Enhancement of Natural and Experimental Respiratory Mycoplasmosis in Rats by Hexamethylphosphoramide

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Hexamethylphosphoramide (HMPA) was given orally (100 mg/kg/day) to: a) conventional rats of Sprague-Dawley and Long-Evans substrains known to have indigenous *Mycoplasma pulmonis* infection, b) uninfected pathogen-free (PF) Fischer rats, and c) PF and axenic Fischer rats inoculated intranasally with *M. pulmonis* strains having a wide range of virulence. Treated rats infected with virulent *M. pulmonis*, either naturally or experimentally, developed severe clinical signs of murine respiratory mycoplasmosis (MRM) with mortalities of 25 to 60% compared to relatively mild MRM and no deaths in untreated, infected controls. Deaths were attributed to unusually severe lung lesions of MRM (extreme neutrophilic exudation into major bronchi and bronchiectasis) with ulceration of respiratory mucosa and hemorrhage. Rhinitis also was increased in severity by HMPA in conventional rats, but not in experimentally infected PF or axenic rats. Severity of otitis media and tracheitis was not affected by HMPA. Incidence of lesions of MRM was unchanged except for increased frequency of gross lung lesions. In the absence of *M. pulmonis* infection, HMPA treatment of rats caused thinning and microulceration of respiratory epithelium in major bronchi without inflammatory lung disease. Other effects induced by HMPA, with or without the infection, were destruction and fibrous replacement of olfactory epithelium, atrophy of testes, and reduced weight gains. It was concluded that HMPA markedly enhances both rate of progression and severity of the pneumonia while inconsistently potentiating the rhinitis of MRM in rats. Previous studies of HMPA are emphasized as an additional example in which the synergistic effects of an experimental chemical and an indigenous pathogen of laboratory rats have given misleading experimental results. (Am J Pathol 82:171–190, 1976)

HEXAMETHYLPHOSPHORAMIDE (HMPA) is an organic solvent used widely in industrial chemosynthesis.[1] This compound also has been shown to possess sterilizing properties when administered to insects [2] and some mammals.[3] Among investigations of the toxicologic properties of HMPA in mammals, there are two reports [4,5] in which a severe inflammatory lung disease was considered its principal toxic effect in conventional rats. A subsequent study [6] confirmed the latter finding and

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suggested, without bacteriologic confirmation, the possibility that the lung lesions were due to chemical exacerbation of naturally occurring *chronic respiratory disease* (CRD).

The so-called CRD of rats is now more appropriately called *murine respiratory mycoplasmosis* (MRM) because of recent evidence [7-11] that *Mycoplasma pulmonis* is the primary etiologic agent of this disease. Because of the known ubiquity of inapparent *M. pulmonis* infection in conventional rat stocks [10] and the morphologic character of the pneumonia described by others [4-6] in HMPA-treated rats, we postulated that oral administration of HMPA might enhance both the natural and experimental disease due to this organism. The purpose of the present study was to test that hypothesis by determining the effects of HMPA on: a) conventional rats known to have MRM due to natural *M. pulmonis* infection, b) uninfected pathogen-free (PF) rats, and c) PF and axenic rats infected experimentally with *M. pulmonis*.

Materials and Methods

Hexamethylphosphoramide

Purity of each lot of HMPA (Aldrich Chemical Co., Milwaukee, Wisc. or Eastman Chemical Co., Rochester, N.Y.) was confirmed by thin layer [12] and gas liquid [13] chromatography.

The compound was mixed with food or drinking water as follows. A weighed volume of HMPA was diluted in an equal volume of ethyl ether, mixed with 10 g of cornstarch, and allowed to air dry at room temperature under an exhaust hood. The HMPA–cornstarch mixture then was mixed thoroughly with previously ground rodent diet (Ralston Purina Co., St. Louis, Mo., or Allied Mills, Inc., Chicago, Ill.) to give a final concentration of 2.0 g HMPA/kg.[14] Alternatively, HMPA was dissolved in sterile distilled drinking water at a concentration of 1.0 g/liter. Fresh aqueous solutions were supplied at 4-day intervals. Based on measurements of consumption, *ad libitum* provision of either the food or water containing HMPA resulted in ingestion by rats of approximately 100 mg HMPA/kg body weight/day (range, 74 to 120). Control diet and drinking water were prepared by these same procedures, except that HMPA was excluded.

Conventional Rats

Three-week-old Sprague-Dawley (SD) rats were obtained from a commercial source (Southern Animal Farms, Montgomery, Ala.) known to have natural MRM. Long-Evans (LE) rats of the same age were obtained from another vendor (Blue Spruce Farms, Inc., Altamont, N.Y.) advertising LE rats as "respiratory disease resistant." Rats of both strains were received on the same day and housed in groups of two or three per cage, according to strain and sex. The cages were of wire mesh type and were suspended 2 inches apart on racks with tiers separated by solid galvanized catch pans. Catch pans were filled to a depth of ¼ inch with hardwood chip bedding (Ab-Sorb-Dri Inc., Garfield, N.J.) that was changed every other day.

HMPA was supplied *ad libitum*, in either the diet or drinking water, beginning at 25 days of age and continuing for 62 consecutive days. Rats not ingesting HMPA ("indirectly exposed" controls) were housed in alternate cages between treated rats to determine

whether the effects of HMPA could be produced through inhalation of its vapors. For controls devoid of any exposure to HMPA, 8 SD and 8 LE rats from the original lots were retained by the breeders until the end of the experiment, then forwarded for immediate sacrifice upon arrival and evaluation along with the other groups.

Pathogen-Free and Axenic Rats

Six- to eight-week-old PF or axenic rats of a Fischer subline (CDF, Charles River Breeding Labs., Wilmington, Mass.) were used. They were fed autoclaved diet formulated for axenic rodents (Ralston Purina or Allied Mills), housed in polycarbonate cages with hardwood chip bedding, and maintained in Trexler-type plastic film isolators throughout the experiments. Both PF and axenic rats remained free of detectable pathogens upon repeated culturing for fungi, mycoplasmas, and other bacteria, and serologic testing for rodent viruses.[10]

In groups treated with HMPA alone, the chemical was supplied continuously in drinking water for up to 100 days. Animals treated with HMPA and inoculated intranasally with *M. pulmonis* were given the chemical beginning 7 days prior to inoculation of the agent and until they were sacrificed at 28 days after inoculation.

