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POSSIBLE INTERRELATIONSHIP OF MYCOPLASMA
GALLISEPTICUM AND THE AVIAN LEUKOSIS COMPLEX.

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Biochemistry

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POSSIBLE INTERRELATIONSHIP OF MYCOPLASMA
GALLISEPTICUM AND THE AVIAN LEUKOSIS COMPLEX

by

Sol Katzen

A Dissertation Submitted to the Faculty of the
COMMITTEE ON AGRICULTURAL BIOCHEMISTRY AND NUTRITION

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1970
I hereby recommend that this dissertation prepared under my direction by Sol Katzen entitled Possible Interrelationship of Mycoplasma Gallisepticum and the Avian Leukosis Complex be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

Dissertation Director Nov 21, 1969

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:

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Nov 21, 1969

Nov 21, 1969

Nov 21, 1969

Nov 21, 1969

*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.
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SIGNED: [Signature]

[Name]
ACKNOWLEDGMENT

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co-piloted on Mooney N 2669 Whiskey when the occasion demanded. Who could ask more?

Perhaps only those who have been through this can know why wives and children are so often honored in the acknowledgment of a doctoral dissertation. During the time we prepare for the writing of this page our wives are widows and our children orphans. Can less be done than to say 'thank you' for the patience, kindness, understanding (sometimes), and love (always) to Gaya, my wife, Dahn, my son, Ronit, my big girl and Tamar, my baby girl.
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ABSTRACT

A neural and lymphoproliferative form of the avian leukosis complex known as Marek's disease causes high mortality in chickens. Three field experiments were instituted to determine whether an interrelationship exists between Mycoplasma gallisepticum and certain forms of the avian leukosis complex.

In experiment 1, day old chicks were inoculated with macerated tumor suspension and various combinations of living mycoplasma, heat killed mycoplasma, killed mycoplasma supernatant and phytohemagglutinin. The results indicated that onset of mortality due to induced Marek's disease could be delayed by application of any of the mycoplasma treatments.

In experiment 2 chickens 13 to 18 weeks of age showing pronounced symptoms of Marek's disease were randomly divided into three treatments. The treatment injected with live mycoplasma had a 50% survival at 4 weeks. Birds of the untreated control and the mycoplasma supernatant treatment had 7% and 0% survivors respectively during the same period. Mortality differences between the live mycoplasma group and the other two groups were significant at the 0.05 level of probability.
The use of a high lysine: arginine ratio diet to possibly simulate the arginine depletion effect of mycoplasma was the purpose of experiment 3. Three hundred chicks were randomly allotted to three treatments of 100 birds each. All of the chicks were inoculated with heparinized blood of Marek's disease infected pullets. Lot 1 received a normal diet. Lot 2 received the high lysine diet. Lot 3 received a normal diet but was infected with live *Mycoplasma gallisepticum*. The high lysine group suffered 0% mortality. The control group mortality rate was 1% per day beginning 2 weeks post inoculation and the mycoplasma group suffered heavy mortality attributed to the mycoplasma infection during this 5 week experiment.

*Mycoplasma gallisepticum* or a high lysine: arginine ratio diet have preventative or ameliorative effects on Marek's disease infection. The mechanisms may be similar since each treatment may exert an effect on arginine at the cellular level.
CHAPTER 1

INTRODUCTION

The avian leukosis complex is generally divided into two major neoplastic-like diseases known variously as lymphoid leukosis, visceral lymphomatosis, myeloid leukosis, nephroblastoma, osteopetrosis, etc., as symptomatically descriptive terminology for what we will denote as lymphoid leukosis. The second form has been called acute leukosis, neurolymphomatosis, ocular lymphomatosis and Marek's disease. Marek's disease (probably the most accepted terminology for the acute form of the leukosis complex), is usually associated with young chickens prior to the onset of egg production and is believed to be caused by a DNA virus. In contrast, lymphoid leukosis is caused by an RNA virus and generally occurs after the birds are sexually mature. Marek's disease is highly contagious and constitutes the major uncontrolled avian health problem in the United States (and possibly the world) today.

A rather arbitrary diagnostic criterion using the age of the bird to differentiate lymphoid leukosis from Marek's disease has become common practice because of the similarity of clinical symptoms. When tumors occur on the liver, spleen, kidney, heart, proventriculus,
and other specifically visceral locations, the two diseases may be indistinguishable except by age of the bird. Tumor involvement of the nerves, skin and gonads are generally associated with Marek's disease while bursal tumors are assigned to lymphoid leukosis.

Considerable overlapping of the two diseases may further complicate the diagnostic picture. Also contributing to the confusion is the fact that various isolates of Marek's disease may affect different organs. Genetic resistance to Marek's disease has been demonstrated by the selection of resistant and susceptible lines. Whether or not these genetic factors are applicable to all variants of the Marek's agent(s) is as yet undefined.

At various times in the short history since Marek's disease has been separated from the rest of the avian leukosis complex, attempts have been made to identify "vectors" of the Marek's agent. At one time, the Darkling Beetle was considered to be the vector because these insects often were present in large numbers during an outbreak of Marek's disease. Their presence, however, now seems to be unrelated to the incidence of the disease.

