THE PREVENTION OF AVIAN LYMPHOID LEUKOSIS TUMORS WITH THE ANDROGEN ANALOG MIBOLERONE: PATHOLOGICAL, VIROLOGICAL AND IMMUNOLOGICAL STUDIES

By

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ABSTRACT

THE PREVENTION OF AVIAN LYMPHOID LEUKOSIS TUMORS WITH THE ANDROGEN ANALOG MIBOLERONE: PATHOLOGICAL, VIROLOGICAL AND IMMUNOLOGICAL STUDIES

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The androgen analog mibolerone (17β-hydroxy-7α, 17 dimethylestr-4-en-3-one) administered in the feed at μg levels during the first seven weeks of life to chickens that had been infected experimentally with Rous associated virus-1 (RAV-1) and RAV-2, or naturally infected with field strains of lymphoid leukosis virus (LLV) prevented the development of lymphoid leukosis.

The administration of mibolerone did not interfere with the normal cycle of infection of LLV's. Mibolerone-fed hens immunologically tolerant to LLV's shed LLV and group specific (gs) antigen in unincubated eggs at the same rate as hens that had been fed a standard diet. Levels of viremia in mibolerone- and standard diet-fed LLV viremic hens were identical. The administration of mibolerone did not interfere with the horizontal transmission of LLV's and did not increase the rate of shedding of LLV's in non-viremic hens. Chickens that had been fed mibolerone developed neutralizing antibodies to LLV's at the same rate

as chickens fed the standard diet.

The androgen analog mibolerone prevented LL by inducing a slow but progressive involution of the bursa of Fabricius, the target organ for LL transformation. Treatment with mibolerone resulted in practically bursa-less chickens at the age of seven weeks. Histopathological examination of the regressed bursae showed that some bursal follicles remained at the end of the seven week feeding period. However, no microscopic lesions reminiscent of LL bursal transformation could be found in these bursae, at an age where microscopic lesions were found in bursae from chickens fed the standard diet.

Chickens in which the feeding of mibolerone had induced regression of the bursa of Fabricius remained immunologically competent in their bursa-dependent and thymus-dependent functions. They reacted with humoral antibodies after antigenic stimulations with sheep erythrocytes, a thymus dependent antigen and with Brucella abortus, a bursa dependent antigen. Moreover, their spleens contained large numbers of antibody-producing cells that were detected in an hemolytic plaque assay and, their peripheral leukocytes reacted similarly to peripheral leukocytes from chickens fed a standard diet in a PHA blastogenesis assay considered to be an in vitro correlate of cellular immunity. More important, mibolerone-fed chickens could be properly immunized by vaccination against

the most economically important avian pathogens such as the agents of Newcastle disease, infectious laryngotracheitis, infectious bronchitis, fowlpox, Marek's disease and fowl cholera, results that indicate that mibolerone-fed chickens remain immunologically competent and develop immunity to these infectious agents.

Transfer studies with spleen cells from mibolerone-fed chickens did not detect the presence of post-bursal stem cells that would react against a stimulation with Brucella abortus. However, partial reactivity was detected against skeep erythrocytes. The spleens of chickens that had been fed the standard diet contained post-bursal stem cells that could be detected at two weeks of age, the earliest time tested and at 20 weeks of age, the latest time tested. Absence of post-bursal stem cells in the spleen of chickens that had been fed mibolerone as assayed by the transfer technique employed, is an indication of cell traffic from the bursa of Fabricius to the spleen and not of immunoincompetence.

The results reported in this dissertation are significant because it has been shown that the androgen analog mibolerone has a potential for the practical control of LL tumors in chickens infected with LLV's.

Although I have not written my 'great book'
I know what kind of book it ought to have been.

Kenneth M. Clark, Another Part of the Wood

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LIST OF ABBREVIATIONS

Ab	= antibody
AGP	= agar gel precipitation
ALV-42	= strain 42 of lymphoid leukosis virus
AMV	= avian myeloblastosis virus
B cell	= bursa of Fabricius derived cell
BH	= Bryan high titer strain
BME	= basal medium Eagle
CAM	= chorioallantoic membrane
CEF	= chicken embryo fibroblasts
CF	= complement fixation
CFU	= colony forming units
chf	= chick helper factor
COFAL	= complement fixation for avian leukosis viruses
CY	= cyclophosphamide
DEAE	= diethylaminoethyl
DEF	= duck embryo fibroblasts
DMSO	= dimethyl sulfoxide
DNA	= deoxyribonucleic acid
DNase	= deoxyribonuclease
EID_{50}	= dose that infects 50 percent of chicken embryos

FA	= fluorescent antibody
FAPP	= filtered air positive pressure
FFU	= focus forming units
FITC	= fluorescein isothiocyanate
g	= gravity
GA-22	= pathogenic strain of MDV
gs	= group specific
^{3}H	= tritiated
HI	= hemagglutination inhibition
HU	= hemagglutination units
HVT-FC 126	= herpes virus of turkey from the RPRL
IB	= infectious bronchitis
IBA	= infectious bursal agent
IBV	= infectious bronchitis virus
I^{e}	= independent autosomal dominant gene that controls resistance to LL viruses of subgroup E
Ig	= immunoglobulin
ILT	= infectious laryngotracheitis
JM	= an acute isolate of MDV
LL	= lymphoid leukosis
LLV	= lymphoid leukosis virus
LLV's	= lymphoid leukosis viruses
Log	= logarithm
MD	= Marek's disease
MDV	= Marek's disease virus

ND	= Newcastle disease
NDV	= Newcastle disease virus
NP	- non producer
PBS	= phosphate buffered saline
PFC	= plaque forming cell
PHA	= phytohemagglutinin
PM	= phenotypic mixing
Pr	= Prague strain of RSV
QEF	= quail embryo fibroblasts
RAV	= Rous associated virus
RDDP	= RNA directed DNA polymerase
RIA	= radioimmunoassay
RIF	= resistance inducing factor
RNA	= ribonucleic acid
RPL	= Regional Poultry Laboratory
RPRL	= Regional Poultry Research Laboratory
RSV	= Rous sarcoma virus
RSV's	= Rous sarcoma viruses
SCWL	- single comb white leghorn
SE	= sheep erythrocytes and standard error
SI	= stimulation indexes
T cell	= thymus derived cell
Texas-GB	= velogenic strain of NDV
TCA	= tribhloroacetic acid
TSTA	= tumor specific transplantation antigen

tva	= tumor virus locus that controls resistance to LLV's of subgroup A
tvb	= tumor virus locus that controls resistance to LLV's of subgroups B, D, and E
tvc	= tumor virus locus that controls resistance to LLV's of subgroup C
tve	= tumor virus locus that controls resistance to LLV's of subgroup E
U	= units
µg	= microgram
uC	= microcurie
USDA	= United States Department of Agriculture
V	= virus, viremia or dominant gene that controls RAV-0 production

INTRODUCTION

Lymphoid leukosis (LL) is a neoplasm of chickens characterized by initial transformation of lymphoid cells in the follicles of the bursa of Fabricius (Cooper et al., 1968) and later metastases of the transformed bursal cells to visceral organs such as the liver, spleen, kidneys, intestines, gonads, etc. Chickens die due to the physical interference by the large tumors with the normal physiological events (Ellermann and Bang, 1908) or possibly because of immunosuppression that makes the chickens more prone to be overrun by other avian pathogens. The disease can suddenly acquire significance in genetically susceptible flocks and can be responsible for severe economic losses (Purchase et al., 1972).

LL is induced by C-type RNA viruses (Temin, 1974) that are currently classified in the leukosis/sarcoma group of viruses. The exogenous viruses of this group which naturally infect the chicken have been classified into four subgroups (A, B, C, D) based on their envelope properties (Duff and Vogt, 1969) and they are known to be responsible for the LL losses in the field (Calnek, 1968; Churchill, 1968; Sandelin and Estola, 1974). The endogenous viruses of the chicken have been classified into subgroup E

(Hanafusa et al., 1970) and are thought to have little if any oncogenic potential (Motta et al., 1975; Purchase et al., 1977). Exogenous and probably endogenous viruses are perpetuated in nature by vertical (congenital) transmission from dams to offspring (Cottral et al., 1954; Burmester et al., 1955). Endogenous viruses are also genetically transmitted integrated in the chromosomes of the chicken cell (Huebner and Todaro, 1969; Temin, 1971). Exogenous LL viruses are ubiquitous in nature (Purchase et al., 1977) while endogenous viruses have been found to be spontaneously produced by only a few inbred lines of chickens (Robinson et al., 1975; Crittenden, 1976). LLV's are also horizontally transmitted from infected shedders to contact exposed chickens (Burmester, 1956; Rubin et al., 1962). However, LL mortality due to horizontal transmission is probably minimal (Purchase and Burmester, 1977).

Several methods for the control of avian LL have been investigated in several laboratories. These methods include eradication (Calnek et al., 1967; Hughes et al., 1963; Zander et al., 1975), breeding for resistance to virus infection (Crittenden, 1975), breeding for resistance to tumor development (Crittenden, 1975), vaccination (Burmester, 1955; Löliger and Hagen, 1974), and control by removing the target cells for LL transformation from the bursa of Fabricius (Burmester, 1969; Purchase and Cheville, 1975).

In the present experimental work, efforts have been aimed at the control of LL tumors by removing target cells in the bursa of Fabricius. Specifically, the efficacy of the androgen analog, mibolerone (17β- hydroxy-7α, 17 dimethylestr-4-en-3-one) (The Upjohn Company, Kalamazoo, Michigan) in the prevention of natural and experimental LL tumors was assessed. The findings on the prevention of tumors are complemented by research aimed at answering questions on the rate of LL virus shedding in mibolerone-fed hens naturally or experimentally infected with LLV's and by research aimed at assessing the immunological competence of mibolerone-fed chickens in which the bursa of Fabricius had regressed by seven weeks of age.

LITERATURE REVIEW

History

Lymphoproliferative neoplasms of chickens have long been recognized. Cases of "fowl leukosis" were documented as far back as 1868 by Roloff, and 1908 by Ellermann and Bang. Experimental studies performed by Ellermann and Bang (1908) indicated that the term leukemia introduced by Virchow (1845) which referred to circulating neoplastic cells did not apply to the chicken in which the common form of neoplastic disease is an extravascular infiltration of malignant cells in the visceral organs causing enlargement and death from functional interference. These workers proposed the usage of the term "leukosis" that did allow for better description of the several forms of leukosis, i.e., myelocytic leukosis, lymphocytic leukosis and erythroleukosis, etc.

This group of diseases rapidly became one of the most important serious threats to the rapidly expanding poultry operations all over the world. Annual losses in Britain were calculated at 7-8 million sterling pounds (Blaxland, 1967) and in the USA probably around 100 million dollars (Campbell, 1961). Much confusion in classification and correct diagnosis of the diseases existed, which were all

included as members of the avian leukosis complex (visceral and neural lymphomatosis, ocular lymphomatosis, osteopetrosis, erythroblastosis, granuloblastosis, and myelocytomatosis) (Jungher et al., 1941). Therefore, it can be assumed that other diseases besides LL were responsible for these estimates. In all likelihood, the then not properly recognized Marek's disease (MD) (fowl paralysis) and LL (big liver disease) were responsible for at least 90 percent of the losses (Biggs, 1964).

The work of Campbell (1945) and Burmester et al. (1946) prompted Campbell (1961) to recognize the need to separate the avian leukosis complex into fowl paralysis and other forms of leukosis. Furthermore, it was also recognized that the term fowl paralysis was unsatisfactory and confusing and Marek's disease (neural, visceral, and ocular forms) was suggested as a substitute (Biggs, 1961). The issue was largely settled at the First Conference of the World Veterinary Poultry Association (Biggs, 1964) when it was definitely established that the avian leukosis in its three forms; lymphoid, myeloid and erythroid was different from Marek's disease. It was also agreed that the sarcomas, endotheliomas, and the renal sarcomas were related to the leukoses.

Today, it is widely accepted that LL and MD are different neoplastic diseases of chickens; LL caused by oncornaviruses (Burmester and Gentry, 1956; Nowinsky et al., 1970) and MD caused by a cell-associated herpes virus

(Churchill and Biggs, 1967; Solomon et al., 1968). Nevertheless, overlapping in the gross neoplastic picture observed in both diseases under field conditions often presents a diagnostic problem that requires further histopathological studies.

Incidence and Distribution of Lymphoid Leukosis

Infections with the viruses of LL are almost certain wherever extensive commercial poultry operations exist. The disease is ubiquitous in nature and it has been reported in the American (Burmester et al., 1946), European (Churchill, 1968; Sandelin and Estola, 1974), African (Morgan, 1973) and Asiatic (Weiss and Biggs, 1972) continents.

The presence of viruses of different subgroups in field flocks is studied by testing the sera of chickens for the presence of antibodies against Rous sarcoma viruses of known subgroups. By the time chickens reach sexual maturity, the disease has usually spread throughout the flock and most birds have antibodies. The disease incidence varies from flock to flock depending on rate of infection, genetic susceptibility, pathogenicity of strains involved and possibly the presence of other avian pathogens (Cheville et al., unpublished results). Heavy losses have been reported in commercial breeder flocks (Purchase et al., 1972), but total mortality due to LL in the United States

is for all practical purposes considered to be approximately one percent of adult chickens per year.

Etiology

The first indication that "chicken leukemia" could be successfully transmitted was provided by Ellermann and Bang (1908) by inoculating an "emulsion" of tumorous tissue intravenously into chickens. This procedure did not distinguish between cellular and cell-free transmission. However, when the tumor emulsions were clarified by centrifugation and then filtered through a candle of infusorial earth, the disease could still be reproduced. This, then, provided the first evidence of a viral entity in the etiology of "chicken leukemia." Unfortunately, experimental work carried out in the following decades cast doubt on the viral etiology of the disease that was then temporarily considered non-transmissible in nature (Engelbreth-Holm, 1942).

Burmester et al. (1946) working with cell-free filtrates of the Olson transplantable tumor (Olson, 1941) were able to induce lymphoid tumors within six months of inoculating baby chicks by various parenteral routes. The response was different from the one obtained with tumor cells where tumors were seen at the site of inoculation with metastases in the viscera within ten days. Conclusive proof of the viral etiology of LL was obtained after six serial passages of cell-free filtrates of the Olson tumor

in chickens. The incidence of tumors and the average survival time were quite consistent for the several filtrate inoculations and passages. Greater than 80 percent of all chickens inoculated showed some form of leukosis and they died in an average of 137 days (Burmester and Cottral, 1947).

The viral etiology of LL is now quite clear (Burmester, 1971). The viruses have been cultivated and serially propagated *in vitro* (Rubin, 1960; Bogt and Ishizaki, 1965; Duff and Vogt, 1969) and leukosis is induced by the inoculation of viruses of subgroups A through D (Purchase et al., 1977). Viruses of subgroup E are yet to be proven oncogenic (Motta et al., 1975; Purchase et al., 1977).

Strains of Lymphoid Leukosis Viruses and Biological Properties

All strains of avian LLV's are currently classified in the leukosis/sarcoma group of viruses. These viruses are all morphologically indistinguishable, contain a single stranded RNA core, replicate by reverse transcription and are released from infected cells by a process of membrane "budding." All strains contain and induce in infected cells the appearance of a group specific (gs) antigen which is common to all known viruses of the leukosis/sarcoma group.