In a further attempt to determine whether HMPA produces any effect as a result of inhalation, undiluted HMPA was placed in a polycarbonate shoe box–type cage containing six 45-day-old PF rats. A 50-ml beaker, covered with fiberglass insect screening and secured by wire inside the cage, provided for continuous evaporation of the chemical and prevented its ingestion by the rats.

Air samples from the isolator which housed the axenic rats given HMPA and *M. pulmonis* were tested periodically for ammonia by the Kitagawa (Unico Environmental Instruments, Fall River, Mass.) method.

Mycoplasma Inoculations

Selection of inocula was based on ability of *M. pulmonis* strains to produce lower respiratory tract lesions in PF rats in a previous study.[10] Inoculum M_2 was a culture of rat origin which produced gross lung lesions in 40% of infected animals. Inoculum M_1 also was of rat origin but had been found to produce only microscopic lung lesions. The least virulent inoculum (N) was a mouse isolate known to cause only rhinitis following intranasal inoculation into PF rats. All inocula were confirmed as pure cultures of *M. pulmonis* by immunofluorescence (IMF).[15]

Intranasal inoculations were made while rats were lightly anesthetized with fentanyl and droperidol (Innovar-Vet, Pitman-Moore, Inc., Washingtons Crossing, N.J.).[7] At each inoculation, 0.05 ml of broth culture of *M. pulmonis* was placed drop by drop on the external nares of rats held with the head in an upright position. This was repeated on the fourth and seventh days following initial inoculation, giving a total dose/rat of 1.3×10^7, 1.2×10^{10}, and 5.0×10^6 colony-forming units (CFU) for M_2, M_1 and N inocula, respectively.

Isolations of mycoplasmas from experimental animals were performed as described previously.[10] In instances where cultures were not taken, the presence of *M. pulmonis* in respiratory tissues was confirmed by indirect IMF.[10,16]

Pathology

Experimental animals were observed twice daily. Dead or moribund rats were removed promptly for necropsy and collection of specimens. Rats which survived were studied similarly after euthanasia at predetermined times with an overdose of pentobarbital sodium. Immediately prior to euthanasia, hematocrits, differential and white blood cell (WBC) counts were performed according to standard hematologic techniques.

Methods used for collection and preparation of tissues for histopathology were reported

previously.[10] Tissue sections were coded and evaluated without knowledge of treatment or strain of rat. Microscopic lesions at each level of the respiratory tract (nasal passages, middle ears, tracheas, and lungs) were scored (0 to 3) according to severity of lesions previously described for MRM.[10] The sum of scores for each organ divided by the sum of maximal scores possible gave the lesion index (LI) for making comparisons of experimental groups. A LI value of 1.0 was the most severe change possible for an organ. Lesions were quantitated only in rats which survived the entire experimental period.

Histologic sections also were prepared from heart, liver, kidneys, testes, ovaries, adrenals, pancreas, spleen, mediastinal lymph nodes, bone marrow, thymus, and brain.

Statistics

For comparisons of nonparametric data such as LI, the Rank-Sum test was used.[17] Data yielding defined parametric values (body weights, hematocrits, and WBC counts) were analyzed according to the Student *t* test. The Chi-square method was used for data giving only "present or absent" information (pneumonia, mortality, etc.)

Results

Conventional Rats Given HMPA

Clinical Finding

By Day 14, many SD and LE rats ingesting HMPA showed dypsnea, ruffled hair, and reluctance to move. "Snuffling" was pronounced in most treated rats, and a few circled with their heads tilted in the direction of circling. Mild snuffling was noted in indirectly exposed controls.

After the first 3 days of HMPA administration, no differences were noted in quantity of food or water consumed by HMPA-treated and indirectly exposed rats. However, rats of both strains and sexes weighed significantly less at the end of the experiment than those of the indirectly exposed and untreated control groups. The average weight for treated males was 252 g and controls 295 g. For treated and control females, the average weights were 188 and 215 g, respectively.

There was no difference in weight between indirectly exposed and untreated control groups. Treatment with HMPA had no effect on hematocrits, or differential and total WBC counts.

Mortality

No spontaneous deaths occurred in untreated control rats or rats exposed to HMPA vapors. However, mortality in groups ingesting HMPA reached 25, 32, and 34% (Table 1). The first death occurred 19 days after beginning HMPA, and additional deaths were seen sporadically throughout the experiment. There were no differences in mortality according to sex, strain of rat, or method of oral HMPA administration.

Respiratory Tract Lesions

Extensive pulmonary disease was found at necropsy in all rats which died. Gross examination of the lungs revealed one or more collapsed lobes,

Table 1—Respiratory Disease in Conventional Rats Given HMPA

Rats						Lesion index			
Strain	No.	HMPA exposure	Isolation *M. pulmonis* (%)	Mortality (%)	Gross lung lesions (%)	Lungs	Trachea	Middle ears	Nasal passages
SD	8	—	100	0	13	.22	.35	.48	.40
	32	I	88	0	16	.17	.29	.90	.30
	33	F	96	32*	50*	.37†	.28	.78	.56†
	12	W	ND	25	67	.44	.38	.71	.61
LE	8	—	75	0	0	.14	.27	.01	.29
	22	I	92	0	9	.08	ND	.21	.08
	35	F	87	34*	68*	.48*	.38	.11	.63*

SD = Sprague-Dawley, LE = Long-Evans, ND = no data. — = No exposure to HMPA (rats maintained at vendor's facility until termination of experiment), I = Indirect exposure (rats housed in open wire mesh cages adjacent to cages housing rats given HMPA), F = HMPA mixed in food (2.0 g/kg diet), W = HMPA dissolved in drinking water (1.0 g/liter). Lesion index = sum of scores of microscopic lesions for each organ divided by maximum possible score for the organ (1.00 = lesions of maximal severity).

No significant difference was observed due to giving HMPA in food or water. Therefore, results from both groups were combined and compared statistically with the SD indirect control group. Similarly, LE rats given HMPA were compared with LE indirect controls.

* $P \leq .01$.

