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Infectious Bronchitis and Mixed Infections of *Mycoplasma synoviae* and *Escherichia coli* in Gnotobiotic Chickens

I. Synergistic Role in the Airsacculitis Syndrome

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The synergistic role of infectious bronchitis virus (IBV) and mixed infections of *Mycoplasma synoviae* (MS) and *Escherichia coli* (EC) in the airsacculitis syndrome was evaluated in gnotobiotic chickens. Relative air sac lesion score indexes, in descending order of severity, from various combinations of organisms were: 9.5—IBV, MS, EC; 6.8—IBV, EC; 4.5—IBV, MS; 2.7—IBV; and 0.5—MS, EC. Infectious bronchitis virus caused a mild fibrinous inflammation. *M. synoviae* combined with IBV increased heterophilic and follicular lymphoid infiltration and mortality. *E. coli* combined with IBV increased exudation and prolonged airsacculitis. Concentrations of fibrinogen, gamma globulin, and total plasma proteins were elevated significantly by combined infections of IBV, MS, and EC ($P < 0.01$).

Inflammation of the air sacs of domestic fowl often accompanies infections of the upper respiratory tract. Severe and prolonged airsacculitis is attributed to the synergistic action of known and unknown combinations of microbial agents. Frequently, microorganisms of the indigenous host flora are among those isolated from chronic lesions (3, 12, 18).

Relatively nonpathogenic species of *Mycoplasma* and *Escherichia coli*, isolated from air sac lesions, have been shown to enhance and prolong inflammatory processes initiated by viral agents (5, 6, 7, 18). Infectious bronchitis virus has been incriminated most often as the inciting agent that permits infections by secondary invaders (1, 4, 7).

This study was initiated to evaluate the pathogenic and synergistic role of infectious bronchitis virus (IBV), *Mycoplasma synoviae* (MS), and *E. coli* (EC) in the etiology of airsacculitis in the absence of the indigenous host flora.

MATERIALS AND METHODS

Experimental chicks. Fertile eggs obtained from a commercial supplier (SPAFAS, Inc., Norwich, Conn.) were the source of specific pathogen-free White Leghorn chicks for the study. Chicks were housed in plastic film isolation chambers (Standard Safety Equipment Co., Palatine, Ill.) using gnotobiotic procedures previously described (13). The commercial ration (Ralston Purina Co., St. Louis, Ill.) was sterilized by irradiation with 3,000,000 rads from a Co^{60} source. Six experimental groups were used (Table 1).

Microbial treatments. All microorganisms were isolated from field outbreaks of respiratory disease in broiler chickens. The microbial association of all agents with chicks was established by the intranasal route, with bilateral administration of a drop of infective fluids.

M. synoviae, isolate F-10-2, was provided by H. W. Yoder, Jr., Athens, Ga., for this study. Suspensions of the organism in Frey medium, with a titer of $10^{4.5}$ colony-forming units per ml after 18 passages, was administered on the first day.

E. coli, serotype O111ab:K58:NM, with more than 10^8 living cells per ml of nutrient broth medium, was given on day 3.

A Massachusetts type infectious bronchitis virus with a mean lethal dose (LD_{50}) of $10^{7.8}$ after approximately 30 chicken embryo passages was given on day 5 in infected allantoic fluids.

Plasma proteins. Heparinized blood samples were obtained by cardiac puncture for plasma protein fraction evaluation from all chicks prior to necropsy. Total plasma fractions were separated by cellulose acetate electrophoresis using a constant voltage of 300 V for 45 min (17).

Necropsy. Five or more chicks per group were examined on day 10 and weekly thereafter for 4 weeks for clinical signs, gross lesions of the respiratory system, and histologic examination of the lungs, trachea, and air sacs. Air sac lesions were classified as follows: score 0, absence of grossly discernible pathological changes; score 1, mild serous or frothy exudation and/or mild clouding of the thoracic air sacs; score 2, small accumulation of caseous exudate in thoracic air sacs and/or extensive frothy exudation in abdominal air sacs; score 3, extensive accumulation of caseous exudate and/or thickening of air sacs.

Tissues were fixed in 10% formalin, sectioned in paraffin, and stained with hematoxylin and eosin (14).

TABLE 1. *Clinical signs and mortality in gnotobiotic chickens with mixed infections of IBV, MS, and EC*

Group no.	Microbial agents	No. of chicks per group	Day of each death	Clinical signs	
				First day	Last day
1	None	35	0	0	0
2	IBV	45	0	8	12
3	IBV, MS	33	11, 12, 12	8	15
4	IBV, MS, EC	30	13	8	16
5	IBV, EC	31	12	8	15
6	MS, EC	30	0	0	0

Lesion score index. A lesion score index was calculated as a measure of duration and severity of air sac lesions produced by each combination of microbial agents. The lesion score index for each experimental group is the sum of the products of each weekly lesion score mean and the week evaluated, i.e., week 1 lesion score mean × 1 + week 2 lesion score mean × 2, etc.

Statistical analysis. Analyses of variance with specifically designed orthogonal comparisons between treatment group means were conducted to determine statistical significance for synergistic effect of microbial agents on levels of plasma proteins.

RESULTS

Clinical signs. Clinical signs were evident only in groups infected with infectious bronchitis virus. The duration was prolonged by mixed infections (Table 1). Overt signs in all groups included snicking with a mild serous nasal discharge and an occasional but infrequent gasping reflex accompanied by extension of the head and neck. Some dyspnea with abdominal breathing was observed, more frequently in groups with mixed infections. Mortality occurred earlier and was higher among chicks infected with IBV and MS.

Gross pathology. The incidence of mild airsacculitis was higher in chicks infected with only IBV (Table 2). When chicks were infected with either MS or EC and IBV, airsacculitis was characterized by increased exudation. Increased inspissation of the exudate and thickening of the air sacs accompanied infections of EC and IBV.

Higher initial lesion scores with earlier resolution of lesions resulted in chicks receiving IBV and MS than in chicks receiving IBV and EC. An additive effect on severity and duration of lesions resulted from the combined infection of three microorganisms (Table 3).

