Experimental paraquat poisoning — histological, electron microscopic and autoradiographic changes in the lung

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Summary

Paraquat is a potent and widely used herbicide which acts as a specific pulmonary toxin and causes lung fibrosis in man and animals. Some controversy still exists concerning the details of the morphogenesis of the pulmonary lesions.

The lungs of rats exposed to intravenous injections of paraquat and sacrificed 6 - 24 days later were examined by light and electron microscopy. Autoradiography was used to detect possible paraquat accumulation in the lung 5 hours after a single intravenous injection.

The findings on microscopy suggested an acute phase of damage to alveolar lining epithelium followed by epithelial regeneration. The most pronounced light and electron microscopic findings were: (*i*) signs of disruption of the alveolar wall; (*ii*) type II alveolar epithelial hyperplasia; (*iii*) mobilization of mononuclear cells, and (*iv*) migration and accumulation of fibroblast-like cells in the intra-alveolar and interstitial spaces. After three equally spaced intravenous injections of paraquat signs of interstitial connective tissue proliferation could be seen.

Autoradiography showed low-grade radioactivity over the alveolar wall, indicating possible active uptake of paraquat by alveolar epithelium; this coincides with *in vitro* evidence of an active transport mechanism for paraquat by alveolar epithelial cells.

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Paraquat (1,1-dimethyl-4,4 dipyridylium dichloride) is a potent, widely used herbicide toxic to animals and man.[1] The pathological findings in experimentally induced and human paraquat poisoning have focused attention on its pulmonary specificity.[2-5] Many morphological investigations have been undertaken in different animal species and on biopsied and postmortem lung tissue of patients, but some controversy still exists regarding the exact details of the morphogenesis of the pulmonary lesions.[6-12] Paraquat is rapidly removed from the blood of rats after intravenous administration,[13] and Rose *et al.*[14] showed that the lung was clearly able to accumulate paraquat to levels in excess of the blood concentration. Since the response of the human lung to paraquat is delayed, similar accumulation may occur in man.[15] It is therefore very important to direct efforts towards determining the precise cytological target and the ensuing cytological interactions in the development of 'paraquat lung'.

Differences in experimental design, species used, the dose and route of paraquat administration as well as the time interval of exposure have led to diverging and often conflicting interpretations of the morphological changes induced by paraquat poisoning. A number of nonspecific pulmonary lesions have been described as being the prelude to the development of fibrosis. Great differences in opinion still prevail as to the intra-alveolar or interstitial origin of the well-known diffuse interstitial fibrosis which develops later on.[12] Furthermore, some authors consider that the fibrosis is not simply the result of damage to alveolar cells, since it does not occur after exposure to paraquat aerosols.[1] Recently, however, Popenoe[16] demonstrated that inhaled aerosol paraquat solutions do produce pulmonary fibrosis morphologically similar to the lesions produced by systemic administration.

The purpose of the present study was to analyse by various morphological techniques the effects of repeated low-dose intravenous injections of paraquat on the lungs of Long-Evans and Wistar rats. Firstly, we were interested in finding out whether continuous high levels of circulating paraquat might create some form of tolerance to pulmonary injury. Secondly, we wished to follow the systematic progression of the pulmonary lesions in order to identify on morphological examination the various cells most prominent in the different stages of the development of interstitial pulmonary fibrosis. In addition, we undertook an autoradiographic investigation in an effort to localize the paraquat in the lungs of different strains of rats.

Materials and methods

For ordinary light and electron microscopy a total of 20 rats (equal numbers of Long-Evans and Wistar rats) weighing between 180 and 250 g were used. Table I shows the experimental protocol. The animals were anaesthetized with ether and injected via the femoral vein with sterile disposable syringes and needles. After each injection they were allowed to recover from the anaesthetic. The control animals in each group were given intravenous injections of 0,15M saline (similar in volume to the injections given to the experimental animals). They were all kept in standard cages for laboratory animals, with water and maintenance food available *ad libitum*.