† $P \leq .05$.

dark red in color and often marked with multiple 1-to 3-mm abscesses. Microscopic changes in conventional rats which died were not different from those in PF rats which died following HMPA administration and *M. pulmonis* inoculation; these changes will be described in detail below.

When rats were killed after 62 days of HMPA administration, there was a significantly higher incidence ($P < .01$) of gross lung lesions in treated rats than in indirectly exposed and untreated controls (Table 1). No qualitative difference was observed between gross lung lesions in the HMPA groups and those in affected controls. For example, lesions associated with end-stage MRM (bronchiectasis and pulmonary abscesses) were recognized in individuals from all SD and LE groups but were seen far more frequently in rats ingesting HMPA. Microscopically, animals in all groups had lung lesions of at least early stages of MRM—neutrophilic exudate in bronchi, hyperplasia of respiratory epithelium, and peribronchial lymphoid hyperplasia with cuffing. Lungs of HMPA-treated rats had significantly higher LIs (Table 1). The distinctive feature of microscopic lesions in rats ingesting HMPA was that large conducting airways often had extensive ulceration of mucosa in association with extraordinarily large quantities of purulent exudate in the lumina.

No qualitative differences in pulmonary disease were attributable to strain of rat or sex in HMPA-treated groups, but gross lung lesions were more frequent in untreated SD rats than in untreated LE rats (Table 1). However, when microscopic lesions were quantitated by LI and compared statistically, there was no difference between controls of the two strains.

Frequency and severity of microscopic lesions in the trachea were similar in both strains and in all groups (Table 1). Acute and chronic otitis media occurred with greater frequency and severity in all groups of SD rats. All tracheal and ear lesions were charasteristic of MRM[10] and were not influenced by HMPA in either strain.

Changes in nasal passages were significantly more severe in rats ingesting HMPA (Table 1). All groups had varying degrees of rhinitis typical of MRM,[10] characterized by epithelial hyperplasia and dysplasia, increased mucus production, subepithelial accumulations of lymphocytes and plasma cells, and purulent exudate in the nasal cavities. In addition, rats ingesting HMPA consistently had severe ulceration of both respiratory and olfactory epithelium with scarring and distortion of turbinates. Squamous metaplasia occurred commonly in the remaining intact epithelium. In the olfactory region, the cavity often was occluded completely by scar tissue and exudate.

Despite the increased incidence and severity of respiratory disease in rats ingesting HMPA, the rates of recovering mycoplasmas from treated

and untreated groups were not different. *M. pulmonis* was isolated from tracheal aspirates of 92% of HMPA-treated rats and 87% of controls.

Lesions Outside the Respiratory Tract

Lesions in other organs were observed only in HMPA-treated rats. Moderate to severe testicular atrophy was present in all rats ingesting HMPA, but not in indirectly exposed or untreated controls. Microscopically, there was marked hypospermia, with presence of numerous "spermatid giant cells" in seminiferous tubles. Sertoli and interstitial cells appeared normal.

PF Rats Given HMPA Alone

Clinical Findings

All PF rats given HMPA alone were asymptomatic throughout the experiment. However, treated rats gained less weight than untreated controls. Average weight for 12 (6 females, 6 males) randomly selected rats killed after 62 days on HMPA was 210 g, compared to 233 g for 12 untreated controls of the same age and sex distribution ($P < .05$). Hematologic values obtained for the treated group were not significantly different from those of controls.

Mortality

Of the 50 rats given HMPA continuously up to 100 days, only 1 died spontaneously. This individual died after 77 days of HMPA administration due to causes which could not be determined by necropsy and histologic studies.

Respiratory Tract Lesions

Rats were killed and evaluated after 35, 62, and 100 days of HMPA ingestion. Histologic sections of lungs from PF rats given HMPA for 35 days could not be differentiated from untreated controls except for occasional microulcers in the respiratory epithelium (Figure 1). Similar changes were seen in rats given HMPA for 62 days but not in those treated for 100 days. Microulcers occurring in the main stem bronchi and rarely in small bronchi accounted for the slightly elevated LIs in the lung scores for the 35 and 62 day HMPA groups (Table 2).

Tracheas and middle ears from HMPA-treated rats were not different from untreated controls except for the infrequent occurrence of a few small focal ulcerations in the distal end of the trachea of rats treated for 35 and 62 days.

Table 2—Respiratory Tract Lesions in PF Rats Given HMPA and/or *M. pulmonis*

Exp. No.	No. rats	HMPA*	*M. pulmonis*†	Mortality (%)	Rats with gross lung lesions (%)	Lesion index‡ Lungs	Trachea	Middle ears	Nasal passages
I	38	(35 to 100)		0	0	.04	.06	.00	.05
	14	+ (35)		0	0	.12	.07	.00	.36
	22	+ (62)		0	0	.11	.07	.00	.39
	14	+ (100)		7§	0	.04	.05	.00	ND
II	10	(28)		0	0	.05	.03	.00	.04
	28	(28)	M_2	0	39	.40	.72	.54	.52
	15	+ (28)	M_2	53¶	93‖	.80‖	.79	.41	.52
III	8	(28)		0	0	.05	.05	.00	.04
	13	(28)	M_1	0	0	.21	.51	.74	.48
	10	+ (28)	M_1	60¶	90¶	.82¶	.83	.05¶	.38
IV	8	(28)		0	0	.03	.02	.00	.05
	12	(28)	N	0	0	.03	.03	.00	.18
	19	+ (28)	N	0	5	.16‖	.21‖	.00	.44‖

ND No data. Statistical comparisons were made only between those groups receiving (1) HMPA and *M. pulmonis*, and (2) *M. pulmonis* alone.
* No exposure to HMPA, + HMPA given in drinking water (1.0 g/liter), numbers in parentheses are length of experiments in days.
† no *M. pulmonis* inoculation, M_2 most virulent inoculum, M_1 moderately virulent inoculum, N least virulent inoculum.
‡ Lesion index sum of scores for each organ divided by maximum possible score for the organ (1.00 lesion of maximal severity).
§ Represents a single death due to unexplained causes.
¶ P .01.
‖ P .05.