The finding that the Bryan-high (BH) titer strain of Rous sarcoma virus (RSV) was defective led to the

classification of the leukosis/sarcoma into subgroups on the basis of cross neutralization, interference and host range patterns (Hanafusa, 1965). The first helper virus strain was isolated from a stock of the BH-RSV and was recognized as such by its capacity to interfere with the transforming effect of RSV. This strain was designated Rous associated virus-1 (RAV-1) and was classified within subgroup A of LLV's (Vogt and Ishizaki, 1966a). A second strain (RAV-2) was also isolated from the BH-RSV stock and was identified in studies of mutual interference with RAV-1, host range in chicken embryo fibroblasts (CEF) cultures and antigenicity, as a member of a different subgroup of avian leukosis viruses (Hanafusa, 1965) called subgroup B (Vogt and Ishizaki, 1966a). It was concluded that each helper virus (RAV-1 or RAV-2) conferred host range properties to the RSV so that, if RSV was complemented by RAV-2 it was susceptible to interference by RAV-2 but not by RAV-1. Also, if the RSV was activated by RAV-1, then the pseudotype RSV (RAV-1) was susceptible to interference by RAV-1 but not by RAV-2.

The antigenicity of the pseudotypes as determined by serum neutralization tests was determined entirely by the helper viruses since no immunological cross-reactivity was detected between RSV (RAV-1) and RSV (RAV-2) (Hanafusa, 1965). Extensive studies on the antigenic cross-reactivity of RAV's and resistance inducing factors (RIF) using the fluorescent antibody and the serum neutralization tests

with specific antisera prepared in chickens allowed the classification of the then known strains into subgroups A and B (Ishizaki and Vogt, 1966). Although viruses assigned to subgroups A and B serologically cross-react within subgroups, antigenic types have been recognized on the basis of cross-neutralization studies (Ishizaki and Vogt, 1966) since antisera react more strongly with homologous viruses than with heterologous viruses of the same subgroup. More recent studies have shown that chickens immunologically tolerant to ALV-F42, a subgroup A virus, do react with neutralizing antibodies to the subgroup A viruses RAV-1, RAV-3, and RAV-5 when challenged with RAV-3 or RAV-5 (Meyers, 1976). On the other hand, if RAV-1 or ALV-F42 is used as the challenging virus, no neutralizing antibodies against any of the involved pseudotypes are produced. These results show that there is antigenic variability within subgroups.

Subgroups C and D were then created to accommodate newly isolated helper viruses capable of growing in CEF resistant to subgroups A and B (Duff and Vogt, 1969). Viruses of subgroup C were found to plate with equal efficiency in CEF of the types C/O, C/A, C/B, and C/AB (Table 2) but were excluded from C/BC CEF. Viruses classified in subgroup C interfered with RSV of the same subgroup but not with RSV of subgroups A and B. Similarly, all subgroup D viruses had similar plating efficiencies on C/O,

C/A, C/B, C/AB, and C/BC but demonstrated homologous interference in C/O cells.

More recently, another leukosis virus was isolated from gs antigen positive C/A CEF cultures. This virus provides RSV with helper activity and also possess surface antigens that are different from those of previously described subgroups (Hanafusa et al., 1970a; Vogt and Friis, 1971; Smith et al., 1974). The virus is commonly referred to as RAV-0 and is in its most important characteristics indistinguishable from other non-transforming members of the avian leukosis viruses (Vogt and Friis, 1971). RAV-0 is unusually labile, with a poor ability to interfere with related viruses and a dependency on RSV in order to infect quail cells. The pseudotype is known as RSV (RAV-0).

Induction studies in normal cells using physical and chemical carcinogens have resulted in the release of viruses that resemble RAV-0 in their biological and biochemical properties (Hanafusa et al., 1970b; Weiss et al., 1971; Robinson et al., 1976), and on the basis of host range, interference, and antigenicity they have been classified as members of subgroup E of avian leukosis viruses (Robinson et al., 1976). A list of the virus strains currently classified in the chicken leukosis/sarcoma group of viruses is provided in Table 1.

Table 1: Chicken leukosis/sarcoma group of viruses.

Sub-group	Leukosis	Sarcoma	
		Helper-dependent	Helper-independent
A	RAV-1	BH-RSV(RAV-1)	SR-RSV-A
	RPL-12	BH-RSV(RPL-12)	PrC_2A
	RAV-3	RSV(RAV-3)	
	RAV-4	RSV(RAV-4)	
	ALV-F42	RSV(F42)	
	RAV-5	RSV(RAV-5)	
	MAV-1		
	FAV-1		
	Most of LL field isolates		
B	RAV-2	BH-RSV(RAV-2)	SR-RSV-B
			PrC_2-B
	RAV-6	RSV(RAV-6)	Harris strain RSV
	MAV-2		
	AMV-B		
C	RAV-7	BH-RSV(RAV-7)	B-77
	RAV-49	BH-RSV(RAV-49)	Pr-RSV-C
			MH-2
D	RAV-50	BH-RSV(RAV-50)	SR-RSV-D
	CZAV		CZ-RSV-D
E	RAV-0	BH-RSV(RAV-0)	-
	RAV-60	BH-RSV(RAV-60)	-

References: Vogt and Ishizaki (1966b).
Vogt (1970).

Host Range In Vivo and In Vitro

a. In vivo

Chickens are the only natural hosts of both exogenous (A, B, C, and D subgroups) and endogenous (E subgroup) LLV's. Histopathological changes similar to those of avian LL have been reported in a few isolated cases in the Japanese quail (Coturnix coturnix japonica (Wight, 1963) and in a large flock of Japanese quail in which 8.1 percent of all dead quail had "leudosis" lesions (Löliger and Schubert, 1967). However, no bursa tumors or serological and virological findings were reported. The gross and microscopic lesions resembled those described for a lymphoproliferative disease of quail which is different from LL (Schat et al., 1976). An outbreak of visceral lymphomatosis has also been described in turkeys (Simpson et al., 1957). Both gross and microscopic lesions described are also compatible with either MD or reticuloendotheliosis.

b. In vitro

LLV's are readily propagated in susceptible CEF cultures, but, do not induce cytopathic effect except after prolonged passage (Calnek, 1964). The susceptibility of CEF cultures to infection with leudosis viruses is genetically controlled and is expressed at the cell membrane level. In the case of resistant cells, the block occurs at the penetration stage, since excluded viruses are

absorbed equally well to resistant or susceptible CEF cultures (Piraino, 1967). The host range of chicken lymphoid leukosis viruses of various subgroups in CEF cultures is shown in Table 2.

Chicken LLV's (subgroups A-D) have also been propagated in jungle fowl embryo fibroblast cultures and in embryo fibroblasts of species other than chickens (Vogt, 1970). Bobwhite quail fibroblasts are resistant to viruses of subgroups A through D. Goose, duck, and turkey fibroblasts fall somewhere in between the extremes of susceptivility and resistance to viruses of subgroups A through D (Vogt, 1970). Pheasant, turkey and quail embryo fibroblasts are all susceptible to infection with RSV (RAV-0) and less susceptible to infection with RAV-0. The host range of chicken lymphoid leukosis viruses in fibroblast cultures of several species is shown in Table 3.

Pathology of Virus-Induced Tumors

a. Macroscopic pathology

Ellermann (1922) believed that avian leukosis originated in the lymphoid follicles of the spleen. He did not seem to implicate the presence of "retrorectal" tumors in the pathogenesis of the disease. However, the retrorectal tumors described by Ellermann were in all likelihood tumors of the bursa of Fabricius, a retrorectal organ now known to be the target organ for lymphoid leudosis transformation (Cooper et al., 1968). Although the bursa of

Table 2: Host range of chicken lymphoid leukosis viruses of various subgroups in CEF cultures.

Phenotype	Subgroup A	B	C	D	E
C/0	S	S	S	S	S
C/A	R	S	S	S	S
C/BE	S	R	S	SR	R
C/ABE	R	R	S	SR	R
C/BCE	S	R	R	SR	R
C/E	S	S	S	S	R

Abbreviations: In the abbreviations of the cell types, the excluded subgroup appears behind the bar.
S = Susceptible; efficiency of plating more than 10% relative to C/0
R = Resistant; efficiency of plating less than 1%
SR= Semi-resistant; efficiency of plating between 1 and 10%

References: Hanafusa (1975).
Vogt (1970).

Table 3: Host range of chicken lymphoid leukosis viruses of various subgroups in avian cultures from several species.

Avian Species	Subgroup A	B	C	D	E
Jungle fowl	S	S	S	S	
Goose	R	R	S to SR	S to SR	
Duck	R	R	S to SR	S to SR	
Turkey	S	R	S[1]	R[2]	S
Japanese quail	S	SR to R	SR[1]	SR	S
Guinea fowl	S	SR to R	SR[1]	SR[2]	
Ringneck pheasant	S	R	S[1]	R[2]	S
Bob white quail	R	R	R	R	

S = Susceptible; efficiency of plating more than 10% relative to C/0
R = Resistant; efficiency of plating less than 1%
SR = Semi-resistant; efficiency of plating between 1 and 10%
1. Excludes RAV-7
2. Infected by RAV-50

References: Hanafusa (1975).
Vogt (1970).

Fabricius is the organ where the primary tumor occurs as early as eight weeks of age (Cooper et al., 1968), LL tumors are rarely seen outside the bursa before five months of age.

In LL, all soft organs can be affected but the bursa of Fabricius, liver and spleen are more often involved. Occasionally, the kidneys, mesentery, intestines, lungs, heart, and bone marrow are also affected. The tumors are soft, smooth and glistening and they can be nodular, miliary or diffuse (Purchase and Burmester, 1977), or a combination of all three. The nodular tumors (Figure 1) vary from the size of a pinhead to that of a hen's egg. They are usually spherical but occasionally may be flattened especially when located at the surface of the organ. Nodular tumors vary greatly in numbers. In the diffuse form (Figure 2), the liver is enlarged up to eight times its normal weight (Ellermann, 1922) and it is slightly grayish in color and often friable. The spleen is usually pale and enlarged showing a pronounced follicle pattern when sliced open. In the miliary form (Figure 3) the liver is strewn with numerous, uniformly distributed small tumors less than two mm in diameter (Ellermann, 1922).

b. Microscopic pathology

The earliest microscopic LL lesions have been seen in the bursa of Fabricius eight weeks after experimental infection (Cooper et al., 1968). Both the cortical and

Figure 1. Nodular tumors in liver of chicken with lymphoid leukosis.

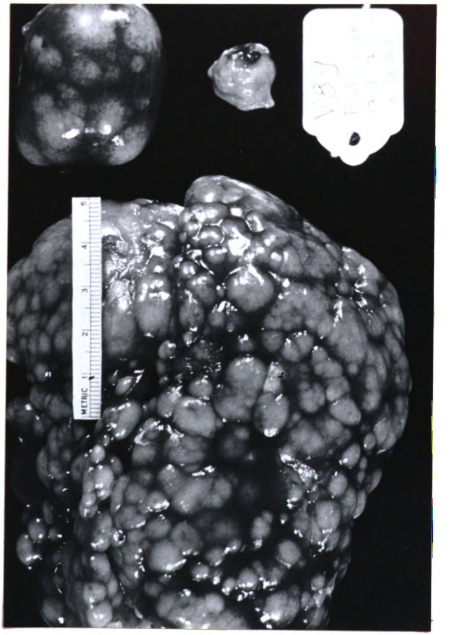
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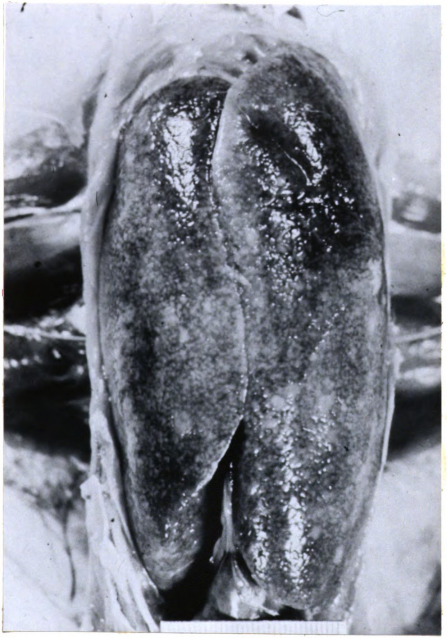
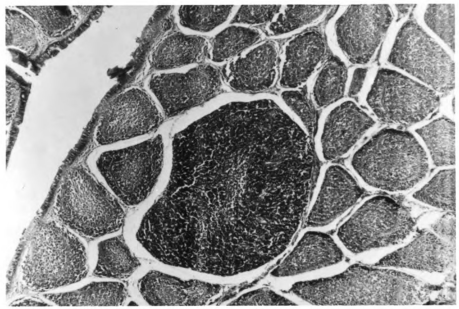
Figure 2. Diffuse tumors in liver of chicken with lymphoid leukosis.

Figure 3. Miliary tumors in liver of chicken with lymphoid leukosis.



medullary areas of affected bursal follicles contained increased numbers of immature "blastform" cells with pyroninophilic cytoplasm. There is an increased number of cells in mitosis, the cortical zones are widened by densely packed immature cells and there is no clear cortico-medullary distinction. Initially, the transformed follicles are surrounded by normal follicles (Figure 4) but as the disease progresses, more and more transformed follicles are seen (Purchase and Burmester, 1977). Bursal tumors are focal and multicentral in origin (Purchase and Burmester, 1977). The tumors grow by profileration and compression in the parenchima of the affected organ, and not by infiltration. Once the transformed cells peripheralize and the metastases are established in the visceral organs, the microscopic lesion observed is basically the same as that seen in a tumor. The tumors consist of aggregates of large lymphoid cells which are consistently uniform in size. These cells are considered to be immature and rapidly dividing malignant lymphoblasts that contain large amounts of RNA in their cytoplasm which stains readily with methyl green pyronin. These malignant lymphoblasts have a poorly defined cytoplasmic membrane, a vesicular nucleus with marginated and clumped chromatin and one or more acidophilic very conspicuous nucleoli. There is no persistent leukemia in lymphoid leukosis except perhaps, at the terminal stage.

Figure 4. Histological section of bursa of Fabricius from chicken infected with LLV. The bursal transformation is characterized by the presence of lymphoblast-type cells throughout a single bursal follicle.



Pathology of Transplantable Tumors

a. Macroscopic pathology

Virus-induced bursa tumors representative of subgroups A through D of LL are homotransplantable and can be serially propagated in susceptible recipients until the transplant consistently kills the host within an incubation period of 6-7 days (W. Okazaki and C. H. Romero, unpublished data). Thus, these transplantable tumors have a much shorter incubation period than their virus-induced counterparts. Established transplantable tumors appear at the site of inoculation, i.e., muscle and subcutaneous tissue (Olson, 1941), anterior chamber of the eye (Burmester and Belding, 1949; Burmester, 1952), abdominal cavity (Ponten and Burmester, 1967). These tumors may regress, continue to grow but remain localized or continue to grow and metastasize to visceral organs (Olson, 1941). The heart and proventriculus are sites of predilection for metastases (Olson, 1941). However, transplantable tumors injected intravenously mainly grow in the liver, spleen, kidneys, bone marrow, gonads, and occasionally in the thymus (W. Okazaki and C. H. Romero, unpublished data). Transplantable tumors in their early passages can be either nodular or diffuse; the nodular form seems to be a property of less rapidly dividing tumor cells before adaptation to continuous growth. Once the transplant is established, the predominant form is the rapidly growing diffuse form in which both liver and spleen are generally increased several

times their normal size. Only rarely are bursa tumors seen in transplantable tumors (Purchase and Burmester, 1977).

b. Microscopic pathology

Microscopically, the transplantable cells are more uniform and more anaplastic than the tumor cells from which they originate (Purchase and Burmester, 1977). These malignant cells are almost exclusively large anaplastic lymphoblasts with a vesicular nucleus and several large nucleoli (Olson, 1941; Purchase and Burmester, 1977). Small or medium lymphocytes that may be observed in virus-induced tumors are never seen in transplantable tumors. Microscopic nodular lesions are characterized by localized aggregates of lymphoblastic cells surrounded by normal tissue (Figure 5). Microscopic diffuse lesions are characterized by infiltration of malignant lymphoblasts throughout the parenchima of the visceral organ involved (Figure 6).