Histopathology. In the absence of other microorganisms, IBV infections of the trachea initially produced severe hypertrophy of the epithelial layer without loss of cilia, but with mild fibrinous exudation. An infrequent diffuse mononuclear cell infiltration increased and by the second week was accompanied by some heterophilic and lymphocytic infiltration. In combined infections of MS or EC and IBV, heterophilic infiltration was more extensive and occurred earlier.

Both a diffuse cellular and follicular lymphocytic infiltration were observed in the mucosa of the bifurcations of the mesobronchi of chicks administered microorganisms. Follicular lymphoid infiltration occurred more extensively in chicks infected with MS and IBV. An infrequent diffuse infiltration of lymphocytic cells was not observed in the mucosa of the mesobronchi in germfree chicks until week 4.

IBV infections resulted in some edema of the air sacs accompanied by mild heterophilic and mononuclear cell infiltration and mild fibrinous exudation. Edema and exudation were enhanced by infections associated with EC and IBV. Epithelial cells lining the air sacs appeared more cuboidal in edematous and thickened air sacs. Heterophilic infiltration was more extensive in air sacs after MS and IBV infections (Fig. 1-9).

TABLE 2. *Incidence of lesions scores[a] and lesion score indices[b] of gnotobiotic chickens with mixed infections of IBV, MS, and EC*

Microbial agents	No. of chicks	No. with lesion score:			Lesion score index
		1	2	3	
None	29	0	0	0	0
IBV	41	12	3	0	2.7
IBV, MS	28	6	4	0	4.5
IBV, MS, EC	25	4	6	1	9.5
IBV, EC	26	5	3	3	6.8
MS, EC	25	1	0	0	0.5

[a] Based on scale of 0 to 3.
[b] Sum of products of weekly lesion score mean and week evaluated.

TABLE 3. *Weekly mean lesion scores[a] of air sacs in gnotobiotic chickens[b] with mixed infections of IBV, MS, and EC*

Microbial agents	Week postinfection evaluated			
	1	2	3	4
None	0	0	0	0
IBV	0.8	0.6	0.2	0
IBV, MS	1.0	1.2	0.2	0.1
IBV, MS, EC	0.8	1.5	0.8	0.8
IBV, EC	0.8	1.0	0.8	0.4
MS, EC	0	0	0.2	0

[a] Arithmetic mean based on scale of 0 to 3.
[b] Five or more chickens examined each week.

Plasma proteins. Levels of plasma protein fractions varied with combinations of microorganisms (Table 4). Levels of fibrinogen, beta globulin, and gamma globulin were significantly higher and alpha-1 globulin was lower in chicks associated with microorganisms than in germfree chicks. Levels of alpha-1 globulin were significantly higher in IBV-associated chicks than in chicks with other microorganisms, but similar to levels in germfree chicks. Levels of albumin and alpha-2 globulin were higher in chicks associated with EC than with

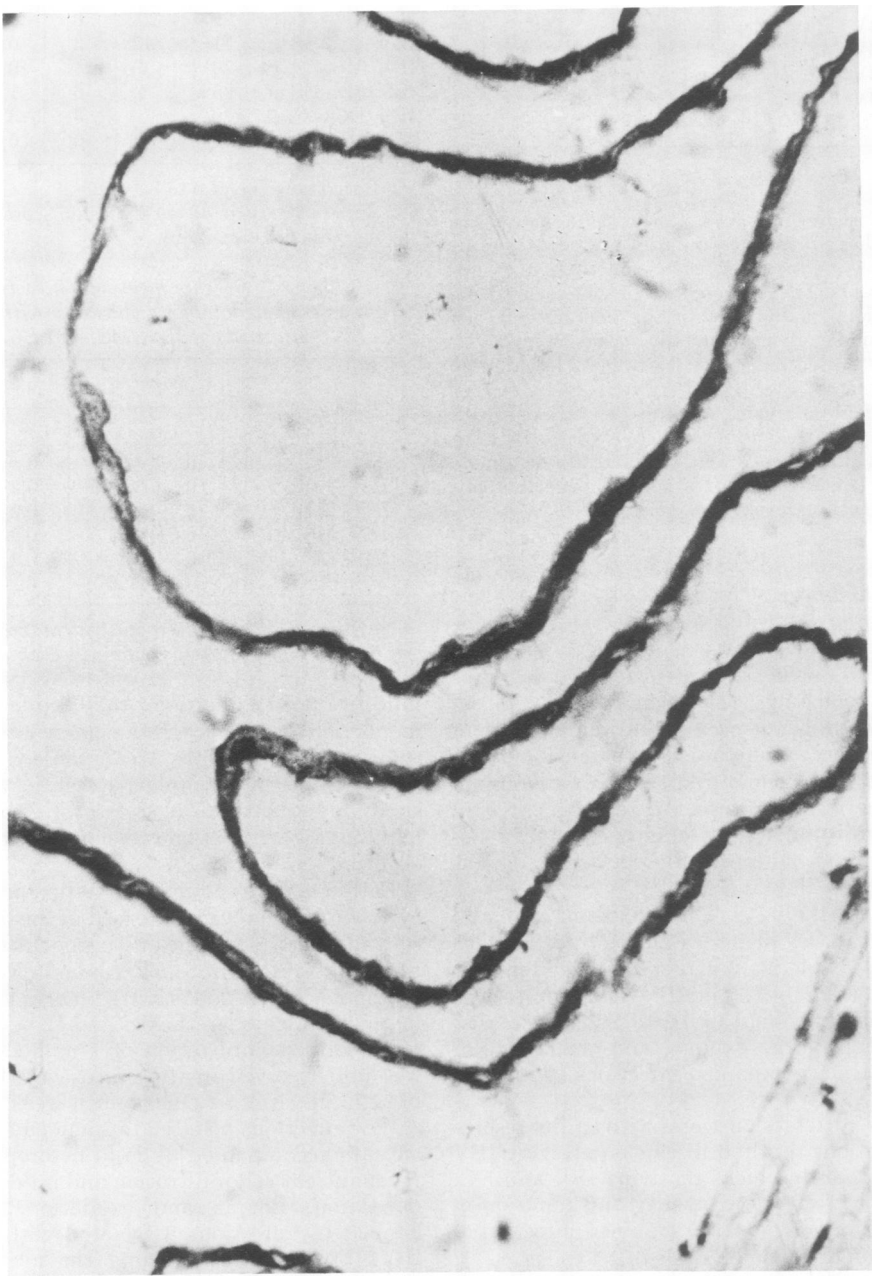

FIG. 1. *Air sac of germfree chicken, day 10, epithelial layers of peritoneal and endodermal sides in close apposition.* ×480.