There were severe microscopic changes in nasal passages of treated rats (Table 2). After 35 days, there was thinning and reduction in number of epithelial cells (Figure 2A and B). The epithelium tended to be low columnar to cuboidal. In severely affected areas, there was patchy loss of cilia and/or olfactory hairs. Focal microulcers were present, particularly on the nasal septum (Figure 2C). The microulcers frequently contained accumulations of macrophages along with proliferating fibrous connective tissue which sometimes was so extensive as to encroach on the medial surface of the maxillary turbinate, obstructing the lumen of the nasal passages at this point (Figure 3). Lesions extended to the cribriform plate and obliterated much of the normal ethmoturbinal architecture, resulting in atrophy of nerve bundles in the olfactory tract (Figure 4A and B). Few neutrophils were present in the remaining lumina of this region, and subepithelial infiltration of inflammatory cells was minimal.

Nasal passages of rats killed after HMPA treatment for 62 or 100 days showed continuing progression of the nasal passage lesions. Changes seen commonly at these later times, but only rarely in rats at 35 days, included squamous metaplasia (Figure 2D) of respiratory epithelium and mineral deposits within the scar tissue. Similar sections of nasal passages from untreated controls killed at the same time showed only mild and inconsistent focal disorganization of olfactory epithelium without inflammation.

PF rats not ingesting HMPA, but maintained as indirectly exposed controls for 45 days, remained clinically asymptomatic. Microscopic examination of respiratory tracts revealed none of the changes associated with HMPA ingestion.

Lesions Outside the Respiratory Tract

As in the conventional rat experiments, organs other than testes were not altered histologically by HMPA ingestion. Changes within seminiferous tubules were the same as described for HMPA-treated conventional rats. PF rats exposed only to HMPA vapors had normal testes.

PF Rats Given HMPA and/or *M. pulmonis*

Clinical

Rats given *M. pulmonis* M_1 or M_2 in the absence of HMPA had mild clinical signs of MRM (snuffling) 14 days after inoculation and continuing throughout the experiment. In contrast, rats given HMPA in addition to *M. pulmonis* showed extreme respiratory distress as early as 7 days after inoculation. Clinical signs of MRM were even more severe than those seen

in HMPA-treated conventional rats, except that no evidence of inner ear disease was seen. Animals inoculated with the least virulent inoculum, N, showed no clinical signs of disease, even when treated with HMPA.

Hematologic values for PF rats given HMPA and/or *M. pulmonis* were not statistically different from those of untreated uninfected controls.

Mortality

No deaths occurred in PF rats given *M. pulmonis* alone or in HMPA-treated rats given inoculum N. When rats were treated with HMPA and inoculated with the M_2 or M_1 isolates, more than 50% died between 10 and 28 days after inoculation (Table 2).

Respiratory Tract Lesions

Asphyxiation was considered the primary cause of death, since complete occlusion of either the major bronchi and/or the lower end of the trachea was demonstrated histologically in more than 70% of cases. Sections through the major bronchi characteristically revealed a severely ulcerated mucosa and a stringy coagulum consisting of mucus, neutrophils, erythrocytes, and fibroblasts plugging the lumen of bronchi (Figure 5) and, sometimes, the distal end of the trachea (Figure 6). The remaining epithelium was tenuous in appearance, being low cuboidal to simple squamous.

Rats which died between 10 and 14 days after inoculation had primary and secondary bronchi consistently filled with purulent exudate and blood. Hemorrhage was associated with erosion of the mucosa and submucosal capillary bed. Lung parenchyma was focally atelectatic and congested with a few neutrophils and macrophages scattered in alveoli.

Rats which succumbed from 15 to 28 days after inoculation showed lesions more characteristic of end-stage pulmonary changes in natural MRM. There was pooling of mucopurulent exudate in distal bronchiectatic airways and squamoid change of respiratory epithelium (Figure 7). Some bronchi were severely scarred, sometimes leaving a residuum of small, nonfunctional airways filled with neutrophils and cellular debris (Figure 8). Alveolar architecture was masked by a cellular infiltrate consisting of neutrophils and macrophages, and large subpleural abscesses were formed by pools of neutrophils with central cores of necrosis. Severe congestion and focal hemorrhage were present in most of the less-consolidated lobes. Diffuse fibrinopurulent pleuritis was seen in 4 of 25 rats. The findings generally resembled closely those in conventional rats which had died after HMPA treatment.

Histologic lesions in the lungs of PF rats sacrificed following 28 days of

HMPA ingestion and *M. pulmonis* infection were similar to those in rats which died 15 to 28 days after inoculation and to those of conventional rats treated with HMPA and killed 62 days later.

It was not possible to differentiate lesions produced by HMPA and inoculum M_1 from those produced by HMPA and inoculum M_2, as virtually every rat in both groups had severe pulmonary disease (Table 2). Such features as stagnation of mucus and cellular debris in airways, abscessation, and bronchiectasis were present in rats receiving *M. pulmonis* only but were much more extensive in groups receiving both HMPA and strain M_2 or M_1 of *M. pulmonis*. Also, consolidation due to cellular infiltration and atelectasis, adenomatoid change of alveolar lining cells, and squamous metaplasia of bronchi were commonly seen in lungs of rats given HMPA and *M. pulmonis*, but only occasionally in rats given the organism alone.

Microscopic lung lesions were seen in only 1 of 19 rats treated with HMPA and inoculated with the N isolate. These lesions were qualitatively identical to those produced by HMPA and either M_2 or M_1.

As in conventional rats treated with HMPA, PF rats given HMPA and inoculated with either isolate M_2 or M_1 developed severe inflammatory lesions in tracheas, middle ears, and nasal passages. However, the LIs calculated for these organs in HMPA-treated, infected groups were not consistently different from those of rats receiving *M. pulmonis* alone (Table 2). The same lesions resulted from HMPA treatment and inoculation of the N isolate, except that changes were much less severe and no middle ear lesions were seen. Animals given inoculum N and HMPA had significantly greater LIs for trachea and nasal passages than untreated, infected controls.

Lesions Outside the Respiratory Tract

Testicular atrophy was seen in rats treated with HMPA, but not in rats given the organism alone. In addition, rats infected with *M. pulmonis* often had lymphoid hyperplasia of mediastinal and cervical lymph nodes as is typical of early MRM.