Pathogenesis

LL is considered a neoplasm originating in the bursa of Fabricius. Several lines of evidence support this idea.

Surgical bursectomy of chickens susceptible to LL significantly reduced the incidence of LL tumors (Peterson et al., 1964). This effect was obtained whether bursectomy was performed at 2 or 29 days of age and whether the chickens were infected at 1 or 28 days of age. It was postulated that the bursa of Fabricius is a focus of

Figure 5. Microscopic nodular lesion in kidney of chicken bearing a RAV-50 lymphoid leukosis transplantable tumor.

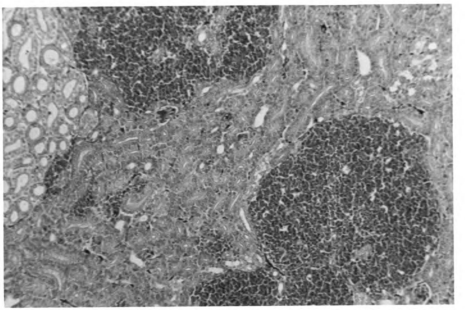
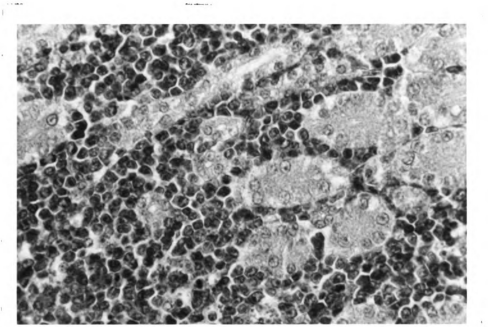


Figure 6. Microscopic diffuse lesion in kidney of chicken bearing a RAV-49 lymphoid leukosis transplantable tumor.



undifferentiated lymphoid cells and that these immature cells are the most susceptible to the transforming effects of the virus. No significant effect of surgical thymectomy on the prevention of tumors was observed. Further studies on the influence of the time of surgical bursectomy on the development of tumors indicated that bursectomy performed during the first 5 months of life significantly reduced the development of LL tumors (Peterson et al., 1966a; Peterson et al., 1966b). Thymectomy alone did not prevent the tumors and the implantation of infected embryonic thymus lobes into surgically bursectomized chickens did not increase the incidence of LL tumors. Furthermore, no additive effect of thymectomy and bursectomy was obtained.

Dipping of embryonated eggs in testosterone propionate solutions, single or multiple injections of testosterone propionate, and feeding methyl testosterone in the diet induced various degrees of ablation of the bursa of Fabricius with the concomitant reduction or complete prevention of LL tumors (Burmester, 1969). Adverse effects related to hatchability, egg production and increase in mortality due to causes other than LL were observed.

Chemical bursectomy with cyclophosphamide (CY) prevented the development of LL tumors presumably by eliminating the target cell for transformation located in the bursa (Purchase and Gilmour, 1975). The transfer of bursal cells from hatchmates into CY-treated chickens resulted in the restoration of susceptibility to LL tumors

most likely because the transferred target cells for LL transformation replaced those destroyed by the CY treatment. Moreover, it was found that CY destroyed the target cells for LL transformation before the stem cells for the immune response since some chickens were resistant to LL tumors but still produced gamma globulins and antibodies (Purchase and Gilmour, 1975).

The infectious bursal agent (IBA), the virus responsible for Gumboro disease, replicates in the bursa of Fabricius causing extensive necrosis of the lymphoid tissue (Cheville, 1967). Chickens infected with RAV-1 at one day of age and with the IBA at two or eight weeks of age developed fewer LL tumors than chickens infected with RAV-1 only (Purchase and Cheville, 1975). Also, IBA vaccines that are bursatrophic, but not the apathogenic non-bursatrophic IBA vaccines significantly reduced the occurrence of LL tumors in RAV-1 infected chickens (Cheville et al., personal communication). The reduction in LL tumors was most probably due to a direct lytic effect of the IBA on the target cells that would have been, otherwise, transformed by RAV-1.

A different line of evidence for the target role of the bursa of Fabricius was provided by the studies of Cooper et al. (1968). Neoplastic transformation of lymphoid cells within isolated bursal follicles was observed as early as eight weeks after inoculation at hatching with the RPL-12 leukosis virus. Histological

evidence of transformation was also seen at 12 weeks in two out of ten chickens examined and at 16 weeks after infection nine out of ten chickens had histological evidence of bursal transformation. These transformed follicles contained an increased number of immature "blastform" cells with pyroninophilic cytoplasm in both the cortical and the medullary areas. Cells in mitosis were also increased in number, the cortical zones were widened by densely packed immature cells and the corticomedullary distinction was often hazy in transformed follicles. In most birds, the time between the occurrence of histological neoplastic changes in the bursa and the occurrence of histological neoplastic changes in the peripheral lymphoid organs such as liver, spleen, thymus, and cecal tonsils was longer than 14 weeks. These results, then, showed that LL tumors arise in focal areas within the bursa and remain latent at this site for some time before metastases occur. Cooper et al. (1968) also confirmed that surgical removal of the bursa before peripheralization of transformed bursal cells prevents the development of LL. It was also shown that even when transformation of bursal follicles does occur within the bursa, spontaneous regression could take place.

A different approach to demonstrating the bursal dependence of LL was provided by studies using bursal cell (B cell) markers. When goat specific antisera to μ, γ, and α heavy chains and antiserum to light chains were used in

direct immunofluorescence on viable transformed cells excised and isolated from LL tumors, it was found that all transformed lymphoid cells contained detectable amounts of μ chains on their surface whose distribution was patchy in appearance (Cooper et al., 1974). Light chain determinants were also present on the surface of the tumor cells. On the other hand, γ and α chain determinants were never found on the transformed lymphoic cells. Trypsinization and further incubation in growth media showed that after stripping the transformed cells from their μ determinants, regeneration occurred. These results suggested a defect in B cell differentiation at the level of the intraclonal switch from IgM to IgG synthesis with the consequent production of large amounts of heterogeneous IgM by the transformed lymphoid cells. Cooper et al. (1974) suggested that it is at this point in cell differentiation that avian LL viruses exert their oncogenic effect, perhaps by integrating viral RNA-directed DNA into the genome in such a way as to preclude the continuation of the natural sequence of gene expression.

A similar approach to detect B cell markers was used by Payne and Rennie (1975). Rabbit antiserum with a high degree of specificity for B cell markers was used in an indirect immunofluorescence technique on transformed lymphoid cells obtained from LL tumors. More than 90 percent of these cells were strongly stained, the surface staining being reported as coarsely granular and global and

similar to the one seen on normal bursal cells. Up to five percent of the tumor cells stained with rabbit anti-thymus (anti-T) serum. The B cell antigen marker is present on normal bursal cells and peripheral B cells and is different from the immunoglobulin markers (Hammer et al., 1974).

Specimens of Choice for Virus Isolation

LLV's are ubiquitous in nature (Purchase, 1969b), and the infection is perpetuated under field conditions by congenital transmission of exogenous viruses through the embryonated egg (Cottral et al., 1949; Burmester and Waters, 1955; Rubin et al., 1961). The understanding of this cycle of infection and the knowledge that chickens of the LLV types V-Ab+ (viremia negative-antibody positive), V+Ab+, V+Ab- and V-Ab- can all be found (Purchase, 1969b) in an infected flock in various proportions depending on the rate of congenital infection have allowed a more scientific choosing of tissue samples from which leukosis viruses or antigens can be isolated. In chickens of the V+Ab- type, virus can be readily isolated from plasma, serum (Rubin, 1960), tumors, most of the soft visceral organs, saliva and feces (Burmester, 1956a). In laying hens of the same type, virus can be isolated from the various parts of the oviduct, especially from the albumen-secreting portion (DiStefano and Dougherty, 1965), from embryo mashes and cloacal swabs (W. Okazaki, personal communication) and from the albumen and yolk of both fertilized and

unfertilized eggs (Spencer et al., 1976). In chickens of the V+Ab+ type, plasma, serum or tumors are probably the best specimens for virus isolation (Rubin, 1960). In chickens of the V-Ab+, embryos and albumen are probably the samples of choice (Spencer et al., 1976), and chickens of the V-Ab- type should be definition not yield virus. Since all LLV's are relatively thermolabile, the specimens from which virus isolation is to be attempted must be stored at temperatures below -60°C.

Methods for Assaying Lymphoid Leukosis Viruses

a. Biological assay

In vivo studies of LLV's were initially severely handicapped by the long incubation period of the experimental disease and because the diagnosis was complicated by the occurrence of MD, another neoplastic disease of poultry which in its visceral form resembles LL. Thus, several studies have been made to develop accurate bioassays that will give a rapid and sensitive response of a quality acceptable for virus assay.

The original method for the assay of LL involved the inoculation of one day-old susceptible chickens by the intra-abdominal route (Burmester and Gentry, 1956). This bioassay was a time consuming procedure that necessitated the rearing of susceptible chickens in isolation over long periods of time that could extend up to 270 days. A second bioassay that exploited the close dose-response relationship

during the early period after experimental inoculation was then developed (Burmester, 1956b). A third bioassay aimed at reducing the experimental period for an erythroblastosis response is performed by inoculation of 11 day-old chicken embryos by the intravenous route. By this procedure, approximately ten percent mortality is expected within 18 hours due to hemorrhages, and of those embryos that survive the inoculation, 70 percent hatch (Piraino et al., 1963). Chicks inoculated as embryos with high doses of virus rapidly develop erythroblastosis with appreciable deaths occurring during the first two weeks of age. Other tumor responses include nephromas, chondromas, fibrosarcomas, and endothelial tumors. An important practical consideration is that an erythroblastosis response of chickens inoculated as embryos with low doses of virus can be obtained 46 days postinoculation, while similar doses inoculated into one day-old or two week-old chickens would necessitate an experimental period of more than 240 days (Piraino et al., 1963).

b. Resistance inducing factor (RIF) test

LLV's replicate in susceptible CEF usually without producing visible cytopathic changes. RSV's on the other hand, replicate in susceptible cells and induce transformation (Manaker and Groupé, 1956). This transformation can be quantitated by end-point dilutions of the RSV in susceptible CEF grown under an agar overlay. Easily countable

foci of transformation are obtained at high dilutions, one focus being the result of infection of a cell by one virus. When susceptible CEF are infected with avian LLV's and virus propagation is allowed, these cells become resistant to the transforming effects of a RSV (Rubin, 1960) of the same subgroup (Hanafusa, 1965) probably by virtue of having the specific cell receptors taken up by the leukosis virus and thus preventing the penetration of the RSV. Tumor cell-free extracts from field cases of LL have been assayed for RIF activity and found to induce complete resistance to RSV infection. It was rather fortuitous that a subgroup A RSV was used in the interference assays with the leukosis virus strains, that were in all probability also of subgroup A, since the interference phenomenon only occurs between viruses of the same subgroup (Hanafusa, 1965). The RIF test has shown unequivocally that LL viruses are present in embryonic cells, supernatants of embryo extracts and allantoic fluids of incubated eggs (Rubin, 1960). The RIF test has also allowed the detection of viruses in the plasmas and the egg yolks of viremic hens (Rubin et al., 1961) and has been used as a test to identify LLV shedders in eradication trials in small commercial hen flocks (Hughes et al., 1963; Zander et al., 1975).

c. Non-producer (NP) test

When CEF were transformed by solitary infection (low multiplicity of infection) with the BH strain of RSV,

infectious virus progeny were not produced from some clones of transformed cells which were referred to as non-producer or NP cells (Hanafusa et al., 1963). Electron microscopic studies of NP cells revealed the presence of viral particles either extracellularly or enclosed in cytoplasmic vesicles which were indistinguishable from those of the avian leukosis viruses (Dougherty and DiStefano, 1965). Robinson (1967), Vogt (1967b), and Weiss (1967) independently isolated a virus from NP cells which was named RSV-0 but later called RSV (RAV-0) to designate its helper dependence. This virus could not be assayed by standard techniques but was detected in cells of rare, susceptible chick embryos, or Japanese quail cells. Super infection with an avian leukosis helper virus activated the release of RSV from these cells with a much broader host range in chicken cells. Knowledge of the need for a helper virus to rescue infectious RSV from NP cells prompted the development of a test for assaying avian leukosis viruses. This test was named non-producer (NP) cell activation test (Rispens et al., 1970; Rispens and Long, 1970). All LL viruses can act as helper viruses and the complete RSV's resulting from this infection have the host range, interference pattern and antigenic specificity of the helper virus as well as subgroup E host range. The test requires the availability of a good stock of NP cells and a feeder layer that can be either chicken or duck embryo fibroblasts. The fibroblasts and the NP cells are cocultivated and infected with the

leukosis sample. After a further growth period of one week (activation phase) the supernatants are harvested, frozen and thawed, centrifuged, and inoculated onto C/E CEF. Positive results in the form of confluent transformed monolayers are obtained between three and five days. The potential use of this technique to identify LLV shedders was rapidly identified and was used in field situations as a first step in leukosis eradication (Rispens and Long, 1970).

d. Phenotypic mixing (PM) test

Phenotypic mixing (PM) is the interaction between genetically different but related viruses infecting the same cell, with the formation of progeny virus which carries coat proteins of both parental types but containing the genome of only one parent. In the case of avian leukosis virus assays, an RSV is always used as an indicator since leukosis viruses are non-transforming in vitro. PM of RSV occurs when viruses of subgroups A and B are simultaneously propagated at high multiplicity of infection (Vogt, 1967c). This PM expands the host range of the viruses since viruses of subgroup A which are normally excluded from C/A cells can penetrate these cells, and viruses of subgroup B, normally excluded from C/B cells can also penetrate the latter cells. This ability to overcome the host range barrier is due to the presence of

envelope antigens of both subgroups on single virus particles (Vogt, 1967c).