combinations of IBV and MS, but were significantly higher only in chicks associated with both EC and IBV when compared with levels from chicks associated with both MS and IBV. When the associated microorganisms were IBV combined with both EC and MS, concentrations of total proteins, gamma globulin, and fibrinogen were elevated significantly ($P < 0.01$).

DISCUSSION

To simulate natural field conditions, a flora of the least pathogenic microorganisms, EC and MS, was established before the IBV infection.

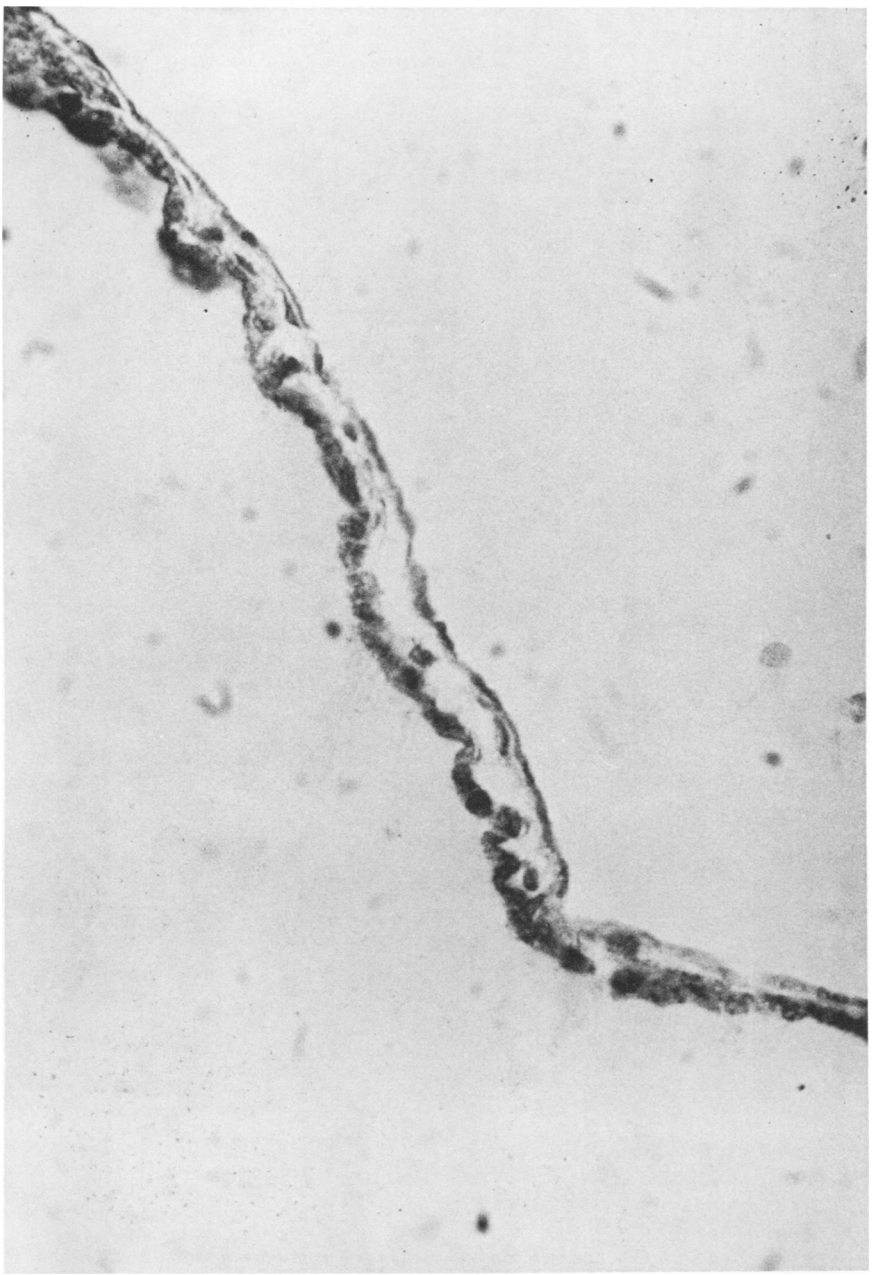

FIG. 2. *Air sac infected with IBV, day 10, some thickening from edema.* ×480.

Previous studies have shown that the inflammatory response is increased if EC and MS are administered shortly after the onset of infection by respiratory viruses (6, 8). A 2-day interval between the administration of each microbial agent was arbitrarily used to reduce stressing of the young chicks.