Axenic Rats Given HMPA and *M. pulmonis*

In order to confirm results obtained using conventional and PF rats, 12 axenic Fischer rats were treated with HMPA and inoculated with *M. pulmonis* M_1. By 20 days after inoculation, 50% of the animals had died. Because the remaining animals were critically ill, the experiment had to be terminated on 20 days rather than after 28 days as originally planned.

At necropsy, all rats were found to have severe bronchiectasis and

pulmonary abscesses. Histologic lesions were identical to those seen in conventional rats treated with HMPA and in PF rats given HMPA followed by inoculation with *M. pulmonis*. Examination of these lesions by IMF confirmed the presence of large numbers of *M. pulmonis* throughout the respiratory tract. At the termination of the experiment, cultures of trachea were negative for bacteria other than *M. pulmonis*, and serum was serologically negative for rodent viruses.[10]

Discussion

The chemical, HMPA, is best known industrially as an organic solvent and biologically for its gonadal sterilizing properties in insects and mammals.[2,3] The present paper describes two additional properties resulting from oral administration of HMPA to rats: a) dramatic enhancement of the pneumonia in natural and experimental MRM due to *M. pulmonis*, and b) extensive destruction of olfactory epithelium leading to replacement by fibrous connective tissue.

Oral administration of HMPA markedly enhanced the clinical and pathologic manifestations and increased the mortality due to MRM in conventional SD and LE rats naturally infected with *M. pulmonis* and in PF and axenic Fischer rats experimentally infected with *M. pulmonis* strains representing a wide range of virulence. These effects of HMPA were attributed to marked potentiation of the lesions of MRM in the lower respiratory tract. With the exception of ulceration and hemorrhage, all of the observed lung changes occur in natural (including untreated conventional rats of this study) and experimental MRM in rats, but always at a much lower rate of progression and severity.[10] HMPA had no consistent effect on incidence or severity of the rhinitis, otitis media, or tracheitis.

In contrast, administration of HMPA alone to PF rats for extended periods failed to cause inflammatory lung disease. Thinning and ulceration of respiratory epithelium were observed in trachea and major bronchi, and rarely more distal than second order bronchi. Similar changes in the nasal passages, heretofore unreported, were especially pronounced. In many cases, normal ethmoturbinal architecture of the olfactory portion of the nasal passages was completely replaced by collagenous connective tissue. These changes were found consistently in all PF rats receiving HMPA as long as 35 days, and except for microulcers in bronchial epithelium, all lesions persisted up to 100 days. No explanation is given for the disappearance of bronchial microulcers in rats on HMPA for the longer time.

The testicle was the only organ outside the respiratory tract to show a

morphologic change due to HMPA treatment. Degenerative changes in seminiferous tubules were more frequent in conventional rats, but even in them, the changes were sporadic and inconsistent. This observation, in addition to that of significantly reduced weight gains, agrees with the findings of Shott *et al.*[6] The renal tubular changes and pulmonary arteritis reported by Kimbrough and Sedlak [5] were not seen.

The lack of enhancement of natural MRM in conventional rats housed in suspended wire cages adjacent to rats given HMPA in the food and the fact that PF rats unable to ingest HMPA but exposed constantly to its vapors failed to develop any anatomic lesions, indicate that HMPA produces its effects as the result of ingestion rather than inhalation. The possibility exists that after ingestion, HMPA is metabolized to a substance with pulmonary irritating properties. Dimethylamide and NH_3 are two such compounds which, at least theoretically, could fill this role. Other investigators [3,5] have shown conclusively that HMPA is not converted and excreted through the lungs as dimethylamide. The question remains unresolved whether metabolism of HMPA leads to respiratory excretion of NH_3, although we determined that the plastic isolators housing axenic rats ingesting HMPA never had detectable (5 ppm or greater) levels of NH_3.

Anatomic findings in rats given HMPA alone or with *M. pulmonis*, and the findings of recent IMF studies [18] suggest that HMPA exerts its effects on *M. pulmonis* infection in the lungs by impairing pulmonary clearance. Studies to be reported separately [19] show that impaired clearance of the agent correlates with reduced cell renewal rates in pulmonary tissues. Presumably, a related mechanism could also explain the olfactory and testicular lesions and the reduced weight gains due to HMPA.

HMPA treatment resulted in more pronounced clinical effects and pneumonias in PF and axenic rats experimentally inoculated with *M. pulmonis* than in conventional rats harboring the natural infection. No clear explanation for this difference can be given, but there are a number of possibilities. It seems likely that the dose of *M. pulmonis* (10^6 to 10^{10} CFU) given to the PF and axenic rats was much higher than that obtained naturally by the conventional rats. Also, previous microbial experience of the conventional rats with mycoplasmas and other organisms may have stimulated partial immunity or microbial interference.[20] Conversely, the presence of a normal flora in the nasal passages of the conventional rats possibly explains why the rhinitis was enhanced by HMPA in them, but not in *M. pulmonis*–infected PF or axenic rats.

The present studies did not substantiate the broad claim of the vendor that LE rats are resistant to MRM. It was found that control LE rats had slightly fewer gross lesions and lower microscopic lesion scores than

control SD rats, but the differences were statistically significant for only the ear lesions. This observation and a previous report by Freudenberger,[21] claiming that LE rats had a much lower incidence of otitis media than Wistar rats housed in the same room, suggest that LE rats may, in fact, be relatively resistant to the otitis media of MRM. It is particularly interesting that despite the differences in ear disease, the LE rats in our study were found to harbor *M. pulmonis* in the respiratory tract just as frequently as the SD rats. When the two strains were given HMPA, there was no difference in their ensuing respiratory disease.