Coccidiosis was also thought to be related to Marek's disease but evidence now seems to indicate that impairment of the development of mature lymphoid cells by the Marek's agent could contribute to a lowered resistance to coccidiosis and other diseases. What was once believed to be a cause may really be an effect.
Attempts to control Marek's disease outbreaks by sanitation have generally been ineffective. The "new house syndrome," where Marek's disease losses are often highest in the first flock in a new house as compared with subsequent flocks in the same house is a case in point. The above observation has led to experimentation with "controlled exposure" or "natural exposure" whereby day old chicks are introduced into a deliberately contaminated poultry house. Often, litter from a house where Marek's disease has been a problem is used to "seed" litter where chicks are being started. Some success in reducing mortality from Marek's disease by the natural exposure method has been reported.

Another method along similar lines and carrying similar risks, involves inoculating breeder hens with a crude inoculum from artificially infected birds thereby inducing higher maternal antibody levels in the chicks. This might afford protection to the chick until the susceptible age has passed.

Avian encephalomyelitis virus vaccine has also been used to induce an immunologic response against Marek's disease in the susceptible chick. Although avian encephalomyelitis virus is not considered to be the causative agent of Marek's disease, it was believed that immunization for avian encephalomyelitis did provide protection against Marek's disease. The results with this technic have been highly variable.
A field observation by the author that the elimination of mycoplasma (PPLO) infection in large flocks of breeder hens was accompanied by an increase in the incidence of Marek's disease suggested that the treatment to eliminate mycoplasma or the elimination of mycoplasma itself could in some way be related to the incidence of Marek's disease.

The work presented here was initiated to determine if an antagonistic relationship could exist between the two diseases. In this respect, it should be mentioned that after completion of most of the experimental work of this research, a report by the United States Department of Agriculture was published* which showed the causes of condemnation of poultry in inspected dressing plants. Although the report did not attempt to relate condemnations due to Marek's disease and mycoplasma infection, the apparent inverse correlation was so striking that a statistical regression analysis was done on the graphs by this author. The graphs are shown in Fig. 1.

With 25 degrees of freedom the student's t value from the table required -0.59 to assume an inverse linear relationship. By regions and for the United States as a whole, the correlations between mycoplasma caused condemnations and leukosis (Marek's disease) condemnations were as follows:

Fig. 1. Changes in incidence of USDA condemnations of broilers due to Marek's disease and *Mycoplasma gallisepticum*. 
South Atlantic — 0.96953
South Central — 0.80441
North Atlantic — 0.87776
United States — 0.95082

These correlations were highly significant within each region and for the United States.

Except for a report made on this research by Katzen, Matsuda, and Reid (1969), the literature contains no direct evidence that mycoplasma can affect the incidence of Marek's disease—a disease presumably caused by a highly cell-associated DNA virus. However, there is considerable evidence that a multitude of microorganisms exist simultaneously on and in living higher organisms and that proliferation of certain pathogenic types can occur when attempts are made to control other pathogens.

The interaction of tumor cells and tumor inducing agents with chemicals or microorganisms acting to suppress proliferation of tumor cells is the main topic of the literature review. Reference is also made to the possible biochemical pathways postulated by the cited authors.
CHAPTER 2

REVIEW OF LITERATURE

The etiology of Marek's disease, a highly contagious form of what was once known as the avian leukosis complex, has been well summarized and described in a recent article by Biggs, Churchill, Rootes, and Chubb (1968). Briefly, this disease affects chickens between the first few weeks of life until approximately 20 weeks of age and is characterized clinically by a proliferation of lymphoid tissue in the peripheral nerves and in many cases, in visceral organs. Biggs et al. (1968) indicated that as the disease progresses, the peripheral nerves become enlarged and lose their characteristic striations. The visceral organs often become gross tumors.

Outwardly, the birds may begin to show paralysis due to the nerve involvement, ruffled feathers, diarrhea, and general weakness. The time from first detection of symptoms until death of the bird is often less than one week. The disease almost invariably leads to the death of the individual.

Kottaridis (1969) described the various factors influencing the incidence and severity of Marek's disease. There is considerable circumstantial evidence that the agent responsible for this disease is a
herpes type virus (HTV). HTV have been demonstrated in tissue cultures from chickens clinically affected with Marek's disease by Churchill and Biggs (1967); Nazerian, Solomon, Witter, and Burmester (1968) and Committee Report (1967). Biggs, Churchill, Rootes, and Chubb (1968) have shown also that there is an excellent correlation between the presence of HTV in cell cultures and the cultures' ability to produce lesions of Marek's disease in susceptible chicks.

Electron micrographs have shown the presence of HTV particles in tissues from chicks showing symptoms of Marek's disease but such particles were not found in tissues obtained from apparently uninfected control chicks grown in isolation, Schidlovsky, Ahmed and Jensen (1969); Eidson, Richey, and Schmittle (1969). Both Schidlovsky et al. and Eidson et al. have pointed out that HTV particles similar to those found in Marek's infected birds have been reported in human cell cultures originating from African Burkitt lymphoma and some other human tumor diseases.

The Committee Report of the leukosis workshop (1967) also emphasizes the difficulty of characterization of the agent of Marek's disease. They conclude that although procedural uniformity of bioassay technic has yet to be achieved the in vivo assay is the mainstay of Marek's disease research. The response criteria suggested in this report include clinical signs, mortality and gross or microscopic lesions. The above, in addition to the recommended administration
of cell tumor suspensions or plasma via intra-peritoneal inoculation and a minimal two week holding period seem to be generally accepted by many investigators in the field of Marek's disease research and have been generally followed in this research also. An exception to the committee report was made in one experiment of these trials where infected birds were held longer than the 8 week maximum suggested to avoid the occurrence of contact-induced response.