For light microscopy rats were sacrificed by intraperitoneal injection of pentobarbitone sodium. The lungs were then inflated via the trachea using Bouin's fixative. The trachea was tied to prevent leakage and the lungs were immersed in fresh Bouin's fixative. After overnight fixation, blocks (1 cm^3) were taken from areas which appeared to be abnormal on macroscopic examination as well as from three predetermined anatomical areas, processed in an automatic tissue processor and embedded in paraffin wax. Sections (5 μm) were then cut and stained with the following standard staining procedures:[17] Harris'

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TABLE I. EXPERIMENTAL PROTOCOL USED IN THE INVESTIGATION OF THE MORPHOLOGICAL EFFECTS OF INTRAVENOUSLY INJECTED PARAQUAT (15 mg/kg BODY WEIGHT) IN RATS

	No. of rats in each group					
Group	Total	Paraquat-treated animals	Saline-treated animals	No. of paraquat or saline injections	No. of paraquat-treated rats that survived treatment	Time from last injection to sacrifice of rats
I	9	7	2	1	6	6 d
II	3	2	1	2	2	6 d
III	3	2	1	3	2	6 d
IV	5	3	2	3	3	24 d
V*	5	3 + 1	1	1	3	5 h

*These animals were used in the autoradiographic experiment. Three received radioactive paraquat and 1 received non-radioactive paraquat.

haematoxylin and eosin, Masson's trichrome technique, Verhoeff's iodine iron haematoxylin procedure for elastic fibres counterstained with van Gieson's method for collagen fibres, Gomori's technique for reticular fibres and the periodic acid-Schiff technique.

For electron microscopy the lungs were fixed *in situ* by inflating them with a freshly made glutaraldehyde solution (2% glutaraldehyde and 1% formaldehyde in 0,1M phosphate buffer at pH 7,4) at 37°C. The entire lung and heart preparation was removed and immersed in the glutaraldehyde solution. Samples of lung tissue (1 mm^3) were taken from areas of interest in several lobes. These cubes were then fixed in the buffered glutaraldehyde fixative for 1 - 2 hours and then fixed in 1,5% osmium tetroxide in Veronal buffer (pH 7,4) at 4°C overnight. After rinsing in buffer solution the specimens were dehydrated in graded acetone solutions and embedded in Spurr's resin. Thin sections stained with uranyl acetate and lead citrate were examined by a Siemens Elmiskop I electron microscope.

For the autoradiographic experiments (group V in Table I) 3 animals were each injected with 200 μl (methyl-^{14}C)-paraquat chloride (specific activity 8 $\mu Ci/\mu mol$) in 0,15M saline via the femoral vein. One control animal each received an intravenous injection of saline or of non-radioactive paraquat in saline respectively, and these 2 animals were then treated like the experimental animals. Five hours later they were all killed by intraperitoneal pentobarbital sodium injection. The lungs were perfused (right atrium to left atrium) with normal saline and then inflated via a tracheal cannula using 6% gelatine in saline, quickly dissected out, wrapped in thin plastic foil, and covered in ice to set the gelatine. After about 3 minutes on ice small samples of lung tissue were removed, frozen in a CO_2 freezing device, and stored in the cryostat at −25°C wrapped in aluminium foil. Autoradiography was carried out using Kodak AR.10 stripping film according to the method of Appleton,[18] except that an aqueous solution of gelatine and chrome-alum was used as a coating solution to prepare coverslips before applying the film, and a sucrose-bromide solution was used to float out the film strips before they were picked up on the coated coverslips. Sections on filmed coverslips were exposed in light-tight boxes at −20°C and removed for developing at 12-week intervals for 18 months. Filmed coverslips (plus sections) from each tissue block were exposed to white light at the start of the experiment as controls for negative chemography.

Results

The excellent survival rate of the paraquat-treated animals (13 out of 14 animals) could be ascribed to the relatively low dosage used (15 mg/kg body weight) compared with an LD_{50} of 25 mg/kg body weight. The animals tolerated the paraquat injections remarkably well, although the groups which received three injections tended to recover more slowly from the anaesthesia. Group IV animals showed a slightly diminished food and water intake and some difficulty in breathing during the 24-day recovery period. The control rats appeared to be clinically unaffected and had no lung abnormalities (Fig. 1).