From the results of these studies, it is clear that HMPA alone does not cause pneumonia in rats as reported previously.[4,5] Instead, it markedly enhances natural MRM or its experimental counterpart produced by intranasal inoculation of *M. pulmonis*, either of which normally causes a slowly progressive respiratory disease without death during experimental periods of up to 2 months.[10]

These studies with HMPA provide an example of the extremely subtle complications which *M. pulmonis* may cause in experiments using rats. In the first two reports on the toxicity of HMPA for rats,[4,5] the resulting pneumonia was attributed exclusively to this compound. This was a logical conclusion based on the reported absence of lesions in untreated controls and the fact that no attempts were made to isolate mycoplasmas. It may also be pertinent that many investigators are not aware that detection of all except the advanced lung lesions of MRM requires microscopic examinations.[10]

It is sometimes said that as long as one uses adequate numbers of controls, there is no need to be concerned about naturally occurring diseases of experimental animal stocks. The present case, involving the synergistic effects of HMPA and a natural infection, argue very strongly against this assumption. Almost certainly there is a large number of additional chemicals (particularly carcinogens,[22,23] immunosuppressants [24] and irritant gases [7]) which, if given to conventional laboratory rats having MRM, will lead to confusing experimental results due to synergistic effects.

Methods presently used for early detection of *M. pulmonis* infection, including the best culture techniques available, generally have serious limitations, and the natural evolution of all lesions of MRM in the rat usually requires months. For these reasons, the oral administration of HMPA may be useful for improving the effectiveness of culture, IMF and pathologic techniques in the detection of inapparent *M. pulmonis* infections. Further studies will be required to prove the feasibility of this potential application of the chemical.

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Legends for Figures

Figures 1 to 4 show the histology of control rats and those given hexamethylphosphoramide (HMPA).

Figure 1—Wall of a large bronchus from a PF rat given HMPA for 35 days. Loss of epithelium has resulted in formation of three microulcers (*arrows*). Note irregular distribution of epithelial nuclei, lack of uniform cell height, and macrophages present in floor of ulcers. (H&E, × 140)

Figure 2—Nasal septum from 4 rats. **A**—Normal PF rat. Note the abundance of nuclei in the mucosal epithelium and the typical pseudostratified pattern. **B**—PF rat given HMPA for 35 days. Mucosal cells are reduced in number, and nuclei are arranged in almost a single row. **C**—PF rat given HMPA for 35 days. A microulcer is present, and its floor contains numerous inflammatory cells. **D**—PF rat given HMPA for 62 days. Squamous epithelium has replaced the columnar epithelium normal to this location. (H&E, × 200)

Figure 3—Transverse section of nasal passages from a PF rat given HMPA for 35 days. Ulcers are present on medial surface of both turbinates (*T*). (H&E, × 80)

Figure 4—Transverse sections through olfactory region of nasal passage and brain. **A**—Normal PF rat. Nasal cavity in this region has a smooth lining of olfactory epithelium supplied by numerous nerve tracts. **B**—PF rat given HMPA for 35 days. There is complete replacement of olfactory epithelium by scar tissue, and nerve bundles have almost disappeared. (H&E, × 100).

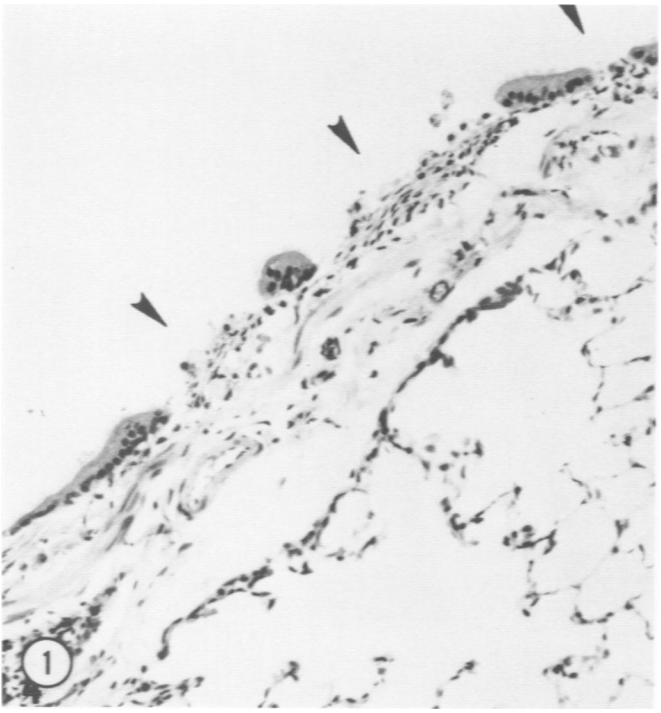
Figures 5 to 8 show the histologic lesions of PF rats given both hexamethylphosphoramide (HMPA) and *M. pulmonis*.

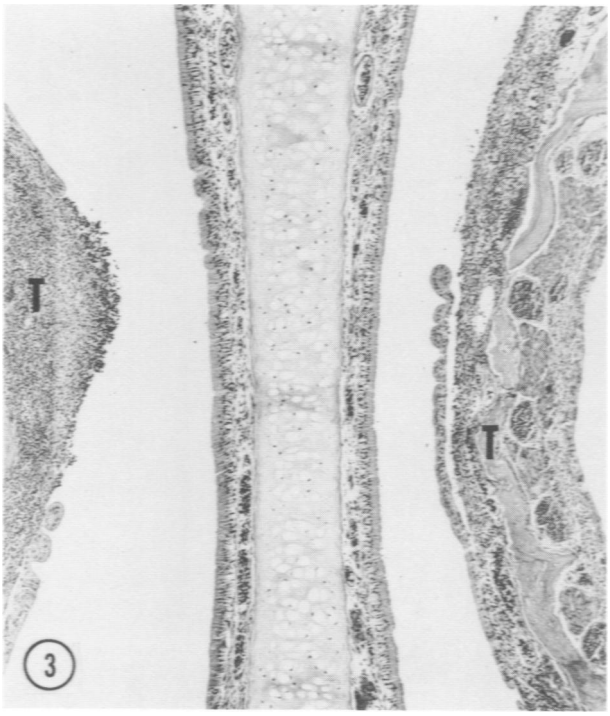
Figure 5—Main stem bronchus from HMPA-treated PF rat which died 11 days after inoculation with 1.2×10^{10} CFU of *M. pulmonis* (M_1 strain). Epithelial lining has been almost completely destroyed. Lumen contains purulent exudate, blood, and immature collagen (at top and left). (PAS, × 60).