Churchill and Biggs (1967) successfully transmitted Marek's disease and since that time extensive studies have been made on the stability of the pathogenic agent. Passage of certain isolates of the Marek's agent as much as 45 times did not diminish pathogenicity according to the studies of Eidson and Schmittle (1967). Their work with a strain of leukosis isolated in Georgia and designated as GA, showed that 73 to 90 per cent mortality with identifiable visceral tumors occurred by 2 weeks of age. Median day of death was 9 to 10 days post inoculation. Fresh tumor or plasma preparations were used as the inoculum since their studies and those of Solomon, Witter, Nazerian, and Burmester (1968) and Biggs, Thorpe, and Payne (1968) suggested that the agent was highly cell associated.

In more recent studies, Eidson, Richey, and Schmittle (1969), found that the agent could be filtered through a 0.45 micron membrane filter. These results provided further evidence that the agent was a virus size particle. In contrast to the apparent stability of the GA
isolate which is still routinely passaged in two to four week intervals. Eidson et al. (1969), Churchill, Chubb, and Baxendale (1969) reported on the attenuation of their Marek's agent isolate in successive passage in cell culture. The possibility of occurrence of a non-pathogenic mutation was discussed and evidence was also presented which indicated that the attenuated form could be used as vaccine for Marek's disease. They did recognize, however, that a contaminant may have influenced the apparent attenuation.

Jawetz, Melnick, and Adelberg (1968) point out the significance of microbial interrelationships in the development of diseases. Resident flora in their normal location may be beneficial to their host by preventing colonization by pathogens and possible disease by "interference." The resident flora may itself be pathogenic if introduced into foreign locations and if predisposing factors are present. A number of reports have appeared which show that mycoplasma can interact with tumor cells or tumor inducing agents. These studies have indicated that inhibition of tumor cell growth (or the inducing agent), can occur because mycoplasma and leukosis agents compete for the same binding sites on the cell, mycoplasma may act as an anti-mitotic agent or mycoplasma may deplete the environment of factors required by the tumor cell or agent.

A possible interaction between the myxovirus and Mycoplasma gallisepticum was indicated from in vitro serological studies by
Gesner and Thomas (1966) which suggested sialic acid as the red cell binding site for both organisms. These authors reported that pre-treatment of red cells with neuraminidase inhibited hemagglutination by *Mycoplasma gallisepticum*. Barile (1967) reviewed interactions between mycoplasma and animal cells.

Spitler, Cochrum, and Fudenberg (1968) in studying the effect of phytohemagglutinin induced DNA synthesis in goat lymphocytes presented evidence that *Mycoplasma arthritidis* may act as an anti-mitotic agent. In this experiment, the mycoplasma were previously killed either by lysis in distilled water or by heating at 60 degrees for one hour. The fact that inhibition of the phytohemagglutinin stimulation occurred with the addition of the mycoplasma as late as 45 hours after the addition of phytohemagglutinin apparently rules out the possibility of competition for combining sites on the cell. The authors suggest that some mechanism of inhibition other than competition for the same cell binding site such as direct interference with the synthesis of DNA and RNA, might be operative. Although the nature of the anti-mitotic agent was unknown, the authors did speculate that an arginine degrading enzyme might be involved.

Kraemer (1964) reported on the specific in vitro inhibition of certain murine lymphoma cells by a non-dialyzable substance in the medium in which certain variants of *Mycoplasma hominis* were grown. The cytocidal effect was prevented by the addition of l-arginine to the
medium, and he suggested that the mycoplasma may act by producing an enzyme that depleted the medium of l-arginine, which was required for growth of the tumor cells. A possible connection between the arginine depleting properties of _M. hominis_ and the anti-lymphoma properties attributed to guinea pig serum asparaginase was discussed. Old, Boyse, and Campbell (1968) conducted experiments with asparaginase on canine lymphosarcoma and suggested that the remission of tumors obtained under their experimental conditions may be due to a hitherto overlooked dependence of these tumor cells on extracellular asparagine.

Reports in the literature relating mycoplasma and bacteria to the metabolism of arginine as an energy source are plentiful. Kenny and Pollock (1963), Rouse, Bonifas, and Schlesinger (1963), Schimke and Barile (1963), van Demark and Smith (1964), Barile, Schimke, and Riggs (1965), and Schimke, Berlin, Sweeney, and Carroll (1966) have noted the presence of enzyme systems in mycoplasma that would tend to deplete the nutritional environment of arginine. Barile and Schimke (1963) developed a rapid test for detecting PPLO contamination of tissue cell cultures by assaying for the mycoplasma associated arginine deiminase activity.

