostrado una situación similar (9,10).

Sin embargo, llamó la atención el que no existiera un aumento en la biosíntesis en los pulmones fibróticos, lo que podría deberse al avanzado estadío de la enfermedad.

Nuestra impresión en este sentido, es que en el depósito anormal de colágena en los pulmones con FPID, participan modificaciones en la biosíntesis y degradación de esta proteína, que probablemente ocurren en diferentes fases de la progresión de la enfermedad. Este será otro de los estudios para los que se utilizará nuestro modelo, ya que la identificación de esta posible secuencia permitirá entender mejor la dinámica del anabolismo y catabolismo de la colágena en el curso del proceso fibrosante y eventualmente intentar su normalización con diferentes recursos terapéuticos.

TABLA 1

CONCENTRACION, BIOSINTESIS Y DEGRADACION DE COLAGENA EN FIBROSIS PULMONAR IDIOPATICA

PACIENTE	CONCENTRACIÓN DE COLÁGENA	SÍNTESIS DE COLAGENA**	DEGRADACIÓN DE COLAGENA***
1	334	3,28	0,24
2	255	3,45	1,776
3	293	2,55	1,334
4	504	1,76	1,536
5	358	1,45	2,736
6	407	1,45	3,24
7	329	2,10	1,944
8	252	1,45	0,48
9	243	1,20	3,336
10	317	3,10	1,128
11	305	2,55	1,152
MEDIA±DS	327±76#	2,21±0,8	1,719±1,03 #

* µG DE COLÁGENA/MG PESO SECO

** % CORREGIDO (VÉASE MATERIAL Y MÉTODO)

*** µG DE COLÁGENA DEGRADADA/MG DE COLÁGENA INCUBADA/24 HORAS.

\# $P < 0,001$ (COMPARADO CON LOS CONTROLES, VÉASE TABLA 2).

LOS RESULTADOS INDIVIDUALES CORRESPONDEN AL PROMEDIO DE 3 A 6 ENSAYOS POR MUESTRA.

TABLA 2

CONCENTRACION, BIOSINTESIS Y DEGRADACION DE COLAGENA EN SUJETOS CONTROL

CONTROL	CONCENTRACIÓN DE COLÁGENA*	SÍNTESIS DE COLÁGENA**	DEGRADACIÓN DE COLÁGENA***
1	178	2,75	4,512
2	167	1,70	5,184
3	202	1,45	7,176
4	180	2,30	5,304
5	212	1,80	5,616
6	171	2,50	4,704
MEDIA±DS	185±18	2,08±0,5	5,416±0,952

* µG DE COLÁGENA/MG PESO SECO.

** % CORREGIDO (VÉASE MATERIAL Y MÉTODO).

*** µG DE COLÁGENA DEGRADADA/MG DE COLÁGENA INCUBADA/24 HORAS.

LOS RESULTADOS INDIVIDUALES CORRESPONDEN AL PROMEDIO DE 3 A 6 SENSAYOS POR MUESTRA.

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FIGURA 1 TRASTORNOS CITOLOGICOS INCIPIENTES

(VER TEXTO)