In the absence of the indigenous host microflora, infectious bronchitis virus was a primary pathogen of the air sacs, and its role as an

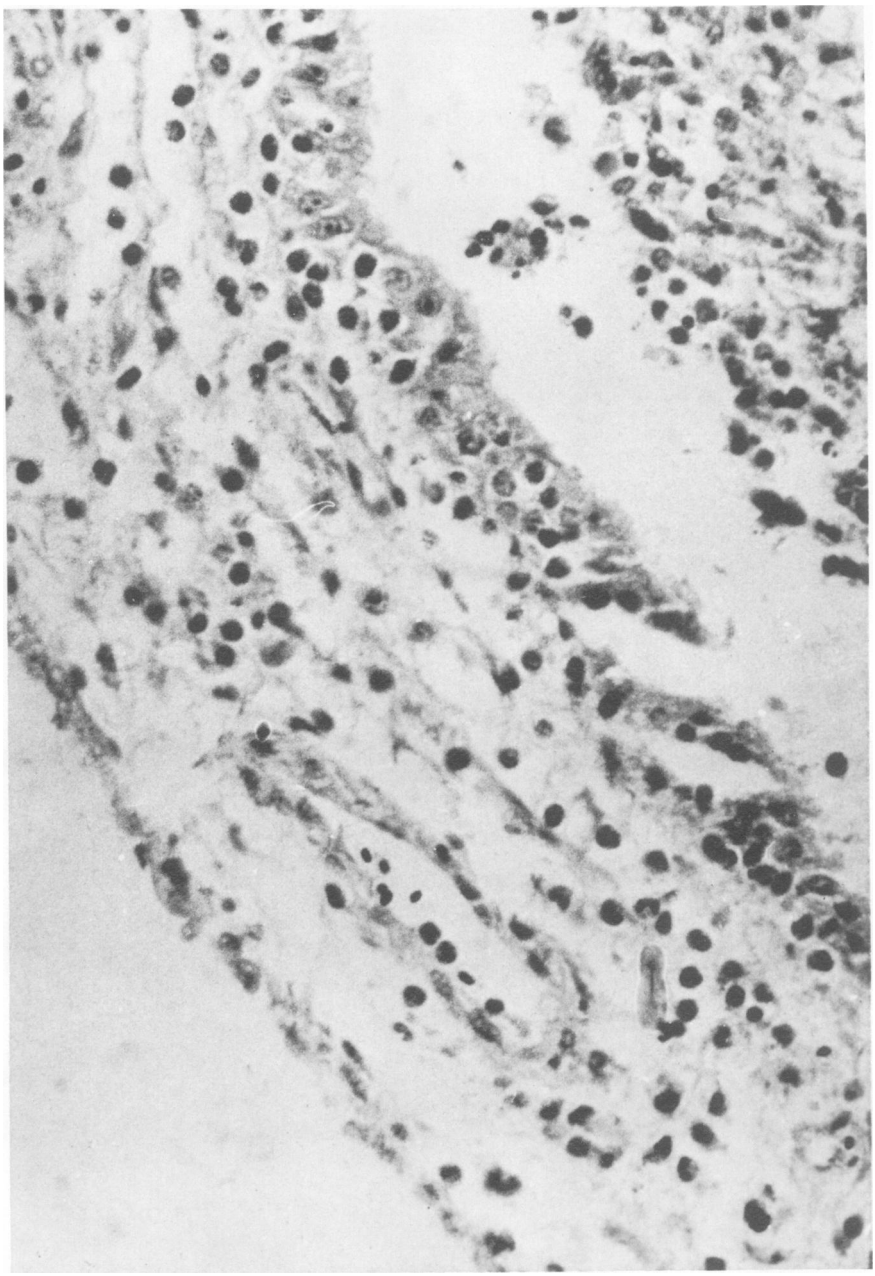

FIG. 3. *Air sac infected with IBV and MS, day 10, distended from edema and a predominant infiltration of heterophiles.* ×480.

inciting agent to allow relatively nonpathogenic organisms to enhance inflammation was confirmed. The ability of EC and MS to exaggerate disease caused by other infective agents has been reported previously (5, 6, 8, 10, 11, 13). A synergistic but different role for both MS and EC in the airsacculitis syndrome was demonstrated.

E. coli serotypes of the somatic antigen groups 1, 2a, and 78, also called the "coli-septicemia" group, are frequently among the coliform organisms isolated from airsacculitis le-