In chicken kidney cells arginine depleting systems could be induced by a nutritional excess of lysine. Smith (1968), Nesheim (1968) showed that lysine: arginine ratios in excess of 1.3:1 in chicken diets
depressed plasma arginine levels. Kidney arginase levels were increased beginning 48 hours after starting a diet with a 2:1 lysine:arginine ratio although the almost immediate depression of plasma arginine levels was thought to be due to competition for a common (lysine, arginine, citrulline) excretion site in the kidney tubule. The effect of inducing an arginase system on the development of Marek's disease is reported in this study.
CHAPTER 3

EXPERIMENT 1. VARIOUS MYCOPLASMA TREATMENTS ON TUMORS INDUCED IN DAY OLD CHICKENS

Introduction

This trial was designed to test the effect of various Mycoplasma gallisepticum treatments on chicks inoculated intraperitoneally (I. P.) with a Marek's disease tumor suspension obtained from naturally infected birds. The basis for relating mycoplasma antagonism to virus induced tumor cell growth has been documented in the review of literature. Experiment 1 was limited to determination of the effect of (a) viable mycoplasma cells, (b) heat killed mycoplasma cells and (c) heated mycoplasma supernatant on the progress of Marek's disease induced in day old chicks.

No specific attempt was made in this trial to determine the mode of action of the mycoplasma. However, an attempt was made to determine if the sequence of injection of killed mycoplasma and tumor had any effect on the number of birds affected by Marek's disease. The studies of Spitler, Cochrum, and Fudenberg (1968) indicated that the timing of exposure of cells (in vitro) to the action of mycoplasma or mitotic agent was of importance. One of the treatments in this
trial involved the injection of phytohemagglutinin to test the effect of this mitotic stimulator in vivo.

Materials and Methods

**Mycoplasma gallisepticum Culture**

In order to secure an adequate supply of *Mycoplasma gallisepticum* for the in vivo experiments, seed culture obtained from A. T. C. C. (Culture #19610) was grown on a modified Hayflick Medium (1965). The initial transfer from the lyophilized state was made into test tubes previously prepared with a diphasic medium as follows:

- agamma horse serum 20%
- yeast extract 1:4 10%
- glucose (20%) 1%
- thallium acetate 1:4000
- procaine penicillin 6000 units/ml
- phenol red indicator

The above is added to the standard dilution of PPLO broth or agar (Difco Laboratories) in a 1:4 ratio. A 2 ml enriched agar slant in a test tube is then covered with approximately 5 ml of the enriched broth and the inoculum is introduced. The tubes are then incubated in a 5% CO₂ incubator at 37°C until a color change of the indicator (loss of the pink color) or a cloudy appearance of the medium indicates growth of the mycoplasma. The cultures are now used for passage to
500 ml culture flasks containing the diphasic medium as above. At this time, 0.1 ml of the broth is plated on a PPLO agar plate, previously prepared, for a colony count. The colonies are viewed and counted under low power of a light microscope after approximately five days of incubation in a high humidity incubator. The colonies show the typical "fried egg" appearance described in the literature.

After obtaining 1500 ml of mycoplasma broth, the culture was harvested by centrifugation in an automatic refrigerated Sorvall Centrifuge Model RC2-B at 20,000 rpm for 1 hour. Both the pellet and supernatant fractions were saved and treated as follows: One liter of the supernatant was heated at 57°C for 1 hour and labelled (a) mycoplasma supernatant. The pellets were pooled and resuspended in a total of 160 ml of supernatant. One hundred ml of the resuspended mycoplasma was heated in a water bath at 57°C for 60 minutes and labelled (b) killed mycoplasma. The remaining 60 ml of resuspended mycoplasma was labelled (c) live mycoplasma cells. All fractions were quick frozen in dry ice and acetone and stored at -20°C.

The original concentration of the live mycoplasma was $1 \times 10^9$ colony forming units (CFU) per ml. After inoculation was completed this figure was $1 \times 10^4$ CFU/ml on the identical PPLO medium. The cell concentration in the heated cell treatment was originally the same as that of the live mycoplasma treatment. Following injection of the birds with the various mycoplasma treatments, the heated culture, the
cell free supernatant and the live cell culture were plated separately on PPLO specific agar and non-specific bacterial nutrient agar. Only the material considered to be live mycoplasma yielded growth attributable to PPLO and all media were apparently free of other bacterial contamination.

Field Trial Studies

Eight hundred PPLO free day old white leghorn pullets were selected at random and divided into 8 treatments of 100 birds each. The chicks were housed in electrically heated batteries with raised wire floors until 3 weeks of age when they were transferred to floor pens allowing 1 square foot of floor space per bird.

All the pens were in the same house except for the pen holding the chicks injected with live mycoplasma. This pen (lot No. 8 in this experiment), was in a separate location to avoid cross contamination of mycoplasma to the other treatments. The duration of this study was 20 weeks and all dead birds were subjected to postmortem examination to determine cause of death. The experimental protocol for this experiment is shown in Table 1.

Results and Discussion

The cumulative weekly mortality values of the various treatments were compared to mortality values of the tumor only treatment (lot No. 2). The results are shown in figures 2 through 8.
TABLE 1. Experimental Protocol for Experiment 1.