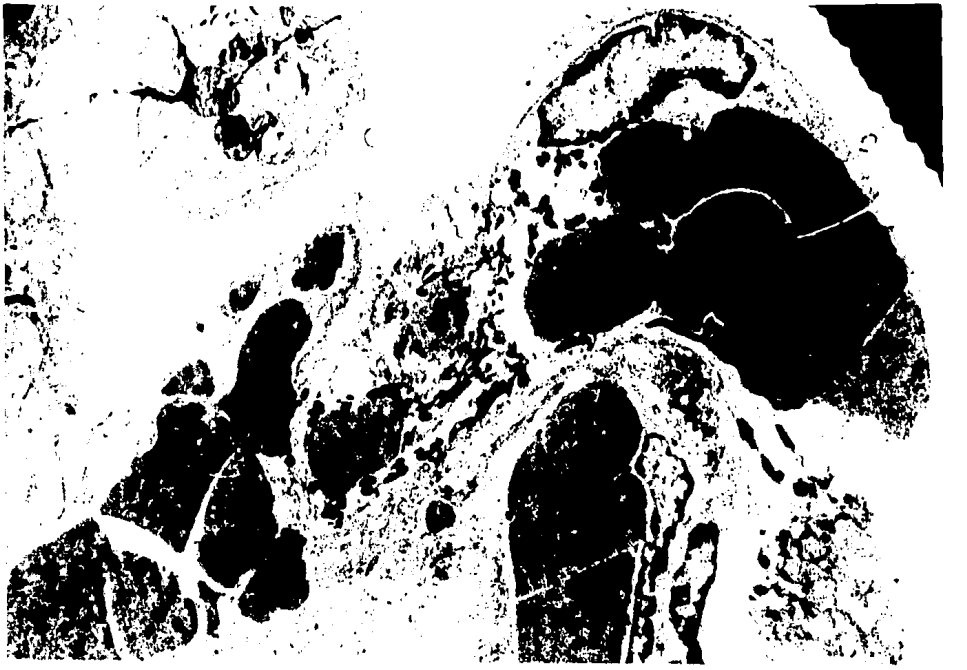

FIGURA 2 FORMACION DE TROMBOS EN LA MICROCIRCULACION

(VER TEXTO)

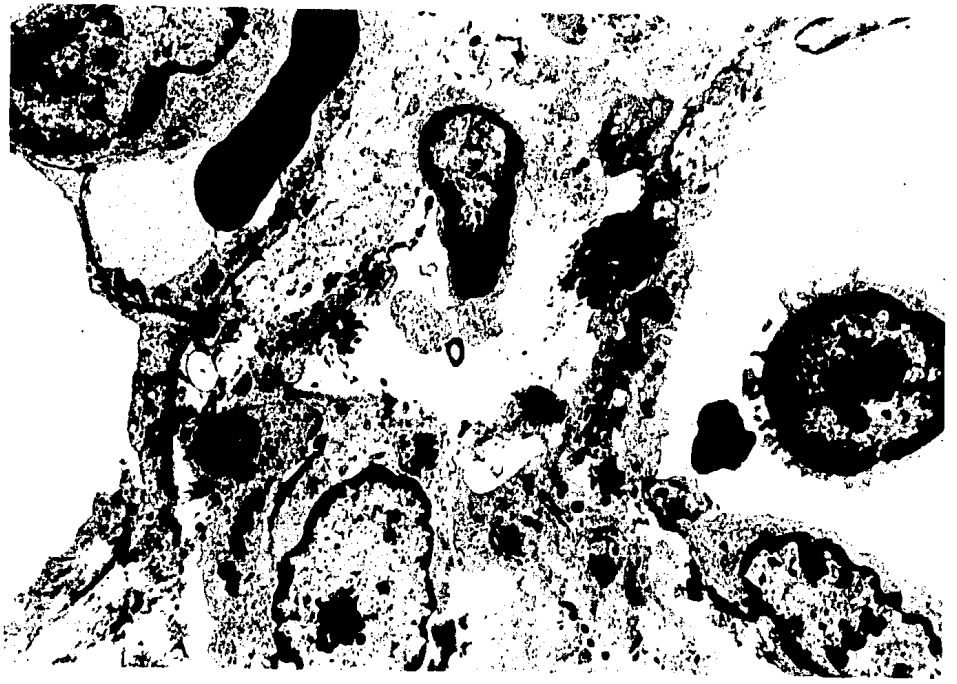

FIGURA 3 INFILTRACION INFLAMATORIA INTERSTICIAL

(VER TEXTO)

FIGURA 4 AUMENTO DE LA COLAGENA INTERSTICIAL

(VER TEXTO)