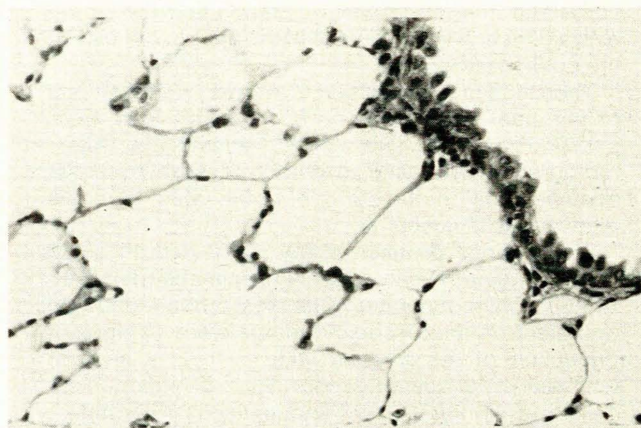

Fig. 1. Photomicrograph illustrating normal distal lung parenchyma of saline-injected control animals. The epithelial lining of a terminal airway is also shown (H and E x 100).

On macroscopic examination the lungs of almost all of the paraquat-treated animals showed (to a greater or lesser extent) signs of focal congestion, subpleural haemorrhages and/or areas of consolidation. On microscopic examination the lungs of the 6 animals in group I, sacrificed 6 days after the first intravenous injection of paraquat, revealed considerable variation in the extent and severity of histopathological changes. One animal had normal lungs. In 4 animals the lungs showed widespread intra-alveolar oedema, foci of increased cellularity consisting of focal interstitial infiltrations of mononuclear cells and in some areas an accumulation of alveolar macrophages, lymphocytes and polymorphs in the alveoli and alveolar walls. The lungs of the 6th animal contained well-demarcated foci of interstitial spindle-shaped cells, which caused pronounced broadening of the interalveolar septa and a consequent dilation of the terminal alveolar ducts and neighbouring alveoli (Fig. 2). However, connective tissue stains showed no increase in stainable collagen or elastic fibres in the interalveolar septa in any of the animals in group I.

An extensive study of a large number of sections from the lungs of group II animals revealed only focal and rather localized lesions, situated mostly in subpleural sites. There was a pronounced proliferation of type II alveolar epithelial cells (Fig. 3). Electron microscopy of these cells showed well-developed nucleoli in prominent nuclei (varying from oval-shaped to spherical), a large number of free ribosomes, a varying number of

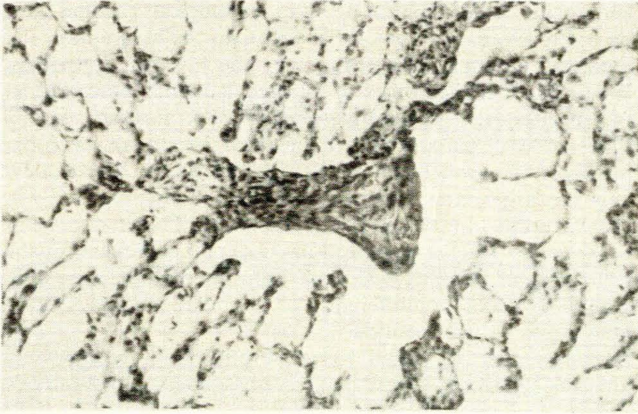

Fig. 2. Proliferation of spindle-shaped and mononuclear interstitial cells caused well-demarcated broadening of alveolar septa and a compensatory dilation of neighbouring ducts and alveoli in a paraquat-treated rat (H and E x 50).