PM was used to recognize that endogenous viruses could be induced by physical and chemical carcinogen (Weiss et al., 1971) treatment of gs- chicken cells, providing evidence that normal chicken cells contain the complete viral DNA genome in a repressed state. Graf (1972) used the PM test to detect latent leukosis viruses of subgroup E by using an RSV of subgroup A, free of subgroup E contaminants as an indicator on CEF under test. Supernatant fluids from these cultures were transferred to chicken cells of the C/A phenotype that were known to be free of subgroup E viruses. The test was described as being relatively simple to perform and having a wide range of sensitivity that with proper manipulation would be able to detect avian RNA tumor viruses from all known subgroups (Graf, 1972). More recently, the technique has been thoroughly described as a method to detect avian leukosis viruses of subgroups A, B, C, and D (Okazaki et al., 1975) in heparinized blood samples, plasmas, and embryo extracts. RSV (RAV-0), a subgroup E sarcoma virus used as a transforming agent, and the sample containing the leukosis virus were cocultivated in C/O cells in order to produce phenotypically mixed progeny that were then assayed on C/E cells, so as to allow exclusion of RSV (RAV-0) and growth of the phenotypically mixed isolated virus. Although the PM was comparable in sensitivity to the complement fixation

test for avian leukosis viruses (COFAL) test, much clearer distinction between a slight positive and a negative sample obtained by the PM test made it superior to the COFAL test.

e. Complement fixation test for avian leukosis (COFAL test)

Hamsters bearing tumors, induced by the Schmidt-Ruppin strain of RSV have antibody in their sera against antigens prepared from materials infected with oncornaviruses (Huebner et al., 1964). Sarma et al. (1964) found that these antibodies specifically react with the group specific (gs) antigen of lymphoid leukosis viruses and that this test was particularly useful since it permits the detection of non-cytopathogenic leukosis viruses growing in cell cultures. Since the technique is a complement fixation test for avian leukosis, it has since been known as the COFAL test. The gs antigen was often detected in cultures within eight days of infection with end points usually attained within two weeks. In these workers hands, the COFAL test appeared to be as sensitive as the RIF test. Subsequent studies centered on the production of COFAL antibodies in pigeons (Sazawa et al., 1966; Sarma et al., 1969; Watanabe, 1970). It was reported that pigeon COFAL antibody had certain advantages over hamster antibody such as; rapidity and easiness in its production, better yields, no need to consider the age factor for infection with RSV. Watanabe (1970) used the pigeon antibody in the COFAL test as an aid in the differential diagnosis of LL from MD.

The gs reactivity was originally thought to be due to a single antigen but, it was later shown to contain several serologically distinct polypeptides (Duesberg et al., 1968; Fleissner, 1971). Final evidence for the serological reactivity of the internal polypeptides of the avian oncornaviruses was provided by preparing specific antisera to internal polypeptides of avian myeloblastosis virus (AMV). It was confirmed that four major internal viral proteins possessed gs determinants, but p19 also possessed type specific reactivity as do the virion surface glycoproteins (Bolognesi et al., 1975; Stephenson et al., 1975).

A comparative study of complement fixation, radioimmunoassay and the reverse transcriptase assay indicates that all three procedures are about equal in sensitivity and, that the use of the appropriate technique is determined by the availability of reagents rather than by the sensitivity of the test (Smith et al., 1977). The high sensitivity and low cost of the complement fixation test make it a desirable test to use in LL epidemiology and research.

f. Radioimmunoassay (RIA)

In the RIA for avian oncornaviruses, a highly purified internal polypeptide usually from AMV is labeled with ^{125}Iodine and reacted with a specific antiserum so as to standardize the number of counts precipitated by the optimal dilution of the antiserum. In the test itself, the optimal

dilution of antiserum is incubated with the unlabeled gs antigen-containing sample for a short time, and then, a specified number of counts of the iodinated probe is added to the mixture and incubated overnight. An antibody to the first antiserum is added to the reaction and a double precipitation is obtained. The results are interpreted depending on the number of counts displaced by the unlabeled gs antigen. A significant reduction in the recoverable counts means that gs antigen is present in the test sample and that it has competed for the specific antibody with the iodinated polypeptide.

A highly specific RIA was developed to detect gs antigen in CEF (Stephenson et al., 1973; Suni et al., 1973). Reactivity was obtained with several strains of the avian leukosis/sarcoma group of viruses but not with mammalian murine leukemia viruses. The technique seemed to be more sensitive than the complement fixation (CF) by about 10-100 fold for detecting gs antigen in normal CEF (Stephenson et al., 1973). Moreover, low level CF positive or CF negative CEF may be found to contain detectable levels of gs antigen by RIA. In other workers hands, the RIA detects as little as 1 ng/ml of the major gs antigen (p27) and was calculated to be 1000 times more sensitive than the CF test (Suni et al., 1973). Epidemiological studies with the RIA performed on tissues and cultured cells from a leukosis-free flock of Brown Leghorn chickens indicated that gs antigen could be detected at lower levels

than in tissues from infected chickens (Vaheri and Ruoslahti, 1973). Furthermore, cell-free media collected from experimentally infected CEF contained high levels of gs antigen, independent of virus subgroup or whether the virus used was a transforming or a non-transforming agent (Sandelin et al., 1974). In these experiments, neigher RIF nor RIA correlated well with the COFAL test. Bolognesi et al. (1975) used the RIA to demonstrate that p27, p19, p15, and p12 internal polypeptides of AMV each contains gs reactivity. Antisera raised in rabbits against individual virus polypeptides reacted at higher dilutions in the RIA than in the CF test. A comparative study of RIA, COFAL, and reverse transcriptase assays showed no significant differences in the sensitivities of these techniques (Smith et al., 1977).

g. Reverse transcriptase or RNA-dependent DNA polymerase (RDDP) assay

The existence of an RNA-dependent DNA polymerase in the virions of RSV-or AMV-infected cells was independently demonstrated by Temin and Mizutani (1970) and Baltimore (1970). The significance of these findings is that the enzyme catalyzes RNA tumor viruses replication through a DNA intermediate and not through an RNA intermediate as do other RNA viruses. Thus, Temin's hypothesis (1971) that the central dogma of molecular biology (DNA→RNA→Protein) does not hold in the case of RNA tumor viruses was supported. The findings also support the DNA "provirus" hypothesis of

Temin (1964) that RNA tumor viruses replicate through a DNA genome integrated in the cell DNA, but have an RNA genome inside the virions (Temin and Mizutani, 1970). The RNA-dependent DNA polymerase is also referred to as reverse transcriptase and can be assayed by studying either the endogenous or the exogenous reaction (Temin and Baltimore, 1972). In the endogenous reaction, disrupted virions are incubated with substrates such as deoxyribonucleosides triphosphates and magnesium in the absence of any added template, and synthesis of DNA is studied using the RNA present in the virions as template. In the exogenous reaction, synthetic templates are mixed with the disrupted virions and the nucleic acids are usually copied at a rate much higher than the endogenous rate. The RDDP assay was found to be as equally sensitive as RIA or direct CF for detecting virus infected cultures. However, it was noted that cells from virus-free chicken lines may express gs antigen detected by direct CF and RIA and consequently, the RDDP assay would be a more reliable indicator of virus production than the direct assays (Smith et al., 1977).

h. Fluorescent focus assay

CEF cultures infected with RAV's show intense specific fluorescence when stained with labeled antiserum to an RSV stock which contains both RSV and RAV (Rubin and Vogt, 1962). This fluorescence is localized at the cell membrane and in the cytoplasm and is detected on the

second day after infection increasing until the fourth or fifth day. The assay has been modified to localize viral antigens in CEF infected with AMV (Vogt and Rubin, 1963). AMV, like most other LLV's, productively infects CEF without inducing transformation and, consequently, virus multiplication cannot be morphologically recognized. In order to obtain a quantitative response, low multiplicities of infection are used to infect the CEF and the cultures are stained four days after infection. In another group of experiments, it was then shown that the relative sensitivity of the fluorescent focus assay (ratio of fluorescent forming units or FFU to infectious virus) was 24, i.e., one of 24 infectious units registered as a focus former (Vogt, 1964) and that the defectiveness of the BH-RSV could also be demonstrated by the direct fluorescent antibody technique using anti RSV chicken serum, by the failure to stain single foci of transformed cells and by their ability to acquire specific fluorescence when superinfected with a RAV.

Methods for Assaying Lymphoid Leukosis Antibody

a. Neutralization test

The assay relies on the presence of antibodies in the plasma or serum of chickens that will specifically neutralize the transforming effects of RSV. Since avian sarcoma and leukosis viruses are considered to be immunologically interrelated (Rubin, 1962; Solomon et al., 1966)

and the neutralization activity against a sarcoma virus of one subgroup is indicative of the neutralization activity against leukosis viruses of the same subgroup (Vogt and Ishizaki, 1966b). RSV's representative of each subgroup have then been used as indicators of virus infection by induction of transforming since leukosis viruses are usually nontransforming in vitro (Rubin, 1960), and cause neoplasia in vivo only after a long incubation period (Burmester and Gentry, 1956). The first clear indication that the serum of some adult chickens did contain neutralizing antibodies to RSV was provided by Duran-Reynals (1940), who could neutralize some of the in vivo oncogenic effect of an RSV stock by incubating virus-serum mixtures and injecting them under the skin of the breast muscle of adult Plymouth Rock chickens. The sera from chickens that had regressed Rous sarcoma tumors had higher neutralizing activity than sera from adult normal chickens. Later, the development of an efficient and precise in vitro assay for RSV (Temin and Rubin, 1958) and the standardization of a serum neutralization test (Ishizaki and Vogt, 1966) that could be performed in vitro facilitated epidemiological studies on prevalence and distribution of humoral antibodies to LLV's in commercial chicken flocks. Since it was also recognized that neutralizing antibodies react with envelope components of the sarcoma and leukosis viruses and is, therefore, subgroup specific (Ishizaki and Vogt, 1966), numerous studies have been performed to assess the prevalence of leukosis viruses of different

subgroups in various parts of the world (Calnek, 1968; Churchill, 1968; Speck, 1971; Sandelin and Estola, 1974).

b. Fluorescent antibody test (FA)

The FA test, like the serum neutralization test detects antibodies directed to the viral envelope components and it is, therefore, subgroup specific (Ishizaki and Vogt, 1966). The direct FA test was originally used to aid in the immunological differentiation of avian tumor viruses. For technical reasons, the direct FA test is not easily applied to field studies of LL antibodies, since it would necessitate conjugation of every sample with the fluorochrome fluorescein isothiocyanate (FITC). The indirect FA test, then, was developed to detect antibodies in the serum and egg yolk of hens (Aulisio et al., 1967). The test can be performed on RSV or LLV infected CEF and if the cultures are fully infected before the serum or egg yolk samples are available for testing, the results are obtained in a matter of hours. The sera of hens cannot be satisfactorily used at less than 1:40 dilution because of non-specific fluorescence, but, a 1:8 dilution of egg yolk or a four fold dilution of yolk extract as described by Aulisio and Shelokov (1967) are free of non-specific fluorescence and contain adequate levels of antibody. The FA titer in the egg yolk is generally lower than that found in the serum of hens from RIF (LLV)-infected flocks. Antibodies have not been demonstrated in

the serum or egg yolks of hens from RIF-negative flocks using the indirect FA test (Aulisio and Shelokov, 1967).

Epizootiology

LLV's are ubiquitous in nature and commercial flocks are nearly all infected with viruses belonging to one or more subgroups. In the USA, viruses belonging to subgroup A are the most prevalent in the field, while viruses of subgroup B occur less frequently and always in combination with subgroup A viruses (Calnek, 1968). Viruses of subgroup A, probably of different envelope antigen types have been isolated from field outbreaks in England (Churchill, 1968), while antibody testing in Germany has shown a prevalence of subgroup A viruses with subgroup B viruses occurring rarely (Speck, 1971). However, field surveys performed in Finland have indicated that antibodies to viruses of subgroups A, B, C, and D are widespread and are usually found in the same flock (Sandeline and Estola, 1974). No data on the frequency of occurrence or distribution of endogenous viruses, mainly RAV-0 of subgroup E are yet available (Crittenden, 1976).

LLV's are known to spread by various mechanisms. These routes of transmission have been defined as, a) congenital or vertical, b) horizontal or by contact, c) genetic transmission.

a. Congenital or vertical transmission

One of the first lines of evidence for the vertical transmission of LLV's was provided by Cottral et al. (1949), who induced high levels of LL by inoculating chickens with embryo cell suspensions. Further studies confirmed that the agent of LL was present in embryonic and newly hatched chick tissue and that the hens laying these eggs or producing such chickens were carriers of the disease agent (Cottral et al., 1954; Burmester et al., 1955). Burmester and Waters (1955) found that although the virus of LL is transmitted vertically, this did not necessarily result in high incidence of meoplasms in infected progeny. However, the progeny were a source of horizontal infection to chicks lacking specific antibodies.

The breakthrough in the understanding of the epidemiology of LL was facilitated by the development of the RIF (an acronym for resistance inducing factor and synonym with LLV) test for LLV's (Rubin, 1960), and by the elucidation of the pattern of congenital infection in chicken flocks (Rubin et al., 1961). It was shown that in an infected flock, hens could be RIF-viremic or non-viremic and that those that were viremic did not produce RIF antibodies and consistently transmitted the infection through the egg to their progeny. Congenitally infected progeny, then, developed a permanent viremia and were immunologically tolerant to the congenitally transmitted LLV. Many chickens that acquire the infection congenitally

developed normally suggesting a relative avirulence of RIF at the cellular level. LL tumors were not produced until the infected progeny reached sexual maturity and then, only a fraction of the infected birds died. Rubin et al. (1961) suggested that this relatively benign behaviour of the virus would favor its perpetuation in nature by repeating the cycle of vertical transmission and by being a rich source of continuous virus for the spread to contact susceptible chickens. Viral multiplication can also persist indefinitely in the germ cells of certain hens that possess high levels of neutralizing antibodies in the serum. These hens will also infect a certain proportion of their progeny by the congenital route (Rubin et al., 1962). Congenitally infected progeny have a higher death rate from neoplasms than progeny from non-viremic hens (Rubin et al., 1962). Viremic tolerant roosters consistently failed to infect their progeny when mated to negative hens (Rubin et al., 1961). This failure occurred in spite of the fact that the testicular cells were heavily infected with leukosis viruses. DiStefano and Dougherty, (1968) in a detailed electron microscopic study of the reproductive organs of congenitally infected male embryos and roosters found that there was viral multiplication in all connective tissue elements, in smooth muscle within walls of tubular structures and in non-germinal epithelial cells. No evidence of viral

multiplication could be found in the germinal cells of both congenitally infected male embryos and adult roosters.

b. Horizontal or contact transmission

Chickens that have not been infected through the egg may become infected after contact with congenitally infected chickens (Rubin et al., 1962). Infected chickens are known to excrete LLV in the saliva, feces or droppings (Burmester, 1956a), and to contaminate their surroundings. The resulting contact or horizontal infection probably takes place by a variety of routes (Burmester and Gentry, 1954) including any mucous membrane normally exposed to the external environment. The upper respiratory tract appears to be particularly susceptible. Infection by the natural routes usually requires larger doses of virus to produce tumors than required for artificial exposure. Rubin et al. (1962) reported that the antibody levels found in the egg yolk or serum of one day-old chicks obtained from antibody positive dams was only one-tenth to one-hundreth that of the dams. However, naturally occurring antibody of maternal origin delays the onset of infection in chickens exposed to viremic contacts (Witter et al., 1966). This passively transferred antibody could be detected up to three weeks of age in 20 percent of the progeny of antibody-positive RIF-negative dams. Horizontal transmission is presently considered of minor significance in the epidemiology of LL (Purchase and Burmester, 1977).

c. Genetic transmission

Genetic transmission occurs when the information that codes for the expression of virus or partial viral products is transferred vertically at a site within the cell chromosomes in the form of integrated DNA.

The first indication that genetic transmission occurred was obtained when embryos from LLV-free hens were found to contain gs antigen when tested by the COFAL technique (Doughe-ty and DiStefano, 1966; Dougherty et al., 1967). Cross-breeding experiments between the Reaseheath I line (gs+) and the Reaseheath C line (gs-), both highly inbred, LLV negative lines confirmed the expression of gs antigen in normal chicken cells and established that this expression was controlled by a single autosomal Mendelian locus with a dominant allele for gs antigen expression (Payne and Chubb, 1968). Soon, it was realized that a second genetic marker did exist, when it was found that cells from certain normal chick embryos could complement the defectiveness of BH-RSV (Hanafusa et al., 1970a) by acting like a helper virus and providing envelope antigens in which the BH-RSV is defficient. This agent was named chick helper factor (chf) and is in all probability the envelope antigen for subgroup E viruses (Hanafusa et al., 1973). Thus, gs antigen and chf are partial expressions of endogenous viral genomes.