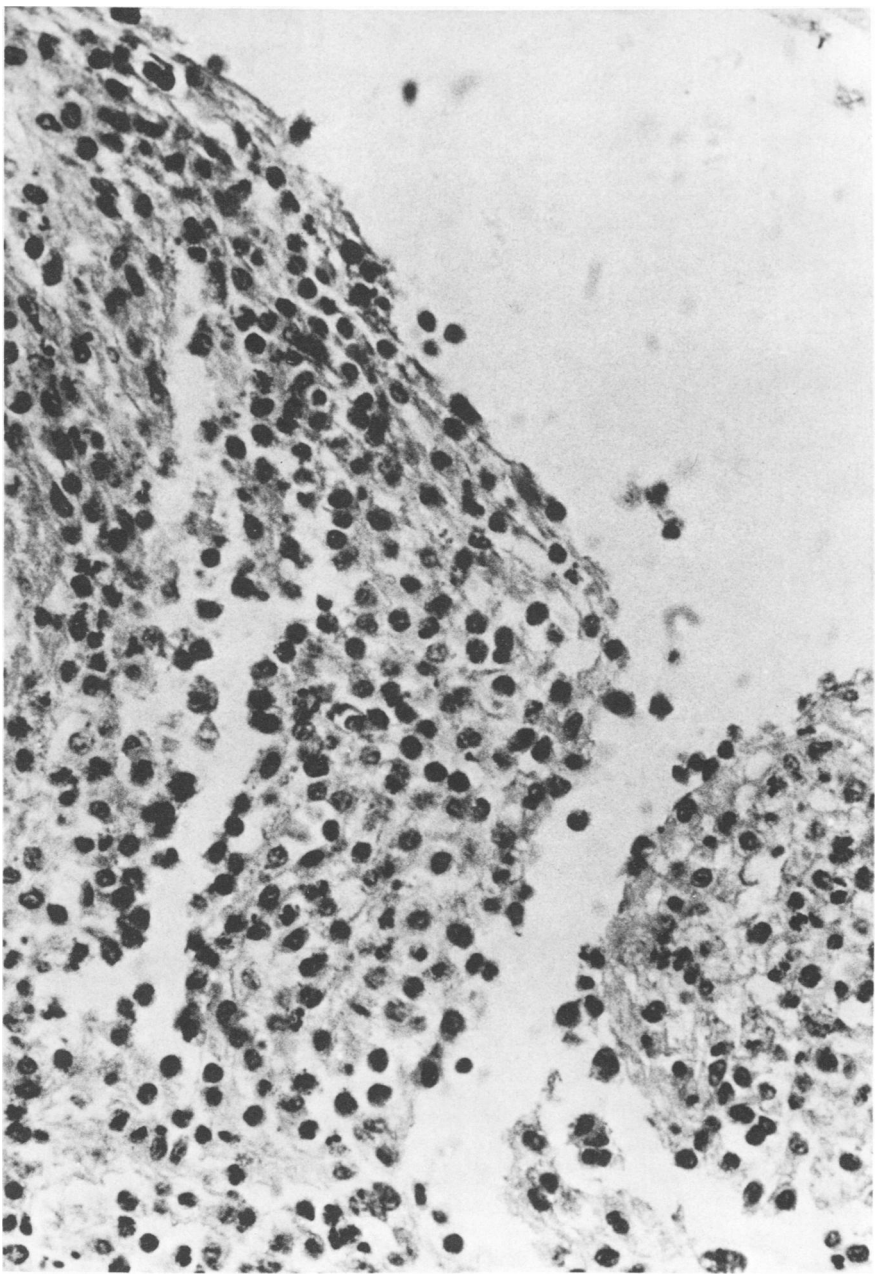

FIG. 4. *Air sac infected with IBV and MS, day 17, edematous with extensive infiltration of heterophiles and some mononuclear cells.* ×480.

sions. Infections by the coli-septicemia group are often systemic and characterized by a pericarditis and perihepatitis in addition to air sac lesions. Concomitant infections by these serotypes with *Mycoplasma gallisepticum* (MG) and/or viruses of the respiratory tract enhance inflammation of the respiratory tract.

The EC serotype used in this study has not been evaluated with MG. The low incidence of pericarditis and perihepatitis that occurred in mixed infections with IBV and MS indicates that it seldom causes a systemic infection and

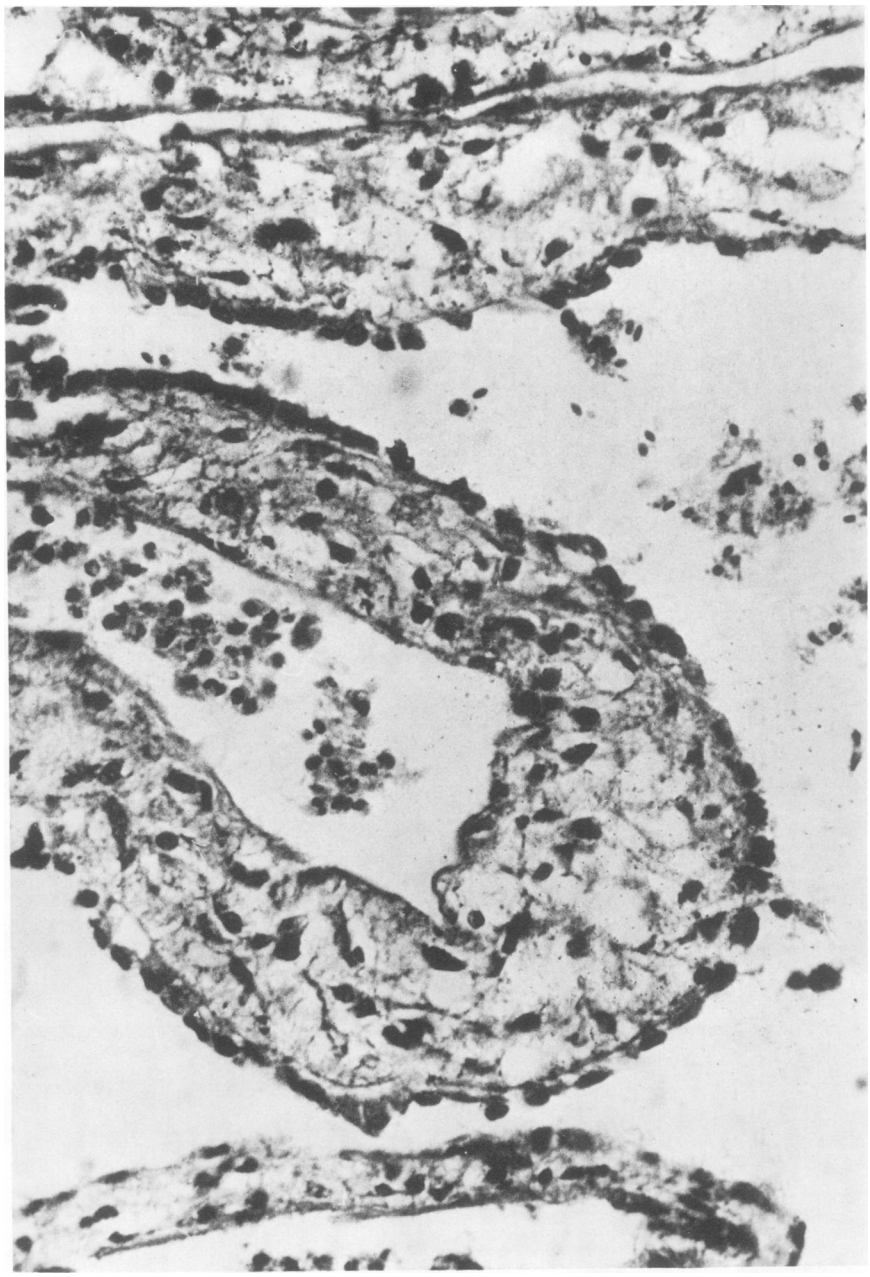

FIG. 5. *Air sac infected with IBV and EC, day 10, distended from edema and a predominant infiltration of mononuclear cells.* ×480.

would not be categorized in the coli-septicemia group. The synergistic role of enhancing inflammation of the respiratory tract initiated by a viral and mycoplasma agent as observed by others with the coli-septicemia group occurred with the serotype used in this study.