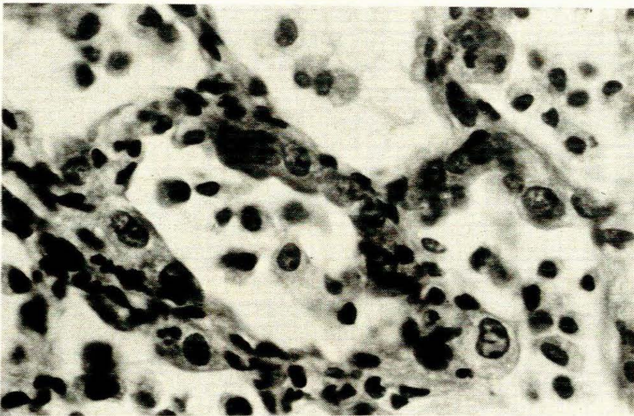

Fig. 3. Pronounced proliferation of type II alveolar epithelial cells in rat lung after a second intravenous injection of paraquat (Masson's trichrome x 200).

cytosomes with different densities, cell-to-cell adhesions and short, irregular surface microvilli (Fig. 4). In some places these cells constituted the entire lining of an alveolus and in other areas they were arranged in clumps, seemingly attached to the disrupted alveolar walls. In such areas several morphologically distinct cells were lying free in the fragmented architecture of the lung parenchyma (Fig. 5). These cells could be identified on ultrastructural examination as alveolar macrophages, leucocytes, type II alveolar epithelial cells and fibroblast-like cells with some large irregular and poorly differentiated cells.

The lungs of group III animals showed marked and widespread pathological changes. The alveolar architecture in some areas was obliterated by the presence of mainly three types of cells —actively proliferating type II alveolar epithelial cells, alveolar macrophages and spindle-shaped fibroblast-like cells (Fig. 6). The hyperplastic and hypertrophic type II alveolar epithelium showed the ultrastructural characteristics of similar cells illustrated previously (Fig. 4). Electron microscopic examination also revealed isolated foci of disruption of the alveolar walls and the presence of fibroblast-like cells in the alveoli (Fig. 7). Most of the fibroblasts were, however, confined to the interstitial spaces and covered by a layer of cuboidal type II alveolar epithelial cells, this constituting the phenomenon of cubic metaplasia of alveoli so often encountered at the peripheral zones of interstitial pulmonary fibrosis. The connective tissue stains revealed an increased amount of fine collagen fibres throughout these lesions.

The lungs of rats sacrificed 24 days after three intravenous injections of paraquat (group IV in Table I) basically showed a similar histological picture, but there was substantially less type II alveolar epithelial proliferation, more fibroblast infiltration of the interstices and a pronounced increase in the connective-tissue fibres stainable by the appropriate techniques. The degenerative and regenerative epithelial change therefore regressed, but the interstitial infiltration of fibroblasts and associated fibrosis persisted.

The sections for examination by autoradiography were developed, stained and studied at 12-week intervals for a total period of 18 months. Although not very prominent, there was an increase in grain density over the alveolar walls after 6 - 9 months but no further increase over the next 9 months. No negative chemography or false-positive results were obtained in the control experiments.

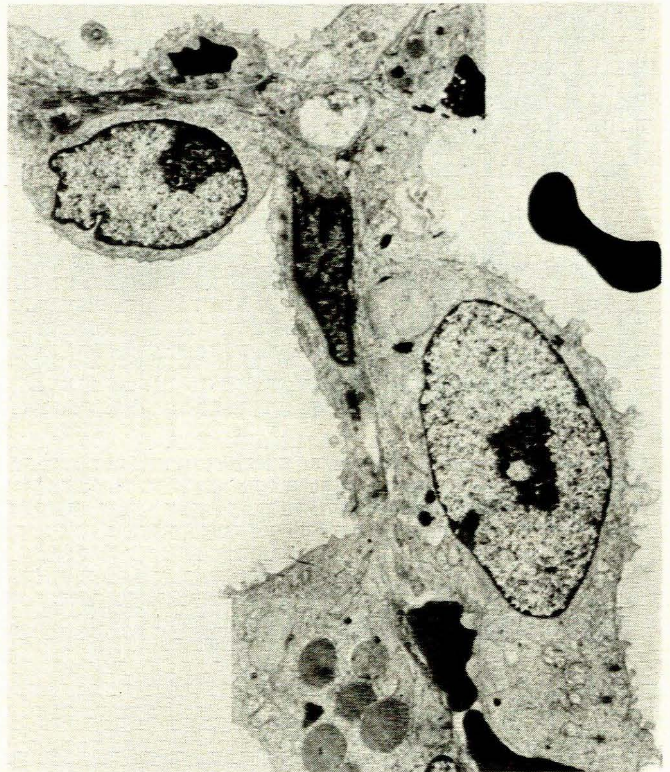

Fig. 4. Montage of electron photomicrographs illustrating the ultrastructural features of proliferating type II alveolar epithelial cells at different stages of maturation (x 4 000).