The production of RAV-0, now recognized as an endogenous virus with subgroup E characteristics was first

observed by Vogt and Friis (1971). These workers suggested that RAV-0 represented the spontaneously activated form of the genetically transmitted genome. Weiss et al. (1971), used pheasant cells in order to amplify the titer of virus released from gs+ non-producer cells after treatment with X-rays. It was found that chemical mutagens and carcinogens as well as ionizing radiation could induce the release of endogenous RAV-0. Additional evidence for the presence of viral DNA genome of LLV's has been provided by nucleic acid hybridization studies. Viral DNA has been found in normal uninfected cells apparently, integrated into the host cell genome (Baluda, 1972; Neiman et al., 1975). It has now been shown that, at least, in the RPRL line 7, that a single dominant gene (V) controls RAV-0 production (Crittenden and Robinson, 1976).

Relevant to the genetic transmission of LLV's are the viral oncogene (Huebner and Todaro, 1969) and the protovirus (Temin, 1971) hypotheses. The viral oncogene hypothesis proposes that all cells contain in their DNA the information necessary to specify the complete genome of an RNA tumor virus (the virogene). Part of the virogene is the oncogene, which is the segment responsible for transformation. The protovirus hypothesis proposes that reverse transcription by a cellular reverse transcriptase allows cellular RNA to serve as a template for the new DNA. This new DNA could become integrated into the DNA of the same or adjacent cells. Cancer, then, would result from

variation from the normal physiologic evolution of protovirus DNA either through its mutation or integration at an incorrect site in the cellular genome. A major prediction of the protovirus hypothesis is that type-C viruses will ultimately be produced as a consequence of the mistranscription.

Control

Several methods for the control of lymphoid leukosis have been suggested and they are discussed below.

a. Eradication

By our present knowledge, eradication consists of the elimination of LLV's by extensive testing of dams to identify virus shedders that will be subsequently eliminated from the flock. Since the biological infection cycle of LLV's is reasonably well understood (Purchase, 1969b) the feasibility of eradicating LLV's from commercial operations is becoming practical. The most important source of LLV is the congenitally infected tolerant dam (Rubin et al., 1961). These dams are hatched viremic through vertical transmission, probably remain viremic for life and are immunologically tolerant to LLV. Thus, they do not produce neutralizing antibody and are unable to combat the infection. These hens also perpetuate the infection through vertical transmission and are a constant source of horizontal infection by excreting virus in the saliva and droppings (Burmester, 1956a). Virological techniques designed to

identify these shedders are currently used in eradication efforts. Using dams with circulating antibodies as the source of embryonated eggs, pretesting the embryos from selected dams by the RIF test to demonstrate a non-shedder state, and avoiding indirect spread of virus to eggs or chicks (Hughes et al., 1963), a small flock free of LLV's was developed in a single generation. Serum neutralizing tests performed when the oldest progeny were 26 weeks old were all negative. Zander et al. (1975) reported the eradication of LLV's of subgroups A and B from a small nucleus of breeder chickens selected from a large breeding flock of White Leghorns. The testing of serum samples for RIF activity on dams from successive generations was used as a tool to identify viremic hens. These viremic hens were then eliminated from the flock, and progeny were only obtained from the remaining negative dams. This flock was considered to be free of LLV after four generations of intensive testing. Failures in eradication occur if viremic dams are missed or if non-viremic dams are shedding (Zander et al., 1975). Serum neutralization tests for detecting antibodies in successive generations of commercial stocks maintained in conventional environments is mandatory to determine the ultimate success of present eradication procedures. It has been suggested that eradication is probably the method of choice for the control of LL but it is still prohibitively expensive (Calnek et al., 1967). The technology presently available

to achieve a virus-free flock is, time consuming, complicated, expensive, and is not yet applicable for large scale use (Purchase and Burmester, 1977).

b. Breeding for resistance to infection

A cell is resistant to infection when LLV fails to penetrate it. This type of resistance is commonly referred to as "first line of resistance" (Burmester and Purchase, 1970) and is basically a block to viral envelope functions since virus adsorbs quite readily to the cell membrane of resistant cells but fails to penetrate (Piraino, 1967). However, if the viral genome is introduced into the cell, the resistant cells can produce progeny of the excluded viral subgroup (Crittenden, 1968). This cellular resistance has been shown to have a genetic basis (Prince, 1958) and not to be due to humoral antibodies when chorioallantoic membranes from susceptible and resistant embryonated eggs were challenged with an RSV stock.

Waters and Burmester (1961) inoculated crosses and backcrosses of susceptible and resistant chickens by the intracranial route and demonstrated that genetic susceptibility to RSV was a dominant trait over resistance, and that this dominant trait was dependent upon a single pair of autosomal genes for expression. Crittenden et al. (1963) showed that genetic variability to susceptibility to RSV not only occurs in chicken embryos and in chickens but also CEF, and that there was a correlation between

genetic susceptibility as measured by all three methods. These workers confirmed by crossing and backcrossing susceptible and resistant lines that susceptibility to RSV was a dominant trait and that a single dominant gene influences *in vivo* and *in vitro* susceptibility to RSV (Crittenden et al., 1964). Presently, it is known that four genetic loci are involved in the inheritance of resistance to the five subgroups of RSV (Crittenden, 1975). Three of the loci are called tumor virus (*tv*) loci and possess recessive alleles for resistance to infection by subgroups A through E. The fourth locus has a dominant allele for resistance. Two single autosomal recessive genes control resistance to infection by RSV belonging to subgroups A and B respectively (Crittenden et al., 1967). The *tva* locus controls resistance to subgroup A, the *tvb* locus resistance to subgroups B, D, and E (Motta et al., 1973; Crittenden, 1975; Pani, 1975) and the *tvc* locus resistance to subgroup C (Payne and Biggs, 1970). An independent autosomal dominant gene (I^e) controls resistance to subgroup E alone (Payne et al., 1971). Pani (1976) believes that an independent *tve* locus exists. Further experiments must be conducted to differentiate between the hypotheses proposed by Pani and Crittenden (1975). The practicability of breeding for resistance to infection in breeder flocks depends on whether alleles for resistance occur at a reasonable frequency in the breeding stock.

In a survey of commercial lines, Crittenden and Motta (1969) found that chorioallantoic membranes (CAM) from heavy breeds had a relatively high degree of resistance to BH-RSV (RAV-2), a subgroup B virus, while CAM's from white-egg stocks were consistently susceptible to BH-RSV (RAV-1), a subgroup A virus, and CAM's from heavy breed stocks showed genetic variability to BH-RSV (RAV-1). Most white-egg stocks were susceptible to subgroup C (Pr-RSV) while the heavy breeds were more resistant. These results also suggested that unidentified sources of genetic variability may exist in non-inbred commercial lines and that there may be a variety of alleles for susceptibility or resistance at each locus (Crittenden and Motta, 1969). CAM's of crosses and backcrosses of commercial white-egg stocks with the double recessive line 7 showed a bimodal distribution of pock counts for both RSV (RAV-1) and RSV (RAV-2) and confirmed previous findings that white-egg stocks are more resistant to subgroup B viruses than to subgroup A viruses and that single gene segregation probably occurred in these stocks (Motta et al., 1973).

Resistant alleles to subgroup A viruses, the most common exogenous LLV's naturally found under field conditions can be introduced into chicken stocks with the resulting reduction in the rate of infection and the incidence of LL (Crittenden, 1975). The frequency of these alleles will determine the proportion of dams that will be resistant. These dams will not be infected by subgroup A

viruses and consequently will not form antibodies against viruses of the same subgroup (Crittenden, 1975). If these dams are mated to sires carrying dominant alleles for susceptibility, chicks which are highly susceptible to subgroup A virus infection and free of maternally derived neutralizing antibody will be produced, perhaps resulting in a higher rate of LL mortality (Rubin et al., 1962; Crittenden, 1975). In view of this, it has been said that chickens that are completely susceptible or completely resistant to infection are likely to show a lower incidence of LL than chickens in which viremia can be acquired, maintained and reestablished because of the occurrence of maternal-antibody negative susceptible chickens (H. C. Löliger and D. Harris, quoted by Crittenden, 1975).

c. Breeding for resistance to tumor development

Genetic resistance to tumor development (Gyles et al., 1968) must be differentiated from genetic resistance to viral infection (Rubin, 1965). Technical difficulties inherent to the long incubation period of LL tumors have hindered research to elucidate the mechanism of genetic resistance to the development of LL tumors. Therefore, Rous sarcoma tumors have been used as models to study genetic resistance. On the basis of host reactivity to RSV tumors, chickens have been classified into, a) those with no tumors, presumably genetically resistant to infection, b) those with low genetic resistance that develop

progressive tumors, c) those with high genetic resistance that retrogress their tumors to normal tissue and, d) those that achieve a reduction in tumor size, namely regressors (Gyles et al., 1968).

Studies performed by Gyles and Brown (1971) have shown that selection within a closed flock may substantially increase the percentage of chickens exhibiting a retrogressive tumor response. Regression studies with RSV-induced tumors have suggested that regression could be the result of an immunological response induced by antigens common to tumor tissues (Cotter et al., 1973b). In these studies, line 6 proved to be susceptible to infection by viruses of subgroups A, B, and C but also showed high levels of resistance to tumor development. Lines 15 and 6 from the RPRL are both inbred lines susceptible to virus infection (First line of defense). However, line 6 shows higher levels of regression (second line of defense) than line 15, apparently by resisting tumor development (Crittenden, 1975). Line 6 was also shown to be resistant to the development of the transplantable tumor RPL-16 (El Dardiry et al., 1952). The understanding of genetic resistance is far from adequate. Preliminary work indicates that this resistance may be mediated by an immune response to tumor associated antigens localized on the membrane of the transformed cells (Sjögren and Jansson, 1970). This immune response against tumor development is believed to be cellular in nature and is probably directed against tumor

specific transplantation antigens (TSTA) of glycoprotein in nature and of 100,000 daltons molecular weight (Bauer et al., 1976). More knowledge on the frequency of regression in various lines of chickens may be useful in developing breeding programs designed to increase resistance to neoplastic disease (Cotter et al., 1973a).

d. Vaccination

Burmester (1955) showed the value of specific passive immunity to LL as a result of vaccination of dams. The progeny obtained from LL susceptible dams that had been immunized at eight months of age by multiple injections of live LLV, were 3000 times more resistant to the development of LL tumors than the progeny obtained from the same dams before immunization (Burmester et al., 1956). When formalin-killed or beta propiolactone-treated virus were used as immunizing agents, the increase in resistance was in the range of 200-600 times. In another group of experiments it was shown that chickens that are genetically resistant to subgroup A viruses respond very poorly or not at all with neutralizing antibodies to challenge with subgroup A viruses (Crittenden and Okazaki, 1966) while most of the known susceptible chickens respond well. These results indicate that for neutralizing antibodies to be produced, viral multiplication is necessary. Löliger and VanDem Hagen (1974) have confirmed that the progeny of LLV-immunized dams from a susceptible line were highly

protected against a challenge with the same virus strain. The results were dependent on viral susceptibility and competence of the chickens. More recently, vaccination with virulent LLV of subgroups A and B has been used to control LL in the progeny of dams selected from an initially infected commercial flock (Rispens et al., 1977). Non-shedder dams were selected after extensive testing of pedigreed embryonated eggs using the NP test. Chicks hatched from negative dams were reared in isolation until eight to ten weeks of age, at which time they were vaccinated with a leukosis virus and then transferred to a conventional environment. Rispens et al. (1977) claimed that if this procedure was repeated for at least two generations, elimination of LL was achieved.

e. Elimination of target cells

Since the bursa of Fabricius is the target organ for LL transformation (Cooper et al., 1968), procedures to remove or induce regression of the bursa have been practiced in order to control the disease. Unfortunately, most if not all procedures used have disadvantages that preclude their application under field conditions. Surgical bursectomy (Peterson et al., 1966a) is impractical because of the problems inherent to the technique and also because there is an increase in oeteopetrosis and non-specific mortality, and decrease in body weight (Purchase and Burmester, 1977). Treatment of embryos or newly hatched chicks with

testosterone propionate and methyl testosterone (Burmester, 1969) induces significant bursa regression but results in poor hatchability, increase in non-specific mortality and permanent masculinization of hens with serious adverse effects on egg production. Although the role of naturally occurring IBA in the prevention of LL tumors in the field is not known (Purchase and Cheville, 1975; Purchase and Burmester, 1977) recent experiments have shown that vaccination with pathogenic bursatrophic IBA of RAV-1 infected chickens significantly reduced or eliminated LL tumors (Purchase and Cheville, 1975; Cheville et al., personal communication). However, IBA-infected chickens in which extensive destruction of bursal lymphoid tissue had occurred did not develop a good vaccination immunity to other avian infectious agents (Allan et al., 1972; Giambrone et al., 1976). This temporary state of poor immunological reactivity could jeopardize the health of an entire flock in the presence of endemic infectious agents.

MATERIALS AND METHODS

Chickens for In Vivo Studies

The chickens used were a cross of inbred lines 15I by 7 ♀, line 6 subline 1 (6_1), and line 15I (Stone, 1975) maintained at the Regional Poultry Research Laboratory (RPRL) free of most common poultry pathogens or, commercial Single Comb White Leghorns (SCWL) and Spafas (Spafas Inc., Norwich, CT) chickens.

Isolation Facilities

The housing of chickens varied according to the experiment in question and it is defined under the experimental design of each experiment. Generally, chickens were reared in filtered air positive pressure (FAPP) plastic isolators, negative pressure Horsfall Bauer type isolators, chick brooder batteries in rooms with partially controlled air flow or in commercial type rearing or laying cages.

Cell Cultures for In Vitro Studies

Chicken embryo fibroblasts (CEF) were of the C/O and C/E phenotypes. Japanese quail embryo fibroblasts (QEF) were of the C/BCD phenotype. Duck embryo fibroblasts (DEF) were used for the propagation of the Herpes virus of turkeys

(HVT FC 126) and the JM strain of the Marek's disease Herpes virus (JM-MDV). All embryos were from a flock free of congenital infection with exogenous LLV's.

Embryonated Eggs

Embryonated hen's eggs of the RPRL were used to propagate and titrate avian viruses other than lymphoid leukosis (LLV) or Rous sarcoma (RSV).

Tissue Culture Dishes

Tissue culture plastic dishes (Falcon, Oxnard, California) for primary ce-1 cultures were 150 x 25 mm; secondary cultures were grown in either 60 x 15 mm plastic dishes or in 35 x 10 mm dishes.

Tissue Culture Media

Tissue culture media for the propagation, growth and maintenance of CEF, QEF and DEF were a mixture of medium 199 and Ham's F10 medium (Microbiological Associates, Bethesda, MD). Basal medium Eagle (BME) with Earle's salts and L-glutamine (International Scientific Ind. Inc., Gary IL) was used as a nutritive medium for splenocytes in the Jerne's hemolytic plaque assay. RPMI 1640 medium with L-glutamine (International Scientific Ind. Inc., Gary, IL) was used to culture blood leukocytes in the blastogenesis assay.

Trays for the Jerne's Hemolytic Plaque Assay

Trays for the hemolytic plaque assay were rectangular in shape and were made of solid plastic. The trays measured 15 1/2 x 7 1/2 inches. Each of the two areas in one tray for the lodging of slides was 15 1/2 inches long, 2 1/4 inches wide, and 1/32 of an inch in height.