<table>
<thead>
<tr>
<th>Lot</th>
<th>Treatment</th>
<th>Mycoplasma 0.2 ml I.P.</th>
<th>Tumor suspension 0.2 ml I.P.</th>
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<tr>
<td>1</td>
<td>Control—no tumor no mycoplasma</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>Tumor</td>
<td>---</td>
<td>3rd day</td>
</tr>
<tr>
<td>3</td>
<td>Killed mycoplasma</td>
<td>1st day</td>
<td>---</td>
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<tr>
<td>4</td>
<td>Killed mycoplasma plus tumor</td>
<td>1st day</td>
<td>3rd day</td>
</tr>
<tr>
<td>5</td>
<td>Mycoplasma supernatant 1st day plus tumor</td>
<td>---</td>
<td>3rd day</td>
</tr>
<tr>
<td>6</td>
<td>PHA(^b) plus killed mycoplasma plus tumor</td>
<td>1st day</td>
<td>2nd day</td>
</tr>
<tr>
<td>7</td>
<td>Reverse order (tumor at 1st day, killed mycoplasma at 3rd day)</td>
<td>3rd day</td>
<td>1st day</td>
</tr>
<tr>
<td>8</td>
<td>Live mycoplasma</td>
<td>1st day</td>
<td>3rd day</td>
</tr>
</tbody>
</table>

\(^a\)Tumor suspension was made by homogenizing tumorous spleen and liver tissue from Marek's diseased 12 week old leghorn pullets. The homogenate was suspended in normal saline solution with a tumor:saline ratio of 1:3. 6000 units of procaine penicillin per ml added. The suspension was frozen until used.

\(^b\)Phytohemagglutinin P 0.2 ml at 1st day (Difco Laboratories).
Fig. 2. Comparison of mortality due to Marek's disease, lot 2 (tumor) vs. 1 (no tumor, no mycoplasma).
Fig. 3. Comparison of mortality due to Marek's disease, lot 2 (tumor) vs. 3 (killed mycoplasma).

LOT NO. 2 INCLUDING AUTODYSED MORTALITY

LOT NO. 3

CUMULATIVE PERCENT MORTALITY DUE TO MAREK'S DISEASE
Fig. 4. Comparison of mortality due to Marek's disease, lot 2 (tumor) vs. 4 (killed mycoplasma plus tumor).
Fig. 5. Comparison of mortality due to Marek's disease, lot 2 (tumor) vs. 5 (mycoplasma supernatant 1st day, plus tumor).
Fig. 6. Comparison of mortality due to Marek's disease, lot 2 (tumor) vs. 6 (PHA plus killed mycoplasma plus tumor).
Fig. 7. Comparison of mortality due to Marek's disease, lot 2 (tumor) vs. 7 (reverse order, mycoplasma 3rd day plus tumor 1st day).
Fig. 8. Comparison of mortality due to Marek's disease, lot 2 (tumor) vs. 8 (live mycoplasma).
At 8 weeks, mortality due to Marek's disease occurred only in the treatment given tumor tissue alone. The deaths in the tumor only (lot No. 2) treatment occurred from the fourth week post-inoculation but did not exceed 6%, (3% confirmed, plus 3% probable), due to the low virulence of the tumor suspension inoculum. In treatments given tumor plus mycoplasma (Fig. 4), no tumor, no mycoplasma (Fig. 2), or mycoplasma alone (Fig. 3), no deaths occurred until the 10th week post-inoculation. Onset of mortality was delayed until the 11th to 15th week in the other groups Figures 5, 6, 7, and 8. Death loss at the 20th week post-inoculation varied from 17% in the live mycoplasma group (Fig. 8), to 46% in the tumor only group. The low virulence of the tumor suspension inoculum did not permit definite conclusions regarding the effectiveness of the various treatments.

It appears that cross contamination was responsible for the rapidly ascending mortality curve of lot 1 (Fig. 2), starting at the 10th week since lot 1 was not inoculated with tumor tissue. These data are in agreement with the leukosis workshop Committee Report (1967), that showed that natural cross contamination of birds might occur when experiments are carried out beyond 8 weeks.

At 20 weeks of age all lots except lots 6, 7, and 8 had approximately 40% mortality. Lots 6, 7, and 8 corresponding to Figures 6, 7, and 8 had final mortality figures of approximately 20%. The treatment where tumor was given at the 1st day and killed mycoplasma cell
at the 3rd day (lot No. 7, Fig. 7) did however, exhibit a delayed onset of Marek's disease and a lower total mortality than the mycoplasma at 1st day tumor 3rd day treatment in lot 4, Fig. 4.

The lack of high death loss within 8 weeks in the tumor only treatment (lot 2 of experiment 1) indicated the need for a more effective method of inducing Marek's disease tumors in baby chicks. A trial was conducted to test the effectiveness of transmitting Marek's disease by intra-peritoneal injections of 0.2 ml of whole heparanized blood from Marek's diseased birds to baby chicks. In this case blood samples from diseased chickens were collected and pooled just prior to injection. With the genetic line of poultry available for this study and the type of tumor inducing agent present in the donor chickens a mortality rate of 1% per day was reached between the 2nd and 8th week post inoculation. Total mortality in the test birds reached 40% at 8 weeks.
CHAPTER 4

EXPERIMENT 2. EFFECT OF MYCOPLASMA GALLISEPTICUM ON ADVANCED MAREK'S DISEASE TUMORS

Introduction

This study was designed to determine if mycoplasma cells or the medium in which they were grown contained a substance which would favorably affect birds with advanced Marek's disease.

Materials and Methods

Twenty white leghorn pullets were selected at 13 weeks of age from a 15,000 bird flock showing a daily mortality of thirty to forty birds due to Marek's disease. All of the birds selected were sufficiently advanced in the course of the disease as to be unable to stand, or to stand only with difficulty.