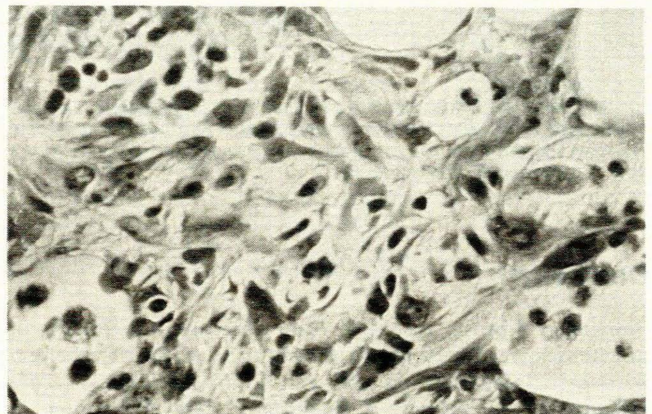

Fig. 5. A heterogenous population of cells in an area in which the alveolar architecture was disrupted. Note the cells with the large vesicular nuclei which appeared to be poorly differentiated connective tissue cells (Masson's trichrome x 200).

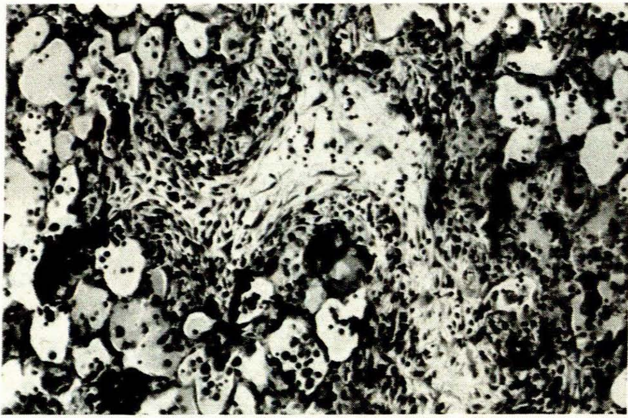

Fig. 6. Obliteration of the alveolar architecture by proliferating and desquamated type II alveolar epithelial cells, alveolar macrophages and spindle- and star-shaped fibroblast-like cells 6 days after the third intravenous injection of paraquat (H and E x 50).

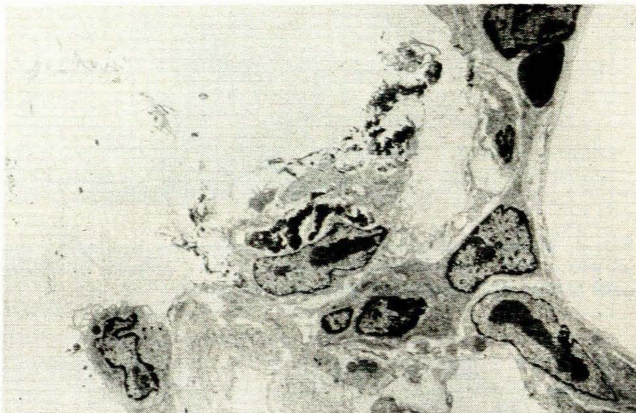

Fig. 7. Electron photomicrograph illustrating disrupted alveolar walls and fibroblast-like and macrophage-like cells free in the alveolar lumen. Note the extremely well-developed nucleoli in the spindle-shaped nuclei (x 2 400).