Mibolerone

The androgen analog mibolerone (17β- hydroxy-7α, 17 dimethylestr-4-en-3-one) was obtained from the Upjohn Company, Kalamazoo, MI. The androgen anolog was in a crystalized form and was dissolved in 100 percent ethyl alcohol immediately before treatment of the feed.

Viruses

a. Rous associated virus-1 (RAV-1)

RAV-1 was a subgroup A LLV originally obtained from P. K. Vogt as an end-point purified virus.

b. Rous associated virus-2 (RAV-2)

RAV-2 was a subgroup B LLV originally obtained from P. K. Vogt as an end-point purified virus.

c. Bryan High titer-Rous sarcoma virus Type 1 (BH-RSV(RAV-1))

RSV(RAV-1) (Rubin and Vogt, 1962) was a stock of the Bryan High (BH) -titer strain of Rous sarcoma virus (RSV) activated by RAV-1 from RSV^- cells and belonged to subgroup A of the leukosis/sarcoma group of viruses (Crittenden, 1976).

d. Bryan High titer-Rous sarcoma virus Type 2 (BH-RSV(RAV-2))

RSV(RAV-2) (Hanafusa, 1965) was a stock of the BH-RSV activated by RAV-2 from RSV^- cells and belonged to subgroup B of the leukosis/sarcoma group of viruses (Crittenden, 1976).

e. Rous sarcoma virus Type 0 (RSV(RAV-0))

RSV(RAV-0) (Vogt and Friis, 1971) was a stock of RSV activated by RAV-0 from RSV^- cells and belonged to subgroup E of the leukosis/sarcoma group of viruses (Crittenden, 1976).

f. Viruses for the Marek's disease trial

The herpes virus of turkeys (HVT) FC 126 (Witter et al., 1970; Purchase et al., 1970; Okazaki et al., 1970) was a cell associated vaccine obtained from the RPRL virus stocks. The JM strain of Marek's disease herpes virus (JM-MDV) (Sevoian et al., 1962; Witter and Burmester, 1967) was used to challenge HVT-vaccinated chickens.

g. Viruses for the Newcastle disease trial

The B1-LaSota strain (Hitchner and Johnson, 1948; Winterfield et al., 1957) of Newcastle disease virus (B1-LaSota-NDV) (Abbott Laboratories, N. Chicago, IL) was a chicken embryo propagated vaccine. The Texas GB strain (Hanson, 1972) of NDV (Texas-GB-NDV) was propagated in chichen embryos and was supplied by Dr. S. B. Hitchner, New York State University, Ithaca, NY.

h. Viruses for the infectious laryngotracheitis trial

The vaccine against avian infectious laryngotracheitis (ILT) was a modified live virus (Gelenczei and Marty, 1964) of CEF origin (American Scientific Laboratories, Madison, WI). The challenging strain of ILT (Hitchner and White, 1958) was the Boudreau virulent strain and was obtained through the courtesy of Dr. S. B. Hitchner.

i. Viruses for the infectious bronchitis trial

The infectious bronchitis virus (IBV) vaccine (American Scientific Laboratories, Madison, WI) contained the Massachusetts and Connecticut types (Jungherr et al., 1956) and was a live vaccine of chicken embryo origin. The IBV Massachusetts 41 (Hofstad, 1972) virulent strain was used to challenge vaccinated chickens and was obtained from Dr. S. B. Hitchner.

j. Viruses for the avian pox trial

The pigeon pox vaccine (Seeger and Price, 1956) was live virus of chicken embryo origin (Abbott Laboratories, N. Chicago, IL). The virulent fowl pox virus (Seeger and Price, 1956) for the challenging of vaccinated chickens was obtained from Dr. D. R. Wenger, Veterinary Services Labs., Ames, IA, and was of chicken embryo origin.

k. Fowl cholera trial

The fowl cholera bacterin contained strains 1059, 1662, and X-73 of Pasteurella multocida (Salsbury Inc.,

Charles City, IA). The virulent strain X-73 (Heddleston, 1961) of Pasteurella multocida for the challenge of vaccinated chickens was obtained through the courtesy of Mr. M. Chengappa, Michigan State University, East Lansing, MI.

Procedures for Preparation of Reagents

a. Cell cultures

Cultures of CEF, QEF, and DEF were prepared and maintained by procedures described by Solomon (1975).

b. Propagation of sarcoma viruses

RSV(RAV-1) and RSV(RAV-2) were propagated in Spafas CEF which were of the C/E phenotype. The supernatants of infected cultures showing advanced transformation were harvested and stored at -70°C in a Revco (Revco Inc., West Columbia, SC) ultra low temperature freezer. The infectious supernatants were thawed, titrated and diluted to the right concentration for neutralization tests. RSV(RAV-0) was propagated in line 100 CEF of the RPRL (C/0 phenotype) or in QEF (C/BCD phenotype). Cultures showing extensive cell transformation were trypsinized and stored at -70°C in tris buffer saline containing 15 percent calf serum. The cell extract was thawed out, titrated and adjusted to the right concentration before infecting line 100 CEF or QEF in the phenotypic mixing (PM) test.

c. Propagation of leukosis viruses

RAV-1 and RAV-2 were propagated in CEF of Spafas embryos (C/E phenotype) and the supernatants were harvested 7-9 days after infection and titrated in the PM test.

d. Propagation of viruses for the Marek's disease trial

HVT FC 126 was propagated as cell associated virus in DEF and when the cultures showed extensive cytopathic changes, the cells were harvested by trypsinization and were frozen slowly in F10-199 medium containing 10 percent dimethyl sulfoxide (DMSO) and 15 percent fetal calf serum to a temperature of -180°C in liquid nitrogen. The JM-MDV strain was propagated as for HVT and the cells were harvested and stored in a similar manner.

e. Propagation of viruses for the Newcastle disease trial

The B1-LaSota strain of NDV was reconstituted in its diluent and was administered following the instructions provided by the manufacturer's. The Texas GV-NDV was propagated in the allantoic cavity of 10 day-old embryonated hen's eggs and the allantoic fluid of embryos dying between 48-72 hours after infection was harvested, stored at -70°C and titrated in embryonated eggs to calculate the embryo infective $dose_{50}$ (EID_{50}).

f. Propagation of viruses for the infectious laryngotracheitis trial

The modified ILT virus was reconstituted and administered following the instructions specified by the

manufacturer. The virulent ILT Boudreau strain was inoculated on the chorio-allantoic membrane (CAM) of 12 day-old embryonated hen's eggs. Seven days later, those membranes showing ILT virus plaques were harvested, ground in phosphate buffer saline (PBS) and frozen at -70°C. The virus was titrated on the CAM of embryonated eggs prior to challenge.

g. Propagation of viruses for the infectious bronchitis trial

The IB virus vaccine was reconstituted following the manufacturer's instructions and was titrated in the allantoic cavity of 10 day-old embryonated hen's eggs. The virulent Massachusetts 41 strain of IBV was also propagated in the allantoic cavity of 10 day-old embryonated hen's eggs. The allantoic fluids of embryos dying 4-5 days after infection were harvested, frozen at -70°C, and titrated prior to use to calculate the EID_{50}.

h. Propagation of viruses for the avian pox trial

Pigeon pox vaccine was reconstituted and administered following the manufacturer's instructions. The virulent fowl pox virus was propagated and titrated in the CAM of 12 day-old embryonated hen's eggs. The infected CAMs were harvested 6 days after infection, ground in PBS and stored at -70°C. The fowl pox virus was titrated on the CAM to calculate the EID_{50}.

i. Fowl cholera trial

The fowl cholera bacterin was administered following the manufacturer's instructions. The challenging virulent X-73 strain of Pasteurella multocida was grown in tryptose broth enriched with 0.3 percent yeast extract and standardized to contain 250 colony forming units (CFU) per 0.5 ml of challenging inoculum.

j. Antibody to RSV(RAV-1) and RSV(RAV-2)

Antisera to subgroups A and B viruses to be used in the neutralization test to assay for antibodies to LLV were prepared by injecting RSV(RAV-1) and RSV(RAV-2) in the wing web of line 6_1 chickens. Serum was obtained from chickens that developed tumors at the site of inoculation. The sera were tested for subgroup specificity and only those sera that reacted with viruses of the homologous subgroup were used in the neutralization test as positive controls. All sera were heated at 56°C for 30 minutes prior to the test in order to inactivate complement.

k. Antibody to sheep erythrocytes (SE) and Brucella abortus

Chickens were injected via the wing vein with 1 ml of PBS containing 5×10^8 SE and a 1:50 dilution of the standard Brucella abortus tube antigen, USDA, or with 1 ml of PBS containing only 5×10^8 SE. The sera collected 7 days later were tested for the primary antibody response. A second antigenic stimulation was carried out 7 days

later, and the sera collected after 5-7 days were tested for a secondary antibody response. All sera were heated at 56°C for 30 minutes before testing.

1. Rabbit antibody to chicken Ig, IgG and IgM

Commercial rabbit anti-chicken gamma G immunoglobulin (IgG) serum (Nutritional Biochemicals Corporation, Cleveland, OH) was reacted in immunoelectrophoresis against normal chicken serum. Fifteen precipitin bands between the antigamma serum and the fractionated normal serum were cut out and washed daily for 3 days in PBS containing 1/50000 merthiolate. The precipitin bands were emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) and inoculated into a rabbit by the subcutaneous route in five places. After 1 month, the rabbit was boosted with the same antigen in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI). Antiserum prepared this way reacted strongly with IgG and faintly against the gamma M immunoglobulins (IgM). This antiserum was then considered to react with both IgG- and IgM-producing cells and is referred to as anti-Ig. Specific antiserum for chicken IgM was prepared in rabbits. Chicken serum was obtained from chickens with overt LL. The immunoglobulin fraction was precipitated 3 times with sodium sulfate (Na_2SO_4) and then passed through a Sepharose 6B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) packed column. The fractions containing IgM were concentrated, emulsified in

complete Freund's adjuvant and inoculated subcutaneously into rabbits. After 1 month, the rabbits were boosted with the same antigen in incomplete Freund's adjuvant and bled 10 days later. In absorptions to remove anti-IgG contamination, 1 day-old chicken serum was added in a 3 percent volume to the rabbit antisera, incubated at 37°C for 1 hour, then at 4°C overnight and centrifuged at 3000 rpm x 30 minutes. Three absorption cycles were necessary to make the rabbit antisera specific for IgM.

m. Mibolerone

Feed was treated with mibolerone to contain 1.0 µg of mibolerone/, 1.5 µg of mibolerone/, or 2.0 µg of mibolerone/ per gm of feed and was administered during the first 7 weeks of life of the chickens (1.0 µg/7 weeks; 1.5 µg/7 weeks; and 2.0 µg/7 weeks regimens, respectively), or to contain 4 µg of mibolerone /per gm of feed and was administered during the period of 29-49 days of age (4 µg/3 weeks regimen). Feed was treated with mibolerone in batches of 25 kgs at a time. A 10 percent excess of mibolerone was always added as flush, i.e., if the 1.0 µg/7 weeks regimen was used, then, 25 mgs (1 ug/gm of feed) plus 2.5 mgs (10 percent flush) were weighed and dissolved in 50 ml of 100 percent ethyl alcohol. The solution was then sprayed over approximately 300 gms of soy bean meal, left to dry and added to the 25 kgs of standard feed contained in a cement mixer. Finally, the feed was mixed for 3 hours,

at the end of which it was considered to have been treated with mibolerone. Mibolerone-treated feed was utilized within 2 weeks of being prepared. Assays performed on freshly prepared mibolerone feed have shown a mean recovery of 99.5 ± 1.45 SD for a 1.6 µg of Mibolerone/gm of feed regimen (Fred W. Staten et al., personal communication), and a mean recovery of 90 percent plus, if the feed was stored for 2 weeks at room temperature (Marv Ogilvie, personal communication).

Procedures for Performing Assays

a. Obtaining bursa weights

The bursae of Fabricius were dissected out and removed by cutting them at their stalk. Bursae were weighed in a Mettler P 1200 balance (Mettler Instrument Corp., Hightstown, NJ).

b. Histological sections of bursa of Fabricius and cecal tonsils

Sections were prepared from tissues fixed in formol sublimate. Histological sections were 5 µ thick and were stained with haematoxylin-eosin. The number of lymphoid follicles in sections of bursae of mibolerone-fed chickens was estimated by counting all lymphoid follicles observed in a section cut through the center of the regressed bursa. The number of lymphoid follicles in sections of bursae of chickens fed the regular diet was estimated by counting the follicles seen in a section of one plica, when observed

with a 3.5 X ocular and a 10 X objective and multiplying the figure obtained by the average number of plicae contained in the bursa of Fabricius. The number of germinal centers was calculated by counting the number of morphologically normal germinal centers seen in one intact cecal tonsil for both mibolerone- and standard diet-fed chickens.

c. Microagglutination for SE and Brucella abortus

The tests were run in microtiter U plates (Cooke Laboratory Products, Alexandria, VA) containing 12 x 8 wells as described by Wegmann and Smithies (1967). Eight antibody titrations were performed in each plate. Using a 0.025 ml plastic dispenser (Cooke Laboratory Products, Alexandria, VA) one drop of buffer for serum dilutions was placed in each well. Two-fold dilutions of sera were made by picking up 0.025 ml of each of 8 sera with individual microdiluters (Cooke Laboratory Products, Alexandria, VA) attached to a microdiluter handle and placing the microdiluters containing the sera on the far left wells of the microtiter plate. The microdiluters were rotated vigorously so as to attain a good mixing and then carried over to the next column of wells to the right, and so on until the far right wells. A 0.2 percent SE suspension was prepared by washing SE 3 times in PBS at 2000 rpm x 5 minutes each time, and resuspending the SE to the 0.2 percent concentration in SE buffer. Each well then received one drop (0.025 ml) of the SE suspension, the microtiter plates were shaken gently

and agglutination was allowed to proceed at room temperature (68°F) for 3 hours, at the end of which the end points were recorded. To detect antibodies to Brucella abortus, a 1:2 dilution of the tube antigen was made in buffer for Brucella antigen and one drop (0.025 ml) was dispensed into each well in the microplate containing the sera dilutions prepared as for the SE agglutination test. The microplates were shaken to allow dispersion of the Brucella antigen and agglutination was allowed to proceed at room temperature (68°F) for 3 hours, at the end of which the end points were recorded. For both SE and Brucella abortus antibodies, the titers are expressed as the $\log_2$ of the reciprocal of the highest dilution of serum causing complete allutination of either the SE or Brucella abortus antigens.

d. Jerne's hemolytic plaque assay

The hemolytic plaque assay was adapted to the chicken system and was performed on glass slides. One volume of 1 percent agarose (Fisher Scientific Co., Pittsburgh, PA) was mixed with one volume of 2x BME and maintained at 56°C. A 15 percent SE suspension in BME was kept at 4°C. Single cell suspensions were prepared by gently teasing the spleens in 10 ml of cold BME and by making a 1/100 dilution of these spleen cell suspensions. The agarose-2x BME mixture was pipetted in 0.4 ml volumes into 12 x 75 mm plastic tubes kept at 56°C. Immediately, 0.05 ml of the