The role of MS in the airsacculitis syndrome is apparently of less significance than MG. Resolution of air sac lesions from MS seems to occur earlier in the syndrome than lesions from

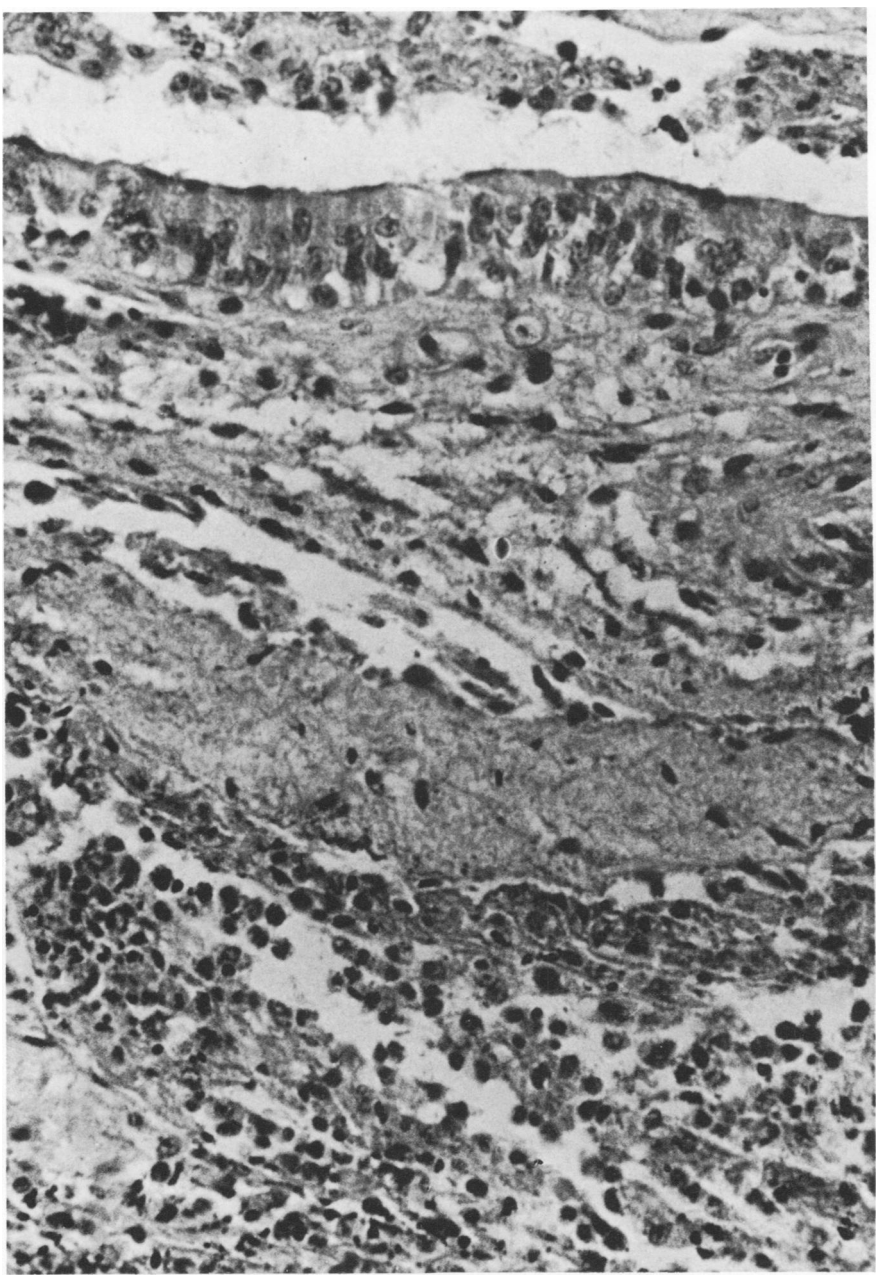

FIG. 6. *Air sac infected with IBV, MS, and EC, day 17, inspissated exudate containing macrophages, degenerating heterophiles, and mononuclear cells surrounding air sac. Note laterally compressed epithelial cells on endodermal side appearing as columnar cells.* ×480.

MG (9). Attempts to reproduce chronic airsacculitis in conventional chickens with MS have been successful only when it was administered by aersol with viral infections (8, 15, 16). MG has been shown to cause severe airsacculitis in combined infections with EC in the absence of IBV (5, 6). MS enhanced and prolonged inflammatory processes only when combined with IBV, or with IBV and EC. It seems probable that bacterial agents contribute to prolonged airsacculitis associated with MS in commercial flocks.

The increased heterophilic infiltration observed in MS-infected respiratory tissues can-