Discussion

Pulmonary fibrosis resulting from paraquat intoxication in humans is preceded by the disintegration of alveolar epithelium with associated oedema and a mild to moderately acute inflammatory exudate.[1,2] A single large dose of paraquat in experimental animals (30 mg/kg body weight) administered intraperitoneally leads to an increase in the thickness of membranous pneumocytes after 4 hours and cytoplasmic oedema of both types of alveolar epithelial cells after 8 hours; at 18 hours the alveolar epithelium shows signs of hydropic degeneration.[3] Witschi *et al.*[13] have demonstrated that the concentration of paraquat in the plasma and lungs of rats after intravenous injection falls dramatically within the first 8 - 12 hours, whereas lung levels rise during the first 4 hours and then stay at a plateau for the rest of the 24-hour period. This accumulation in the lung presumably occurs by means of an energy-dependent uptake mechanism.[4]

The autoradiographic localization of ^{14}C-paraquat over the alveolar walls 5 hours after a single intravenous injection seems to coincide with the abovementioned evidence that the earliest histopathological changes involve the alveolar epithelial cells. Despite the relatively low grain density it seems reasonable to deduce that the active transport mechanism for paraquat is localized in the cells of the alveolar walls. Evidence that the endothelial cells showed no cytopathological changes after paraquat exposure seems to substantiate the assumption that paraquat becomes concentrated in the alveolar epithelium. When much higher doses of labelled paraquat were injected intraperitoneally in nephrectomized rats, autoradiographic localization of paraquat (24 hours after injection) was found mainly in the bronchiolar epithelium.[19] Whether this is due to only the larger mass of bronchiolar epithelium compared with alveolar epithelium remains to be elucidated. The final answer as to the precise cellular or subcellular target for paraquat probably resides in more sophisticated electron microscopic autoradiographic procedures or differential ultracentrifugation techniques for subcellular fractionation.

Our results confirmed those of other reports[1,2] that no morphological changes occur in the bronchiolar epithelium after systemic paraquat exposure. The necrosis and sloughing of the bronchiolar epithelium after exposure to paraquat aerosol[16] probably result from toxic effects of direct exposure via the inspired air. This then provides additional evidence for the hypothesis that the selective pulmonary toxicity of paraquat is owing to the ability of the alveolar epithelium to concentrate paraquat through either the basal (via the bloodstream) or the apical (via the airways) cell membranes.

The prominent hyperplasia of type II alveolar epithelial cells occurring (even after a rather low dose) indicates a high level of regenerative response by the injured alveolar wall. This is, however, a nonspecific reactive process and appears to illustrate the role of type II cells as stem cells for the regeneration of damaged alveolar lining cells. The persisting type II cell hyperplasia demonstrated with repeated low-dose paraquat exposure may represent continuing alveolar damage due to the residual presence of concentrated paraquat within these cells. This excessive epithelial hyperplasia could certainly contribute to the impairment of lung function as well as disruption of the normal alveolar architecture.

Much controversy still exists as to the origin of the intra-alveolar fibroblast-like cells and whether the intra-alveolar fibrosis is pathognomonic of paraquat-induced pulmonary fibrosis. Transformation of macrophages (or other histiocytes which may reach the intra-alveolar spaces) into facultative fibroblasts could account for the appearance of collagen-synthesizing cells in the alveoli. Although we encountered cells which showed some of the ultrastructural characteristics of histiocytes and fibroblasts (Fig. 8), we considered the relatively inconspicuous macrophage mobilization as insignificant or at least as not totally responsible for the presence of the intra-alveolar fibroblast-like cells.

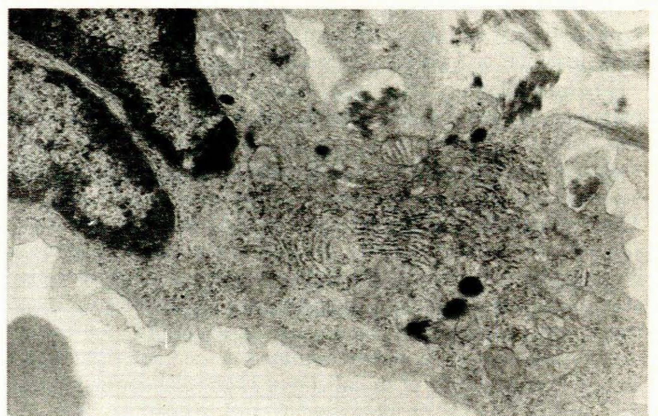

Fig. 8. A higher magnification electron photomicrograph illustrating a cell with the ultrastructural features of fibroblast-like cells (prominent granular endoplasmic reticulum) as well as of histiocyte-like cells (pseudopodia, intermediate filaments, lysosomes) (x 16 000).