Figure 6—Longitudinal section of trachea from HMPA-treated PF rat which died 11 days after inoculation with 1.2×10^{10} CFU of *M. pulmonis* (M_1 strain). The lumen is obliterated by purulent exudate. This was a typical finding in animals which died as a result of HMPA treatment plus *M. pulmonis* infection. (H&E, × 80).

Figure 7—Medium sized bronchus from a HMPA-treated PF rat which died 21 days after inoculation with 1.3×10^7 CFU of *M. pulmonis* (M_2 strain). Epithelial cells of bronchial wall are disoriented, giving a squamoid pattern. Purulent exudate is present in the lumen (*L*). (H&E, × 200)

Figure 8—End stage bronchus from HMPA-treated PF rat which died on day 22 after inoculation with 1.3×10^7 CFU of *M. pulmonis* (M_2 strain). Bronchial epithelium has been partially destroyed and replaced by collagenous connective tissue which almost completely fills the lumen (*L*) except for a small central focus of purulent exudate. (H&E, × 80)

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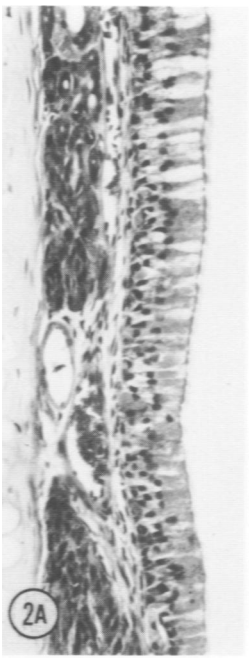