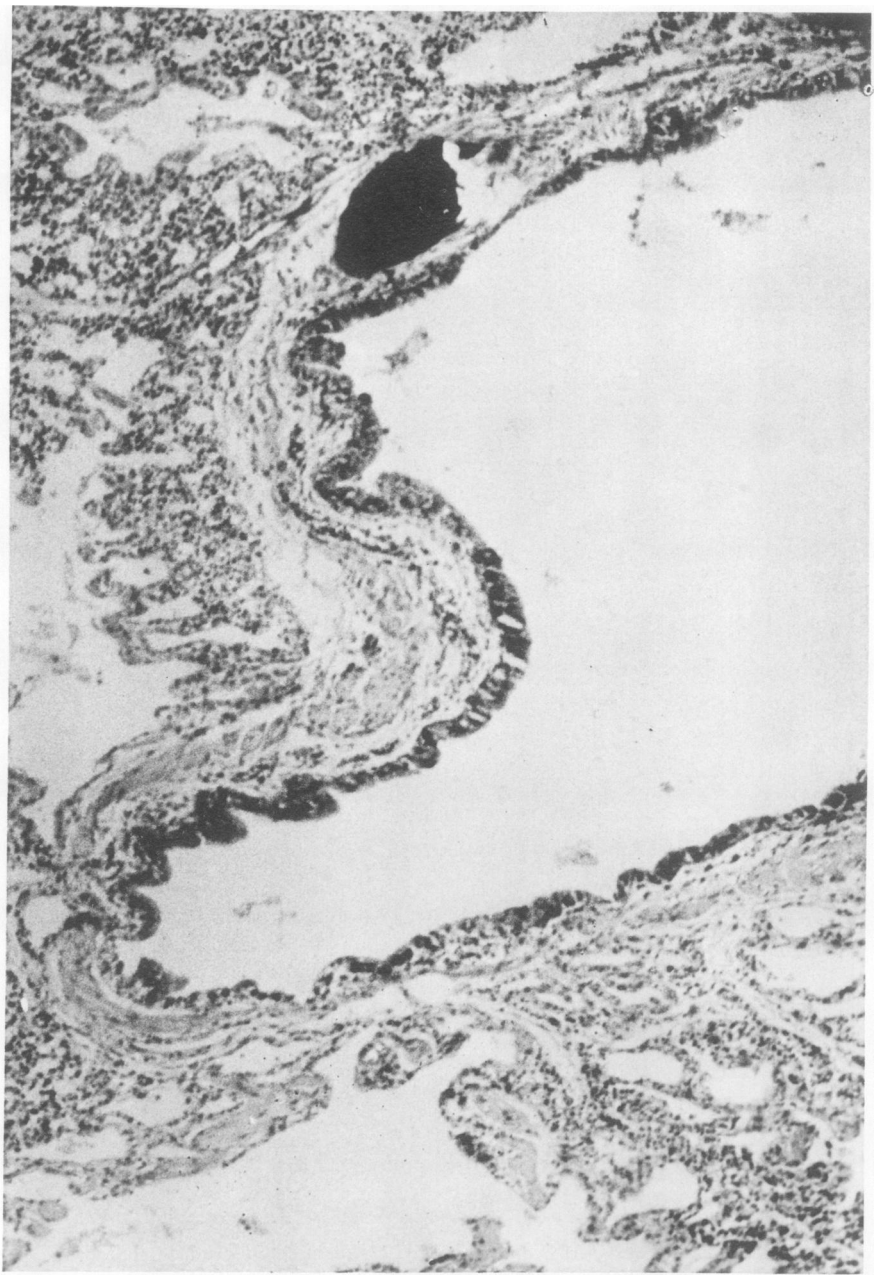

FIG. 7. *Mesobronchus of germfree chick, day 10, without mononuclear cell infiltration of submucosa.* ×120.

not be regarded as specific for MS infections. Others have described increased heterophilic infiltration as characteristic in air sac lesions of chickens infected with *Hemophilus gallinarum* and Newcastle disease (1, 7). Some degree of heterophilic infiltration was observed in lesions caused by all combinations of microorganisms in this study.

A corresponding increase in plasma fibrinogen occurred in groups with increased airsacculitis. Inasmuch as IBV infections are characterized by fibrinous inflammation, a decrease in

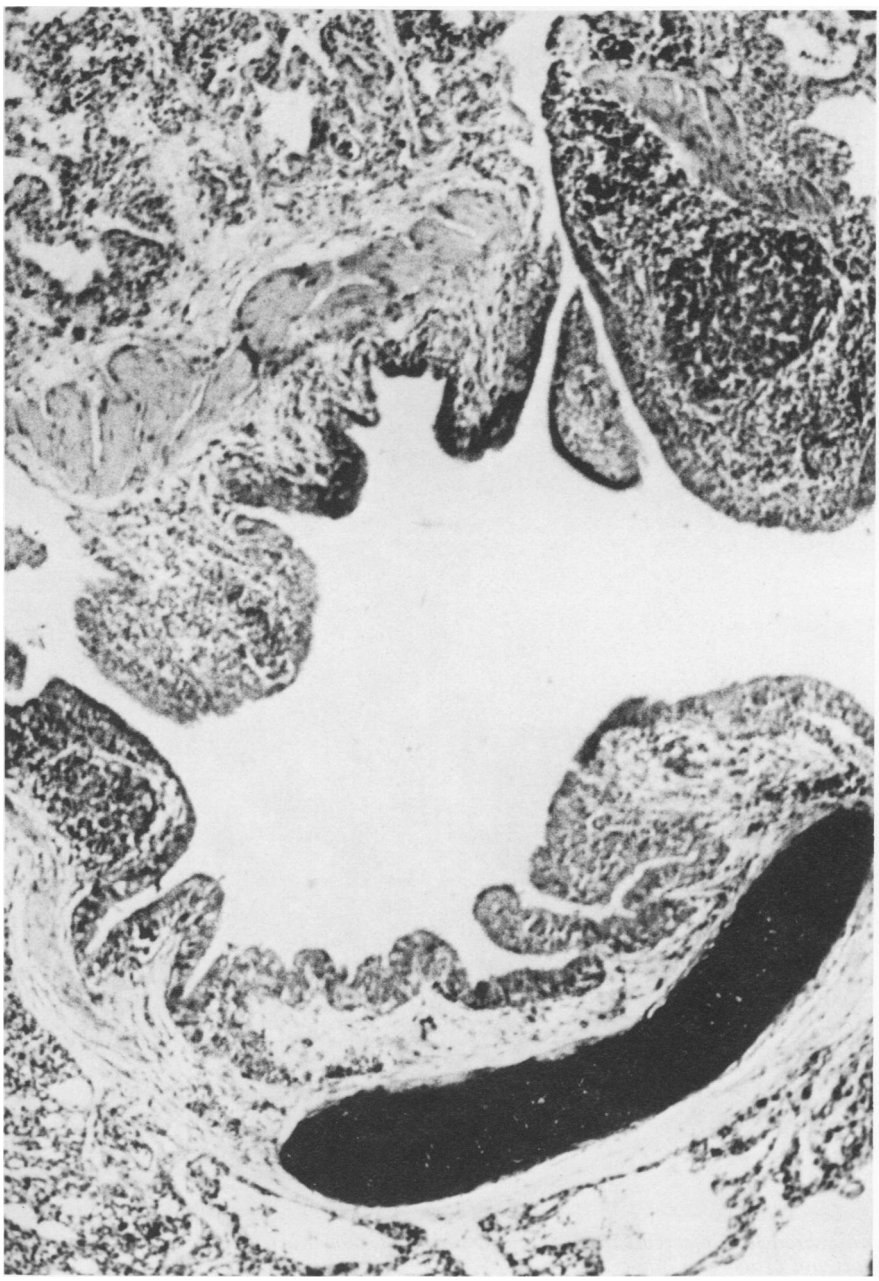

FIG. 8. *Mesobronchus infected with IBV and MS, day 10, extensive mononuclear cell infiltration and lymphoid follicle formation in submucosa.* ×120.