When we followed the progression of lesions by administering booster injections of paraquat, it appeared that the intra-alveolar spindle-shaped fibroblast-like cells did not necessarily arise from some normal intra-alveolar precursor cell. The disruption of the alveolar epithelial layer could result in fragmentation of the alveolar wall, thereby allowing interstitial connective tissue cells to enter the alveolar spaces. As a matter of fact, our experiments showed that the presence of intra-alveolar fibroblast-like cells was inevitably associated with disrupted alveolar walls (Fig. 7).

Vijeyaratnam *et al.*[2] reported that paraquat-induced pulmonary lesions were accompanied by an inflammatory exudative reaction, prominent in which were poorly differentiated connective tissue cells. They speculated that these cells could possibly, by proliferation and maturation, contribute to both the free alveolar macrophage and fibroblastic cell populations. We confirmed the presence of such undifferentiated cells in the intra-alveolar exudate, but considered it to be a consequence of the disrupted alveolar structure whereby interstitial cells gained access to the alveolar spaces. Therefore, we felt that the sequence of morphological events which led to the ultimate pattern of interstitial pulmonary fibrosis did not necessarily imply that the intra-alveolar origin of the collagen-producing cells is a unique or specific feature of paraquat fibrosis. It may just as well represent a very nonspecific morphological response to any form of injury to the alveolar wall.

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News and Comment/Nuus en Kommentaar

Sporadic national lunacy

There is surely a prolific field for psychiatric research into the mass lunacy which periodically strikes whole nations. Vietnam, Lebanon, and Iran are a few cases in point. El Salvador is also high on the list, according to the report of a medical mission which went there to look into the disappearance of doctors, dentists, nurses, members of the medical faculty, social and physical scientists, and members of related health professions (Gellhorn, *N Engl J Med* 1984; **308:** 1043). What they found confirmed their worst suspicions. Murder, torture and violations of human rights were commonplace. The persecution of health professionals started in 1979 and continued until 1981, when a group of doctors and nurses protested against the killing and kidnapping of patients and doctors — a not unreasonable reaction. Retribution was rapid and most of them were murdered or 'disappeared'. Statistics on 'disappearances' are almost impossible to obtain, since even mentioning them can jeopardize whole families.

Those lucky enough to survive are kept in pestilential prisons with minimal sanitation. Health services are in disarray, and the whole impression is of a country which has returned to the law of the jungle. One can only hope that peace and sanity will eventually return to the area.

A few tests

Every year, in England and Wales, the number of requests for haematological tests rises by 7-14%, microbiology tests by 6-10%, and chemical pathology tests by 10-17% (Editorial, *Lancet* 1984; **i:** 1278). This seems to be part of a world-wide trend, but the crucial point is — are all of these investigations really necessary? Probably not, in many cases. Part of the blame can be put on automated machinery, which gives more information than is acutally needed on a blood sample, whether it is requested or not. Request forms have also become more impersonal. It is so easy to casually put ticks on a computerized form rather than write out the name of the test in full. In the hospital setting the attitude of the consultant is crucial. Organization of investigations is left to the most junior member of the team — the intern — and he is not likely to risk the great man's wrath by missing out investigations which may be only marginally necessary. If junior staff are not questioned about the rationale for their investigations, there is nothing to discourage irrelevant or repetitive testing. In one London hospital, a requirement that all out-of-hours requests by housemen be screened by their own registrars resulted in a halving of tests requested. In these cost-conscious days, this is a field which would bear a much closer scrutiny than it appears to have had to date.