SE suspension was added to each tube, and the water bath temperature was lowered to 48°C. A 1/100 spleen cell suspension in BME was then added in 0.1 ml volume to one of the tubes, and its contents swirled gently on a mixer and immediately poured onto a glass slide so as to achieve even distribution over 4/5 of its surface. This final step was repeated for each spleen suspension under test. The glass slides were then placed on a plastic tray and incubated for 2 hours in a humidified incubator with 5 percent CO_2. A suitable dilution of rabbit anti-chicken-Ig of anti-IgM serum in BME was then applied under each slide test in 1 ml quantities and incubated for another 2 1/2 hours. The slides were then transferred to clean trays and a 1/10 dilution of guinea pig complement (Difco Laboratories, Detroit, MI) in 1 ml volumes was added per test and the slides were incubated for another 3 hours. The number of hemolytic plaques was then enumerated immediately or after overnight holding at 4°C. In the first trial, the number of IgG- and IgM-producing splenocytes was calculated per spleen. For each treatment group, the number of IgM plaques was subtracted from the number of Ig plaques and the values obtained were considered to correspond to IgG plaques. All spleen cell suspensions were tested in duplicate and the number of plaque forming cells (PFC) are expressed as the grand means of the means of duplicate assays.

e. Phytohemagglutinin (PHA) stimulation of peripheral leukocytes

The assays were performed following the technique described by Lee (1974). Ten ml of blood were obtained by cardiac puncture with heparinized syringes and the plasma fractions containing the leukocytes were separated by centrifugation at 50 g for 12 minutes. The plasma was diluted with PBS to 13 ml and the total number of leukocytes was calculated by counting in a hemocytometer chamber. The leukocytes were then pelleted by centrifugation at 1500 rpm for 10 minutes and the cells were then resuspended and adjusted to 1×10^7 leukocytes/ml in RPMI 1640 medium containing 10 percent fetal calf serum. Leukocytes were grown in 1 ml vials and 5 cultures were set for each blood sample, each culture containing 1×10^7 leukocytes. Cultures 1 and 2 contained ldukocytes only, and served as negative controls. Cultures 3, 4, and 5 each received 20 µg of phytohemagglutinin (PHA) (Burroughs-Wellcome and Co., London, England) in 50 lambda of PBS, and the cultures were then incubated at 37°C for 48 hours. Then, 1 µC of tritiated (^{3}H) thymidine (Schwaz/Mann, Orangeburg, NY) contained in 50 lambda of PBS was added to each culture, and the cultures were labeled for 18 hours. The cultures were then centrifuged at 2000 rpm for 6 minutes, the medium was removed and 2 ml of trichloroacetic acid (TCA) were added to each vial. The vials were shaken vigorously on a vortex mixer and centrifuged again at 2000 rpm for

6 minutes. The TCA supernatant was removed and 2 ml of sodium hydroxide (NaOH) was added to each culture. The cultures were then shaken on a vortex, centrifuged at 2000 rpm for 6 minutes and the NaOH supernatants were removed. Each culture was solubilized with 0.2 ml of Protosol (New England Nuclear, Boston, MA) at 80°C for 5 minutes and then topped with 2 ml of Aquasol (New England Nuclear, Boston, MA). The cultures were then shaken, placed inside scintillation vials and counted for radioactivity in a Beckman L 5-100 scintillation counter (Beckman Instruments Inc., Fullerton, CA).

f. Phenotypic mixing (PM) test for lymphoid leukosis viruses

The PM test as described by Okazaki et al. (1975) was used with slight modifications. Assays for RAV-1 viremia were performed in secondary Japanese QEF (C/BCD). RAV-2 and natural lymphoid leukosis viremia assays were performed in secondary CEF (C/O). Cultures grown in 35 mm plastic plates were inoculated with 0.05 ml of whole blood or 0.3 ml of plasma, and heparin was incorporated in the tissue culture media to a final 4-6 U/ml during the first 24 hours of culture. Assays for congenitally transmitted RAV-1 either in albumen or yolk were performed in QEF (C/BCD) while the RAV-2 and natural lymphoid leukosis viruses assays were done in CEF (C/O); 0.4 ml of albumen or 0.2 ml of egg yolk were inoculated into cultures on 35 mm plates. The plates were gently swirled to allow

dispersion of the inoculum. No heparin was incorporated in the media for this assay. Plasma infectivity titrations were done in QEF for RAV-1 or CEF for RAV-2 and the naturally occurring LLV's. For the first stage of the test, cells were always plated in media containing 2 μg/ml of DEAE-dextran (Vogt, 1967a) and then infected with RSV(RAV-0) containing approximately 200 focus forming units (FFU). When the foci of RSV transformed cells were confluent (7-9 days) the media were harvested, centrifuged and 1 ml of the supernatant transferred onto C/E CEF. When RAV-2 was being assayed, DEAE-dextran was incorporated in the media at 2 μg/ml. Final readings were usually completed between 7 and 10 days after the transfer. If a titration was being carried out, the cells were overlaid with agar 24 hours after infection. Otherwise, cultures were maintained in a fluid media containing 0.5% DMSO.

g. Assay for group specific antigen in albumen

A direct complement fixation (CF) test for the leukosis/sarcoma group specific (gs) antigen was performed on the albumen of unincubated hen's eggs. The test was performed in microplates (Cooke Laboratory Products, Alexandria, VA) containing 96 U shaped wells. Each albumen was tested and carried out in COFAL buffer to a 1:8 dilution for every sample. Antiserum to gs antigen was prepared in rabbits (Smith, 1977) and was used at a 1:64 dilution (4 units). Guinea pig serum was used as a source

of complement (5 complete hemolytic units) and rabbit anti SE was used as amboceptor. The rabbit antiserum to gs antigen, albumen dilutions and guinea pig complement were incubated overnight at 4°C. The hemolytic system was added after overnight incubation and the plates were further incubated at 37°C for 40 minutes with shaking every 10 minutes. Albumen samples giving 50 percent hemolysis or more were considered negative for gs antigen.

h. Neutralization tests for lymphoid leukosis viruses

The neutralization test has been described by Ishizaki and Vogt (1966). The plasmas or sera were diluted 1:5 in F10-199 medium containing 4 percent calf serum, and their complement was inactivated by heating at 56°C for 30 minutes. The Plasma or serum were then mixed with equal volumes of either RSV(RAV-1) or RSV(RAV-2) that gave approximately 100 foci in an area corresponding to 1/8th of the culture grown in a 35 mm plastic petri dish in the presence of serum free of antibody to subgroups A and B. The serum-virus mixtures were further incubated at 37°C for 40 minutes and secondary CEF (C/E) were inoculated with 0.2 ml of the mixtures. Cultures for assaying of antibody to subgroup B viruses contained DEAE-dextran at a rate of 2 µg/ml of tissue culture fulid. The next day, the media were discarded and the cultures were overlaid with agar media. Cultures were fed with 1 ml of F10-199 medium containing 2 percent calf serum and 0.5 percent DMSO every

2 days. Seven days after inoculation, the number of foci present in an area corresponding to 1/8th of the culture were counted. Sera and plasma samples that reduced the number of foci by 90 percent or more were considered positive. Those that reduced the number of foci between 50 and 90 percent were considered suspicious and a reduction of 50 percent or less was considered negative.

i. Agar gel precipitin test for Marek's disease

The agar gel precipitin test (AGP) (Chubb and Churchill, 1969) modified to be used on glass microscope slides (Sharma and Stone, 1972) was used to detect antibody in the serum of chickens vaccinated with HVT and challenged with MDV. The antigen for the AGP test was prepared by propagating the GA-22 strain of MDV in DEF and concentrating the supernatant fluids 50 times. All sera were inactivated at 56°C for 30 minutes and were tested undiluted.

j. Fluorescent antibody test for Marek's disease

The sera of vaccinated chickens were assayed for HVT antibody by the indirect fluorescent antibody (FA) test (Purchase, 1969a). HVT-infected DEF grown on coverslips and then fixed in cold acetone was used as antigen. The coverslips were divided into 3 sections and each section was reacted with an HVT-antibody positive serum, a serum negative for HVT antibody and the serum under test. All sera were used at a 1:20 dilution and were reacted on the coverslip for 20 minutes. Rabbit anti-chicken serum

conjugated with FITC was used at a 1:10 dilution and was reacted on all coverslips for 30 minutes. After thorough washing and mounting, the coverslips were examined for specific fluorescence with a Leitz fluorescent microscope (Ernst Leitz Ltd., Midland, Ontario, Canada) equipped with an Orthomat accessory for microphotography.

k. Plaque titration for HVT

Spleens of vaccinated chickens were tested for HVT by the technique described by Okazaki et al., (1973). The chickens were killed, their spleens were aseptically removed and spleen cell suspensions were prepared. Secondary DEF cultures grown in 60 mm plastic petri dishes were inoculated in triplicate with 2×10^7 spleen cells. Ten days after infection, the number of plaques seen in each of the 3 cultures infected with spleen cells from 1 chicken was counted and the average was considered to be the number of HVT plaques recovered per 2×10^7 spleen cells.

l. Hemagglutination inhibition for Newcastle disease

The micro hemagglutination inhibition (HI) test as described by Carbrey et al. (1974) was used with slight modifications. The tests were performed in microtiter plates. A suitable dilution of infected allantoic fluid containing 10 hemagglutination units (HU) of the B1-LaSota-NDV strain in a 0.025 ml volumes was used as antigen. Each well was dispensed with 0.025 ml of PBS and twofold

dilutions of the sera under test were made starting at the far left wells and continuing on until the far right wells were done. Then, 0.025 ml volumes containing 10 HU of antigen were added to each well. Finally, 0.025 ml of a 0.5 percent suspension of chicken erythrocytes was added to each well. The tests were incubated at room temperature (68°F) for an hour and the end points were calculated. The titer of a serum was the reciprocal of the highest dilution that completely prevented the agglutination of the chicken erythrocytes.

m. Infectious bronchitis virus recovery test

The virus recovery test was performed as described by Winterfield and Fadly (1971). Tracheal swabs were taken 5 days post-challenge of controls and vaccinated chickens. Each swab was placed in a plastic tube containing 2 ml of F10-199 medium with 4 percent calf serum and frozen at -70°C until the time of testing. On the day of the test, the fluids and swabs were thawed out, swirled thoroughly and 0.2 ml of each sample was inoculated into the allantoic cavity of five 10-day-old embryonated hen's eggs. Samples were considered positive for IBV if any one of the five embryos showed signs of IBV infection such as stunting, curling, kidney urates, clubbed down, or death 4 to 7 days after inoculation. Chickens were considered to be immune if IBV was not recovered from the swabs. Conversely,

isolation of IBV from such swabs indicated lack of vaccine-induced immunity.

Procedures for Restoration of the Immune Response

a. Donors and recipients

All chickens were of the highly inbred line 6_1 of the RPRL maintained free of the most common avian pathogens. Line 6_1 chickens are histocompatible as measured by complete acceptance of skin grafts within or between sire families (Stone, 1975), and they are homozygous (B^2B^2) at the major histocompatibiliby B locus (Pazderka et al., 1975).

b. Housing

Donor and recipient chickens were reared in isolation at all times. Donors were reared in FAPP plastic isolators while recipients were reared in Horsfall Bauer type isolators.

c. Cyclophosphamide treatment

Chicks were injected daily for 4 days, starting on the day of hatching with 4 mg of cyclophosphamide (CY) in 1 ml of phosphate buffer saline (PBS) by the intraabdominal route (16 mg total). CY administered by this route induces chemical bursectomy more effectively than by the intramuscular route (Douglas Gilmour, unpublished results), probably because CY is activated in the liver (Folley et al., 1961).

d. Transplantation technique

Chickens to be used as donors were killed by decapitation and bled out. The spleens were aseptically removed and separated from their capsules. Pools of 2-25 spleens were used to prepare the single cell suspensions from each category of donor. The spleens were expressed through a 21 gauge needle attached to a syringe and then suspended in cold RPMI 1640 media containing 2% vol/vol fetal calf serum, 2.5 I.U. of heparin ml, 0.002% wt/vol of DNase, 100 I.U. of penicillin/ml, 100 µg of streptomycin/ml, and 50 U of mycostatin/ml (complete media). The cell suspension was then passed through a 100 mesh sieve by gently stirring the suspension against the mesh of the sieve with the posterior end of a plastic syringe plunger. The cells were then washed 3 times in complete cold RPMI 1640 media, sieved through a 200 mesh sieve and the cell viability was estimated by trypan blue exlussion.

Since previous studies (Toivanen et al., 1972c) have shown that the spleen of 4-day-old chickens contains less than 2×10^7 cells, and that these chickens respond vigorously to an antigenic stimulation, the recipient chickens in our experiments were always injected via the wing vein with 1 ml of the appropriate spleen single cell suspension containing 2×10^7 viable spleen cells.

e. Serological testing

Chickens were injected via the wing vein with 1 ml of PBS containing both 5 x 10^8 sheep erythrocytes (SE) and 1:50 dilutions of the standard Brucella abortus tube antigen, USDA. The sera collected 7 days later were tested for a primary antibody response. A second antigenic stimulation was carried out 7 days later, and the serum collected after 5 days was tested for a secondary antibody response. All sera were tested by a microagglutination technique (Wegmann and Smithies, 1967).

EXPERIMENTAL DESIGNS

Humoral Immune Response

Two trials were run to determine the effects of mibolerone on the bursa of Fabricius, the humoral immune response to SE and Brucella abortus, antibody producing cells in the spleen, and the mitogenic response to PHA of splenic lymphocytes. All chickens were a cross of the inbred lines 15 and 7 from the RPRL.

Trial 1

Chickens were divided into three groups of 20 each. Group 1 received the 1 μg/7 weeks mibolerone feed regimen, group 2 received the 4 μg/3 weeks mibolerone feed regimen and group 3 received feed without mibolerone. At 8 and 10 weeks, they were immunized with SE, and 7 days later, samples of 7-10 birds were bled for serum for antibody tests and then killed. Their spleens were removed for determining the number of antibody-producing cells and the bursae of Fabricius were removed and weighed.

Trial 2

Chickens were divided into three groups of 24 each. The mibolerone-fed regimens were the same as for the three groups of trial 1. At 8 and 10 weeks, birds were tested as

in trial 1 except that Brucella abortus was also inoculated. At 9 weeks, blood lymphocytes were tested for their response to PHA and the mean stimulation index was calculated. The chickens were killed at 12 weeks and the bursae were removed and weighed.

HVT Vaccination in Mibolerone-Fed Chickens

This trial examined the effect of mibolerone on HVT protection against MD lymphoma formation and on the antibody response to MDV and HVT; 120 chickens of the 15 and 7 cross were divided into eight groups of 15 each. Groups 1-4 received the 1 μg/7 weeks mibolerone feed regimen; groups 5-8 received the regular feed without mibolerone. Chickens of groups 1, 2, 5, and 6 were injected at 1 day of age with HVT vaccine at a dose of 4×10^3 plaque forming units (PFU). Chickens of groups 1, 3, 5, and 7 were challenged, and those from groups 4 and 8 were reared as unvaccinated, unchallenged controls. Serum for antibody determinations was obtained from all surviving chickens at 9, 11 and 13 weeks of age. The agar gel precipitin reaction was used to detect MD antibody. The sera from vaccinated chickens were assayed for HVT antibodies by the FA technique (Purchase, 1969a). All surviving chickens were killed at 13 weeks of age, and the spleens of vaccinated chickens were aseptically removed and tested for HVT by the technique of Okazaki et al. (1973). All chickens that died or were killed at termination were examined for MD lesions.

Tissues were taken from those showing no gross lesions and were examined for microscopic MD lesions as described by Payne and Biggs (1967).