In the first trial, the birds were randomly divided into two treatments. After weighing and leg banding, ten of the birds were given a 6 ml intra-peritoneal (I.P.) injection of the mycoplasma consisting of the cell-free growth medium, heated mycoplasma (57° C for 1 hour) and live mycoplasma in a 4:1:1 ratio. (This material was left over from the earlier experiment.) This combination totaled 120 ml and had been maintained in a -20° freezer prior to use. The live
mycoplasma had $1 \times 10^9$ colony forming units (CFU) per ml on PPLO agar and showed no bacterial contamination when cultured on nutrient agar.

**Results and Discussion**

**Trial 1**

As shown in Table 2, the pullets injected with a combination of *Mycoplasma gallisepticum* cells and supernatant showed a 50% survival rate as compared with less than 10% in the uninoculated controls.

Postmortem findings on the individual birds showed lesions typical of Marek's disease involving nerves of the sciatic and/or brachial plexus plus one or more of the visceral organs. In the case of the one survivor of the eleven-bird control group, no Marek's lesions were detected at the time of sacrifice—four weeks from the start of the test. All of the survivors of the injected group showed the nerve lesions of Marek's disease but no involvement of any of the visceral organs. It was not possible to determine whether the visceral organs had ever been involved or whether regression had occurred.

Aside from the differences in mortality and postmortem findings between the inoculated and control groups, there were obvious improvements in the appearance of the injected birds approximately forty-eight hours post inoculation.
TABLE 2. Mycoplasma\(^1\) and Supernatant Injection of Pullets with Advanced MD Tumor.

<table>
<thead>
<tr>
<th>Lot</th>
<th>Trial</th>
<th>Number birds</th>
<th>Mortality 4 weeks post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (medium and cells)(^2)</td>
<td>11</td>
<td>10 a</td>
</tr>
<tr>
<td>2</td>
<td>Control (supernatant)</td>
<td>10</td>
<td>10 a</td>
</tr>
<tr>
<td>3</td>
<td>Control (medium and cells)(^3)</td>
<td>8</td>
<td>7 a</td>
</tr>
</tbody>
</table>

\(^1\)M. gallisepticum cells (ATCC #19610) grown by successive passage on synthetic media were sufficiently attenuated so as not to cause any noticeable disease symptoms.

\(^2\)6 ml/bird administered intraperitoneally.

\(^3\)10 ml/bird administered intraperitoneally.

\(^4\)Means not having common letter superscripts were significantly different at the 0.05 level of probability.
Trial 2

The injection material used for Trial 1 contained a mixture of live cells, heated cells and supernatant for *Mycoplasma gallisepticum*. Since the possibility existed of a soluble component present in the supernatant being the active material, it was decided to inject only the supernatant in Trial 2. The supernatant being relatively more plentiful than the cellular material, was used in larger amounts and in a larger number of birds.

There were no survivors ten days post inoculation in this trial. All the birds that died showed multiple lesions of Marek's disease and there were no differences between treatments in this trial. All birds showed involvement of some of the visceral organs (liver, spleen, ovary or proventriculus) as well as the brachial and/or sciatic nerves.

Trial 3

The results of trials 1 and 2 suggested that non-heated cellular material rather than a heated soluble extract was required to obtain a favorable response. This was confirmed by a third trial using the remaining mixture of live cells and supernatant.

Trial 3 resulted in a survival pattern very similar to Trial 1. Also, beginning approximately forty-eight hours post inoculation, the injected birds became noticeably more active than the uninoculated controls. There is evidence of a *Mycoplasma gallisepticum* heat labile
cell associated component that induces at least temporary and possibly permanent remission of symptoms of certain forms of Marek's disease in naturally infected white leghorn pullets.
CHAPTER 5

EXPERIMENT 3. EFFECT OF DIETARY LYSINE:ARGININE IMBALANCE ON MAREK'S DISEASE

Introduction

The data of experiment 2 strongly suggested that mycoplasma could in some way act on Marek's disease tumor cells or the agent responsible for this disease. In some respects, the results were analogous to those of Kraemer (1964), who found that certain strains of mycoplasma lysed murine lymphoma cells in vitro. In Kraemer's study, direct contact of mycoplasma to the lymphoma cell was not required for the lytic effect. Some toxic factor which would pass through a VM millipore filter (0.05 μμ) could effect lysis of the lymphoma cells but lysis was prevented by the addition of l-arginine to the cell culture medium.

Rapid metabolism of arginine is associated with mycoplasma growth and this characteristic has, in fact, been utilized as an assay tool for determining if tissue cultures are contaminated with PPLO, Barile and Schimke (1963). The arginine dihydrolase pathway as shown in Fig. 9, was studied by Schimke (1967). Evidence was presented to show that arginine desimidase, ornithine transcarbamylase
and carbamate kinase, the enzymes comprising the arginine dihydrolase system, constituted 16% of the total mycoplasma protein. This amount was considered sufficient to classify this pathway as a major source of ATP formation in mycoplasma.

![Chemical Reaction Diagram]

Fig. 9. The arginine dihydrolase pathway.

If arginase could be induced or derepressed in poultry by a nutritional excess of lysine as reported by Nesheim (1968), arginine would also be degraded to ornithine and thereby depressed in the plasma and at the cellular level. The fact that high levels of kidney arginase have been detected following feeding of excessive lysine: arginine ratios and growth has been reduced proportionately argues strongly for cellular arginine deficiency.