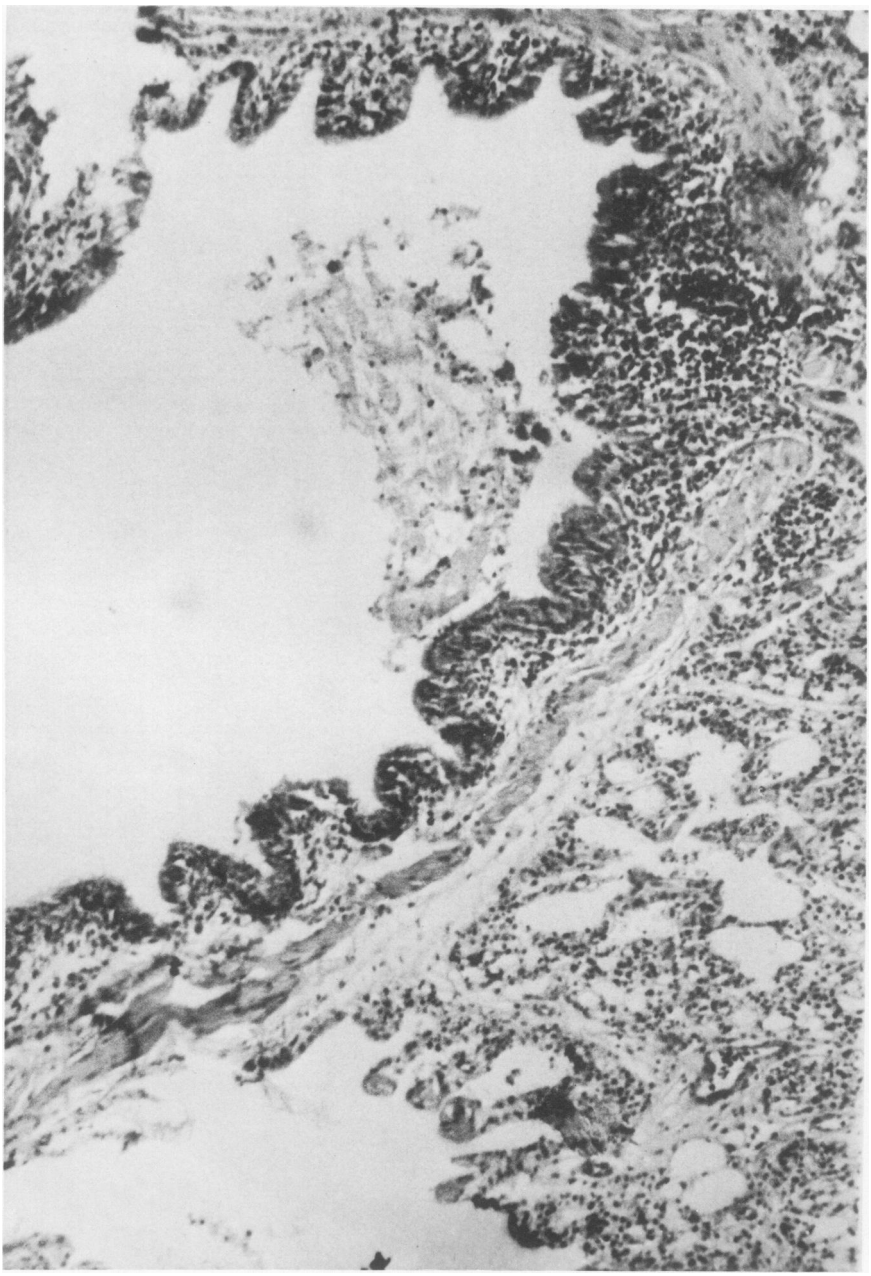

FIG. 9. *Mesobronchus infected with IBV and EC, day 10, mononuclear cell infiltration of submucosa, and cellular debri and exudate in lumen.* ×156.

TABLE 4. *Comparison of the influence of combinations of microbial agents on concentrations[a] of plasma proteins from gnotobiotic chickens*

Groups compared[b]	No. of chicks	Total plasma protein	Albumin	Globulins				Fibrinogen
				Alpha 1	Alpha 2	Beta	Gamma	
1 versus	5	3.5	1.6	0.39	0.27	0.39	0.31	0.59
2, 3, 4, 5, 6	123	3.6	1.5	0.31[c]	0.30	0.41[d]	0.40[d]	0.69[c]
6 versus	22	3.4[c]	1.6	0.28	0.24	0.39	0.30	0.56
2, 3, 4, 5	101	3.7	1.5	0.31	0.28	0.42	0.42[d]	0.72
2 versus	37	3.5	1.4	0.38	0.28	0.36	0.34	0.70
3, 4, 5	64	3.7	1.6	0.28[d]	0.27	0.45[d]	0.47[d]	0.74
4 versus	20	4.1[d]	1.6	0.30	0.29	0.49	0.56[d]	0.88[d]
3, 5	44	3.6	1.5	0.27	0.26	0.43	0.43	0.68
3 versus	23	3.5	1.4	0.24	0.23	0.42	0.46	0.72
5	21	3.8	1.7[c]	0.29	0.30[d]	0.47	0.40	0.64

[a] Adjusted mean values expressed in grams per 100 milliliters.

[b] Microbial agents associated with each group: 1, none; 2, IBV; 3, IBV, MS; 4, IBV, MS, and EC; 5, IBV, EC; 6, MS, EC.

[c] $P < 0.05$.

[d] $P < 0.01$.

levels of fibrinogen would be anticipated. Apparently, an overcompensation of the synthesis of fibrinogen results from protein loss by exudation.

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