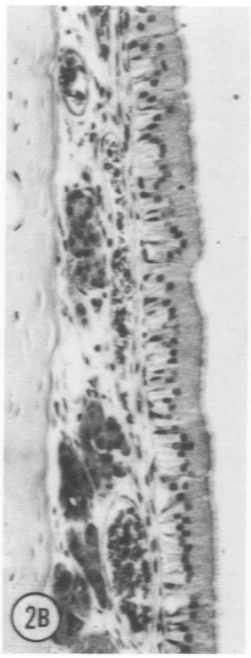

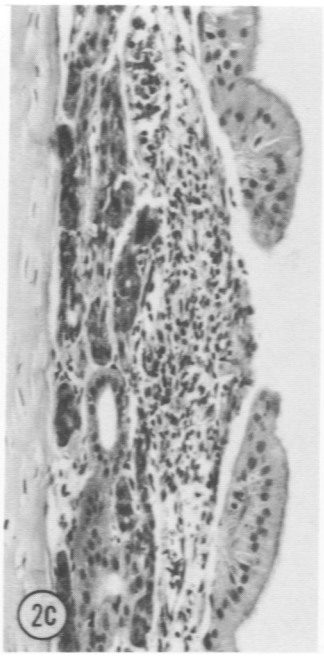

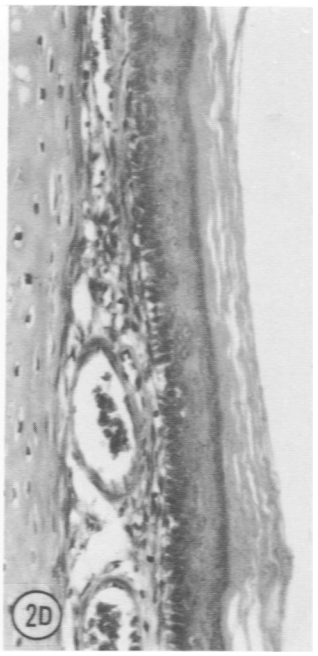

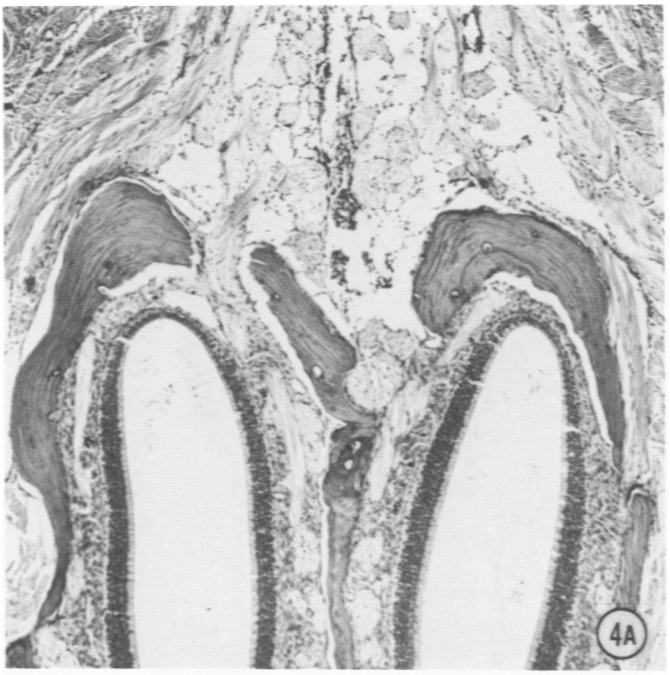
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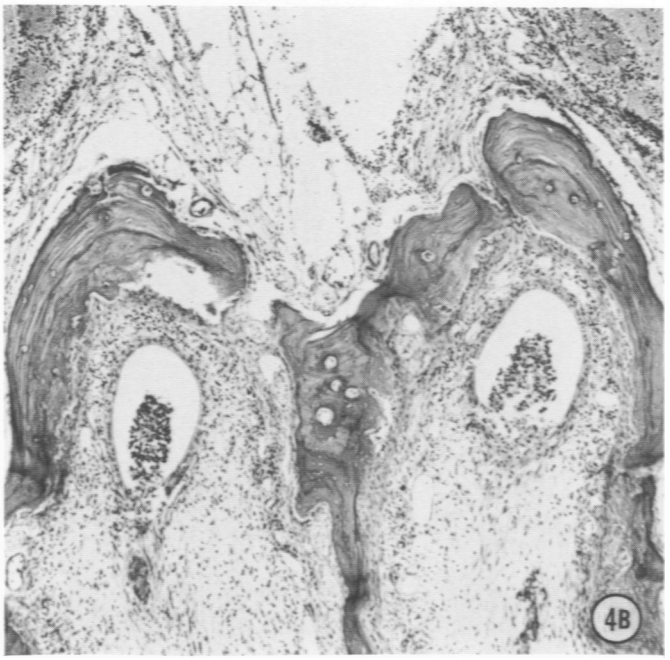
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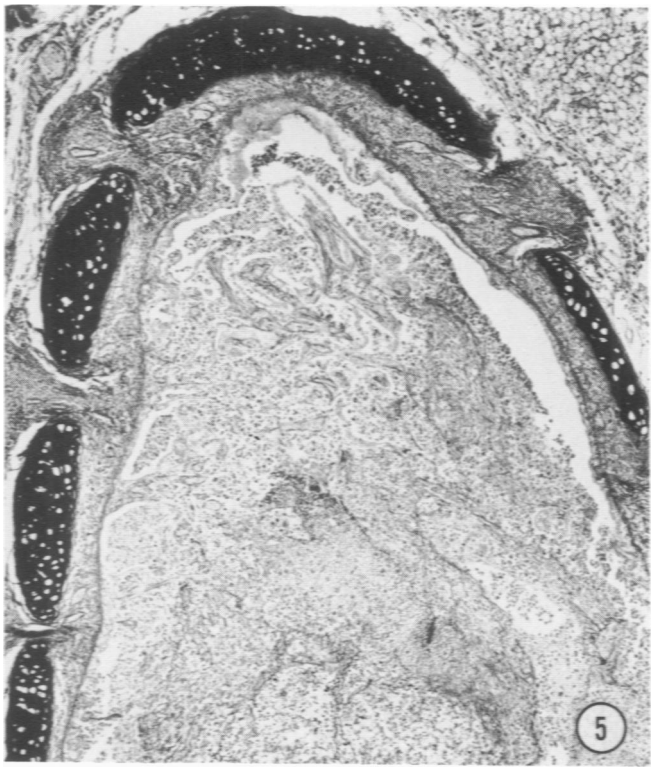
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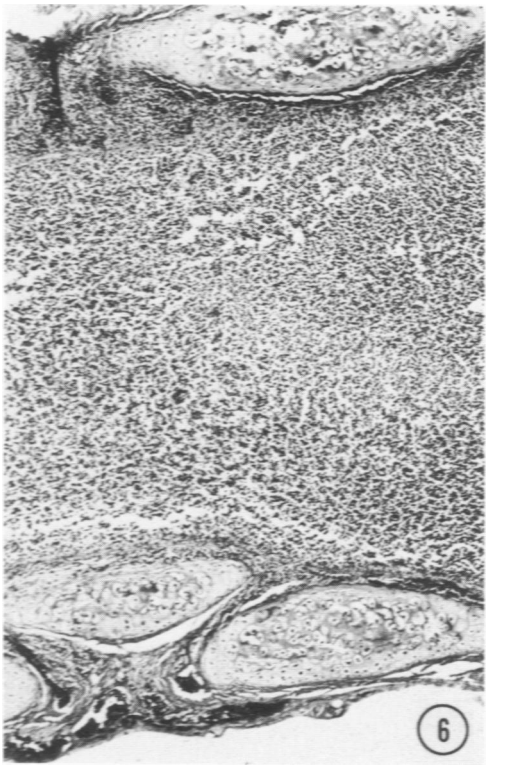
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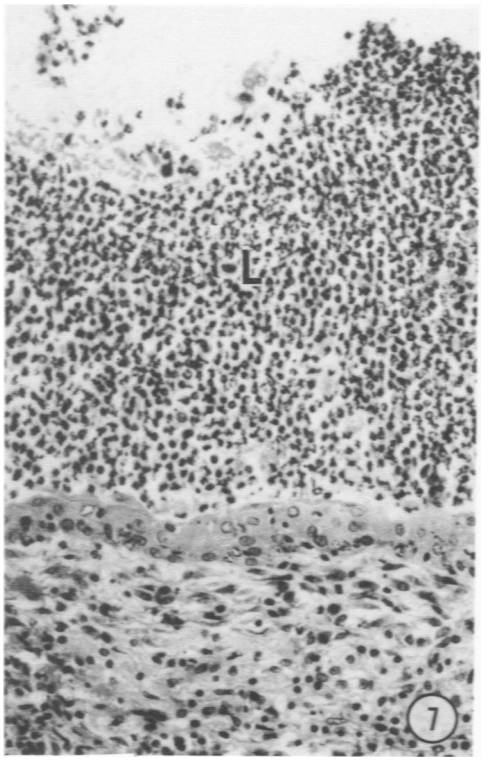
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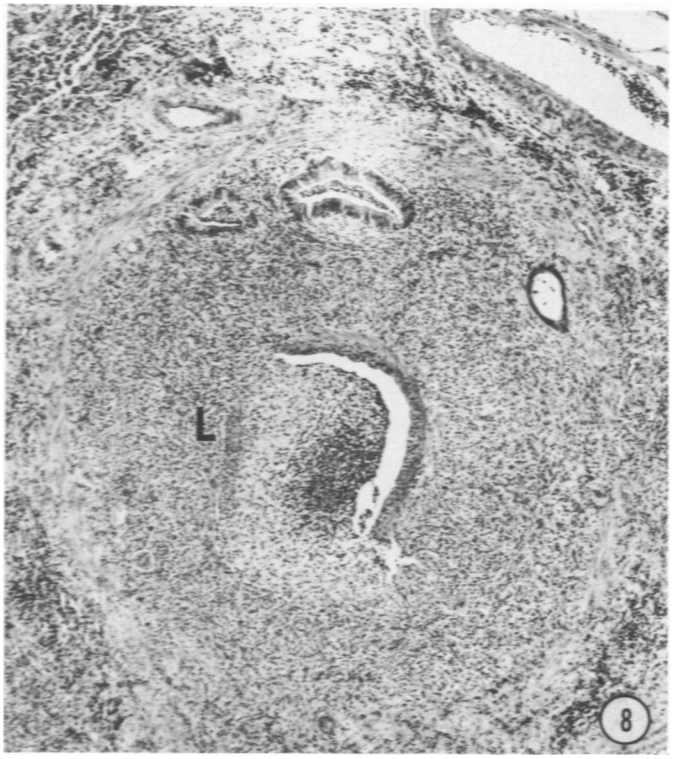
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