Experiment 3 was designed to determine whether decrease in plasma arginine as a result of high dietary levels of lysine would
effect the course of Marek's disease in young chicks inoculated with Marek's disease agent.

**Materials and Methods**

Three hundred white leghorn pullets were randomly allotted to 3 lots of 100 birds per lot. They were housed in floor pens 12' x 8' and brooded under a 1000 watt infra red brooder. Dry sand was used as litter. At 5 days of age all of the birds in the 3 lots were inoculated I. P. with 0.2 ml of fresh whole heparinized blood collected and pooled from Marek's diseased chickens. In the treatment using mycoplasma (lot 3), 0.2 ml *Mycoplasma gallisepticum* was injected I. P. 3 days after the tumor inoculation. Experimental protocol for experiment 3 is shown in Table 3.

**Results and Discussion**

The results of the plasma amino acid assay on the various treatments are shown in Table 4.

If the level of methionine is an indicator of the general nutritional status then it is possible to accept the differences between the treatments as due primarily to the variation of lysine in the diets. A high lysine: arginine ratio tends to depress the plasma arginine regardless of the level of arginine. These results were similar to those reviewed by Nesheim (1968). The birds infected with mycoplasma may have an elevated lysine level due to poor feed consumption.
TABLE 3. Experimental Protocol for Experiment 3--Lysine; Arginine Imbalance.

<table>
<thead>
<tr>
<th>Lot</th>
<th>Mycoplasma&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diet&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>normal feed</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2.5:1 lysine:arginine</td>
</tr>
<tr>
<td>3</td>
<td>0.2 ml</td>
<td>normal feed</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mycoplasma was grown as previously described except that whole cultures passed only 2 times were injected. The mycoplasma cells were not concentrated by centrifugation.

<sup>b</sup>The normal feed contained 1.27% arginine and 1.20% lysine by microbiological assay. The high lysine:arginine ratio feed was made by adding 2.5% of L-lysine monohydrochloride (80% L-lysine) to the normal feed to yield a diet with 3.2% lysine and 1.27% arginine.
TABLE 4. Plasma Amino Acid Levels\textsuperscript{a} of Birds on Normal Dietary Lysine:Arginine Ratios (1:1), High Dietary Lysine:Arginine Ratios (2.5:1), and Birds Injected with \textit{Mycoplasma gallisepticum} while Consuming a Normal Diet.

<table>
<thead>
<tr>
<th>Lot</th>
<th>Treatment</th>
<th>Arginine (mg per 100 ml)</th>
<th>Lysine</th>
<th>Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal feed</td>
<td>40</td>
<td>20</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>2.5:1 lysine:arginine feed</td>
<td>20</td>
<td>40</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>Mycoplasma + normal feed</td>
<td>40</td>
<td>35</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Birds were fasted for 10 hours, given feed free choice for one hour and the blood taken by cardiac puncture two hours after feed was removed. This was done in an attempt to standardize the nutritional status of the birds prior to sampling.
Table 5 shows the mortality, necropsy findings and body weight of the pullets 5 weeks post inoculation with whole heparinized blood of Marek's infected chickens.

The mortality value of treatment No. 3 (M. gallisepticum injected lot) was attributed to PPLO infection. The M. gallisepticum used for these inoculations was passed twice in the laboratory prior to inoculation and probably was not sufficiently attenuated to avoid causing the characteristic disease.

Since first PPLO mortality occurred 1 week after mycoplasma injection in treatment No. 3 and also because light respiratory symptoms were evident in the other two treatments, antibiotic therapy (erythromycin, 1 gram per liter of drinking water), was initiated. The antibiotic was administered only to the control and high lysine birds—not to those injected with mycoplasma.

As seen in Table 5, no mortality attributable to PPLO infection was noted up to 5 weeks in the treatments where mycoplasma was not injected although the lots were in relatively close proximity and the birds were not maintained in isolation. From a practical management point of view this demonstrates the effectiveness of specific antibiotic therapy in the control of specific diseases. Fig. 10 shows the data on Table 5 in graphic form as weekly mortality per lot.

In order to avoid the complications of cross-infection as appears to have occurred in experiment 1, the duration of this
TABLE 5. Results of Varying Lysine:Arginine Ratios and Mycoplasma Infection on Pullets Inoculated with Marek's Disease Agent.

<table>
<thead>
<tr>
<th>Lot</th>
<th>Treatment</th>
<th>Initial no. of birds</th>
<th>Mortality at 5 weeks post tumor inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>1</td>
<td>Normal feed</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>2.5:1 lysine:arginine feed</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Mycoplasma + normal feed</td>
<td>100</td>
<td>23</td>
</tr>
</tbody>
</table>
Fig. 10. Comparison of weekly mortality of tumor inoculated birds fed a normal diet, a high lysine:arginine ratio diet and a *Mycoplasma gallisepticum* infected group.
experiment was limited to 5 weeks. Although the overall mortality was not greatly different between the mycoplasma and control lots, deaths due to Marek's disease in the Mycoplasma gallisepticum infected lot was low or non-existent according to postmortem examination.

The results of feeding a high lysine diet to lot No. 2 (2.5:1 lysine:arginine ratio), indicated that at least for a short period mortality caused by Marek's disease was controlled by this feeding method. Since Marek's disease may be a limited time phenomenon during the growing period of the pullet, this may be a practical method for increasing survival rates.

One reason why reference is made to the short term effect of high lysine levels in the diet is the experience of medical investigators with various promising chemotherapeutic agents that eventually lose their effectiveness in the control of tumor growth. If this treatment does operate on the enzyme induction system as previously mentioned, it is possible that the results would be of a temporary nature.

Any system that would increase the amount of arginase would degrade arginine to ornithine (Fig. 11), and as a consequence more aspartic acid and/or asparagine would be consumed. More citrulline would also be removed but since ornithine transcarbamylase is lacking or deficient in the young chicken, ornithine would accumulate. The question then becomes whether or not, or when, the accumulation
Fig. 11. Possible explanation of asparagine depletion associated with arginine formation.
of precurser (ornithine) or the decrease of product (arginine) will induce or derepress the synthesis of ornithine transcarbamylase and "normalize" the tumor metabolism.

The concept of genetic resistance is generally accepted in reference to Marek's disease in the avian species. It is possible to relate genetics to nutrition if we accept the one gene, one enzyme concept of contemporary molecular biology. This would mean that, as an example, the essentiality of a certain amino acid in the diet of an animal would depend on the presence of an active gene that could produce the necessary enzyme (or enzymes), for the synthesis of that amino acid. Nesheim (1968) evaluated a high or low arginine requirement of certain genetic lines of chickens. This requirement is probably regulated by ornithine transcarbamylase (Fig. 11), although other enzyme systems may be involved. Conceivably, an enzyme that would degrade arginine, such as arginase, would also affect the requirement for that amino acid. The association of nutrition to genetics becomes quite involved.

Genetic resistance may apply to other forms of cancer in other species as well. This would mean that somewhere in the genome an inducer or derepressor was or was not acting to produce or not produce an enzyme or enzymes. The presence or absence of this enzyme(s) would possibly explain some of the in vitro or in vivo phenomena such as:
(a) Kraemer (1964) reported on a non-dialysable substance in a mycoplasma culture that caused lysis of certain murine lymphoma cells in a cell culture. Further, he found that the addition of arginine to the medium reversed the lytic effect of the mycoplasma substance.

(b) Sorof et al. (1967) found a liver protein fraction that bound with an azocarcinogen but would not bind to tumor tissue cells. The properties of this protein fraction were almost identical with arginase.

(c) Old et al. (1968) presented data on regression of lymphosarcoma in dogs (at least temporarily), by the injection of asparaginase. They suggest that the particular lymphosarcoma cells involved could not synthesize asparagine as can a normal cell and therefore asparagine became an essential amino acid for the tumor. Even though enough asparagine to support growth of the tumor was released by the surrounding normal tissue, the addition of asparaginase would so deplete the environment of asparagine that the tumor would regress.

(d) A biochemical pathway, the urea cycle, could be a link between the loss of arginine and the depletion of asparagine in a cellular system as per Fig. 11.

Possibly the effects noted by the various investigators and the author, such as in vitro cell lysis inhibition and the ameliorative effect
of *Mycoplasma gallisepticum* on avian tumors, the lytic effect of mycoplasma on murine lymphoma cells, the remission of lymphosarcoma tumors in the dog and the associative effect of arginase on Sorof's tumor inducing system are all due to depleting the cellular system of asparagine. If the tumors are asparagine dependent and the normal cells are not we would have a selective system for eliminating the asparagine dependent tumor.

Whether arginine depression by high lysine: arginine ratios is due to competition for reabsorption sites on the kidney tubule, induction of an arginase system in the kidney (or derepression of such a system) or some hitherto obscure mode of action may be relatively unimportant. Does lysine excess deplete the cell of arginine? Does this depletion result in using up the asparagine that would be available for tumor growth? Is the arginine or asparagine depression involved in other metabolic effects such as the deprivation of an essential amino acid for the synthesis of viral coat protein without which the infection caused by the virus could not produce a disease? Is arginine such an amino acid? Does the effect of arginase include the increased utilization of carbamyl phosphate for arginine resynthesis (see Fig. 12) and thus deprive the RNA, DNA synthetic pathway of the pre-UMP intermediate? Sorof *et al.* suggested this latter possibility.

One last point on the purely speculative aspects of the leukosis--mycoplasma interaction. Tumors generally have a respiration rate
Fig. 12. Biosynthesis of certain nucleic acids via carbamyl phosphate pathway.
disproportionately higher than corresponding normal tissue. One explanation for this phenomenon might be that viral replication or that part of the tumor DNA derived from the virus (if that is the case) would require relatively large quantities of the basic amino acids to form the histone bearing nucleoprotein and the viral coat protein.

Figure 13 indicates that aerobic glycolysis might become involved in supplying oxalacetate directly from phosphoenol-pyruvate rather than the higher ATP producing tricarboxylic acid cycle. If this were the case, the supply of aspartic acid, asparagine and arginine would become directly dependent on aerobic glycolysis and the utilization of any of these three amino acids could significantly stimulate an increased consumption of oxygen.

While not specifically covered in this work the author considers that it may be worthwhile not to overlook the long range effects of the indiscriminate use of antibiotics and other modifiers of our unseen micro-environment on the human link in the chain of ecological interdependence.
Fig. 13. Pathway of lactate to oxaloacetate.
LIST OF REFERENCES