B1-LaSota Vaccination in Mibolerone-Fed Chickens

The purpose of this trial was to examine the effect of mibolerone on protection afforded by the B1-LaSota strain vaccination against a challenge with the virulent Texas-GB strain of NDV and on the serological response after vaccination and challenge; 360 Spafas chickens were divided into 12 groups. Groups 1-6 contained 35 chickens each; groups 7-12 contained 25 chickens each. Groups 1, 4, 7, and 10 received the 1 µg/7 weeks regimen; groups 2, 5, 8, and 11 received the 2 µg/7 weeks regimen; and groups 3, 6, 9, and 12 received regular feed without mibolerone. Chickens from groups 1-3 and 7-9 were individually vaccinated at 4 weeks of age by the intranasal route with 1×10^5 EID_{50} of the B1-LaSota strain. At 7 weeks of age, groups 1-6 were challenged with $5 \times 10^{5.3}$ EID_{50} of the virulent Texas-GB strain of NDV by the intramuscular route. Chickens from groups 7-9 were left unchallenged, and those from groups 10-12 were reared as unvaccinated, unchallenged controls. At 7 weeks of age, five chickens from each group were randomly selected and killed, and their bursae were removed and weighed. Serum for antibody determination was obtained from six randomly selected chickens of each group at 7 and 10 weeks of age or from unvaccinated challenged survivors

at 10 weeks of age. The micro hemagglutination inhibition was used to assay for antibody. Newcastle disease clinical signs and mortality were recorded on a daily basis during 2 weeks after challenge with the virulent NDV.

ILT Vaccination in Mibolerone-Fed Chickens

The purpose of this trial was to determine if chickens fed mibolerone could be protected by vaccination against challenge with a virulent ILT virus. Seventy-three commercial SCWL chickens were divided into 6 groups. Groups 1, 3, and 5 received the 1.5 μg/7 weeks mibolerone feed regimen and consisted of 15, 12 and 10 chickens, respectively, while groups 2, 4, and 6 received regular feed without mibolerone and consisted of 14, 12, and 10 chickens, respectively. Bursa weights were not recorded in this trial. Chickens of groups 1, 2, 5, and 6 were vaccinated with modified live virus by the eye drop method at 4 weeks of age. Chickens of groups 1, 2, 3, and 4 were challenged at 7 weeks by inoculation of the virulent Boudreau strain of ILT virus into the left infraorbital sinus. All chickens were observed daily during 10 days. Five days after challenge the infraorbital sinuses were squeezed in order to evaluate the presence of exudate, and those chickens that had profuse exudate were considered to be non-immune. Nasal and ocular discharge and swelling of the face were also considered as indicators of a non-immune

status. Absence of all the above clinical signs was an indicator of immunity.

IBV Vaccination in Mibolerone-Fed Chickens

The purpose of this trial was to determine if chickens that were fed mibolerone could be protected by vaccination against a challenge with a virulent strain of IBV. Seventy-four commercial SCWL chickens were divided into 6 groups. Groups 1, 3, and 5 received the 1.5 μg/7 weeks mibolerone feed regimen and consisted of 15, 12, and 10 chickens, respectively, while groups 2, 4, and 6 received the regular feed without mibolerone and consisted of 15, 12, and 10 chickens, respectively. Bursa weights were not assessed for regression in this trial. Chickens of groups 1, 2, 5, and 6 were vaccinated with the Massachusetts and Connecticut mild strains of IBV by the eye-drop method at 3 weeks of age. Chickens of groups 1, 2, 3, and 4 were challenged at 6 weeks of age with the virulent 41 strain of the Massachusetts IBV with a titer of 5×10^6 EID_{50}/ml. Five days after the challenge, tracheal swabs were obtained from 10 chickens of each of groups 1 through 4, and 5 chickens of groups 5 and 6, and the swabs were assayed for virus recovery.

Pigeon Pox Vaccination in Mibolerone-Fed Chickens

The purpose of this trial was to determine if chickens fed mibolerone could be protected by vaccination with pigeon pox virus against a challenge with a virulent strain of fowl

pox virus. Seventy-four commercial SCWL chickens were divided into 6 groups. Groups 1, 3, and 5 received the 1.5 µg/7 weeks mibolerone feed regimen and consisted of 15, 12, and 10 chickens, respectively, while groups 2, 4, and 6 received the regular feed without mibolerone and consisted of 15, 12, and 10 chickens, respectively. Bursae were not weighed in this trial. Chickens of groups 1, 2, 5, and 6 were vaccinated by stabbing the left wing web with a vaccine applicator containing live pigeon pox vaccine at 4 weeks of age. Chickens of groups 1, 2, 3, and 4 were challenged at 6 weeks of age by stabbing the right wing web with an applicator containing the virulent fowl pox virus with a titer of $10^{4.5}$ EID_{50}/ml. Chickens were observed for 10 days and were considered immune if they did not develop pox lesions or scabs at the site of challenge on the right wing web. Chickens developing any of these signs were considered to be non-immune.

Fowl Cholera Vaccination in Mibolerone-Fed Chickens

The purpose of this trial was to determine if chickens that had been fed mibolerone and had been vaccinated against fowl cholera, were immune to a challenge with a virulent strain of *Pasteurella multocida*. Ninety commercial SCWL chickens were divided into 4 groups. Groups 1 and 3 received the 1.5 µg/7 weeks mibolerone feed regimen and consisted of 25 and 20 chickens, respectively. Groups 2 and 4 received the regular feed at all times and

consisted of 25 and 20 chickens, respectively. Chickens of groups 1 and 2 were vaccinated at 14 and again at 17 weeks of age by subcutaneous injection of the fowl cholera bacterin containing strains 1059, 1662, and X-73 of Pasteurella multocida containing 250 colony forming units (CFU). Chickens were observed daily and the development of clinical signs or the occurrence of mortality were recorded for a period of 14 days post-challenge. Survivors at 14 days after challenge showing no signs of disease were considered to be immune. Mortality and persistence of clinical signs were indications of no immunity.

Reconstitution of the Immune Response in Mibolerone-Fed Chickens

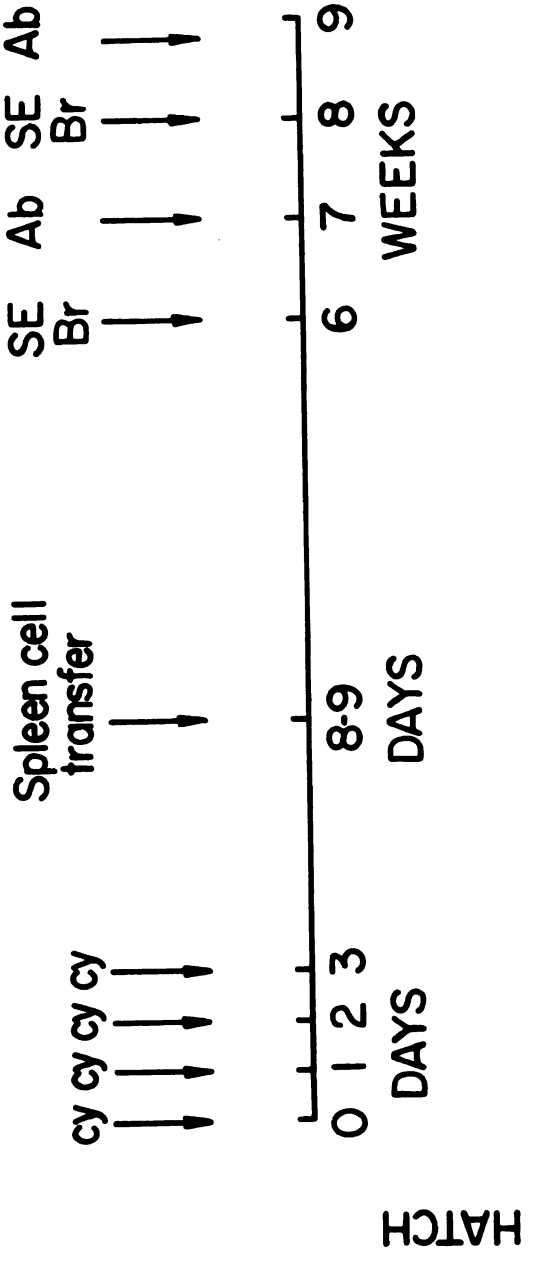
Donor chickens of line 6_1 were hatched and allocated to 3 groups. Chickens of group 1 were fed the standard diet at all times (normal-donors); chickens of group 2 were fed the 1.0 µg/7 weeks mibolerone feed regimen (mibolerone-donors); and chickens of group 3 were treated with CY and fed the standard diet (CY-donors). At 2, 4, 6, 8, 11, 13, 15, 17, and 20 weeks of age, a number of chickens (5-25 chickens) of each of the 3 donor groups were killed, their spleens were removed, processed to prepare single cell suspensions and transplanted by the intravenous route into CY-treated 8-9 day-old recipients. In the case of recipient 6_1 chickens, for each set of transplants, recipients were hatched, and allocated to 5 lots. Lots 1 through 4 were treated with CY, and lot 5 was left untreated and

served as positive control for humoral antibody responses (normal untreated). Chickens of lot 1 were transplanted with spleen cells from normal-donors; chickens of lot 2 were transplanted with spleen cells from mibolerone-donors, and chickens of lot 3 were transplanted with spleen cells from CY-donors. Chickens of lot 4 were not transplanted and served as negative controls for humoral antibody responses (CY-untreated). Five and 7 weeks after the transfer of spleen cells, chickens of all recipient lots were injected with the SE-Brucella abortus suspension and sera for antibody determination were obtained 7 (primary antibody response) and 5 (secondary antibody response) days after the immunizations, respectively. The scheme of this experiment is shown in Figure 7.

RAV-1 Induced Lymphoid Leukosis in Mibolerone-Fed Chickens

Chickens were a cross of the inbred lines 15 and 7. The cross is free from infection by exogenous LLV's but highly susceptible to LL tumor development and infection with viruses of subgroups A and B (Stone, 1975). One hundred and seventy chicks were randomized at one day of age and divided into 4 lots. Lots 1 and 2 consisted of 40 chickens each and lots 3 and 4 of 45 chickens each. Chickens from lots 2 and 4 received the 1.5 µg/7 weeks mibolerone feed regimen. Chickens from lots 1 and 3 received a standard diet throughout the experiment. Chickens from lots 3 and 4 were injected intracardially at

Fig. 7 Scheme of transfer of spleen cells in cy-treated recipients.


HATCH
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Spleen cell
transfer
SE
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Br
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WEEKS

one day of age with 1 x 10^5 tissue culture infective units (TCIU) of RAV-1. Chickens from lots 1 and 2 were infected at 2 weeks of age with 0.2 ml of the same RAV-1 stock contining 1 x 10^5 TCIU by the intraabdominal route. All chickens were vaccinated at 1 day of age with 5000 PFU of the HVT by the intramuscular route. Chickens from the various lots were reared separately in FAPP plastic isolators until they were 18 weeks of age. At 7 weeks of age, 3 males and 3 females were randomly selected from each lot. Heparinized blood was obtained from the wing vein and 0.3 ml of plasma were assayed by the PM test to detect LL viremia. The plasmas were inactivated and then assayed for neutralizing antibodies against RSV(RAV-1). All 24 chickens were then killed and the bursa of Fabricius was carefully dissected out and weighed. Portions of the bursa and cecal tonsils were then taken for histopathological examination. At 18 weeks of age, heparinized blood was obtained from all surviving chickens and the viremia of plasmas, and the neutralizing antibody of the heated plasmas evaluated. All chicks were then moved to cages in a common environment and the experiment was terminated at 150 days. Chickens dying during the course of the experiment or surviving at termination were necropsied.

RAV-2 Induced Lymphoid Leukosis in Mibolerone-Fed Chickens

Chickens were the same as those for the RAV-1 experiment. Seventy chickens were randomized at one day of age

and divided into 2 lots of 35 chickens each. Lot 1 was fed the standard diet throughout the experiment while lot 2 was fed the 1.5 μg/7 weeks mibolerone feed regimen. All chickens were vaccinated at one day of age with 5000 PFU of HVT intramuscularly. At 2 weeks of age, all chickens were injected with 1 x 10^5 TCIU of RAV-2 by the intraabdominal route. Chickens were reared in FAPP plastic isolators until 18 weeks of age and then transferred to cages in a common environment. At 7 weeks of age, 3 males and 3 females selected at random were removed from each lot. Heparinized blood was obtained from the wing vein to test for viremia and antibody. The chickens were killed, the bursae dissected out and histological sections prepared from both bursae and cecal tonsils as before. All chickens were bled at 18 weeks of age and their viremia and antibody status determined. The experiment was terminated at 180 days. Chickens dying during the trial or surviving at termination were necropsied. Tissues for histopathology were taken when necessary to confirm a gross diagnosis. A few females that survived the experimental period were housed in individual cages for shedding studies.

Naturally Occurring Lymphoid Leukosis in Mibolerone-Fed Chickens

A commercial White Leghorn flock naturally infected with LLV's was identified by testing pools of embryos from individual hens after being expressed through a syringe (embryo mashes) by the PM test. Pools of embryo mashes

from individual hens were tested and the infection rate among hens was found to be 40 percent. Consequently, a number of embryonated eggs was obtained from identified shedders, incubated, hatched and the one day-old chicks were randomized and divided into 8 lots each containing 10 chickens. Lots 1 and 2 were fed a standard diet throughout the experiment. Lots 3 and 4 were fed the 1.0 µg/7 weeks mibolerone regimen, lots 5 and 6 were fed the 1.5 µg/7 weeks mibolerone regimen and lots 7 and 8 the 2.0 µg/7 weeks regimen. Since the number of chicks per treatment was relatively small, no attempt was made to assess bursa regression at 7 weeks of age. All chicks were vaccinated at one day of age with 2500 PFU of HVT intramuscularly and against Newcastle disease in the drinking water at 4 days of age. Chicks were reared in chick brooding batteries during the first seven weeks of life and then moved to floor pens. Chickens from each treatment were penned together with only wire separating the pens. All chickens were bled at 3, 6, 10 and 18 weeks of age to assay for LL viremia (wholeblood) and antibody (heated plasmas). The trial was terminated at 220 days. Chickens dying during or alive at termination of the trial were necropsied and the LL diagnosis made on the basis of gross lesions. Females that survived the experimental period were housed in individual cages for shedding studies.

Shedding of RAV-1 and RAV-2 in Mibolerone-Fed Chickens

Female chickens of the RPRL inbred line 15 (highly susceptible to but free of exogenous LLV's) were injected with 1 x 10^4 TCIU of RAV-1 into the thoracic cavity or with 2 x 10^5 TCIU of RAV-2 intraabdominally at one day of age. Female chickens of the 15 and 7 cross were injected intraabdominally at 2 weeks of age with 1 x 10^5 TCIU of RAV-2. In every category, standard diet and mibolerone-fed (1.5 µg/7 weeks) hens were available for shedding studies. At 18 weeks of age, all hens were assayed for viremia and neutralizing antibodies. At 6 months of age, when the hens started to lay eggs they were caged individually. Eggs were collected daily, immersed in 80 percent alcohol for 5 minutes, cut open and the albumen and yolk sampled separately and stored at -70°C until testing. Eggs from which only albumen was obtained were sampled by withdrawing the albumen through a hole in the shell with a syringe with an attached 18 gauge needle. Albumen and yolk were assayed for the presence of LLV's by the PM test. Albumen were assayed for gs antigen by the direct complement fixation test.

Shedding of Lymphoid Leukosis Viruses in Naturally Infected Mibolerone-Fed Hens

Eggs from individually caged commercial White Leghorn hens were collected daily. Albumen was withdrawn with a syringe and needle and stored at -40°C the same day the

egg was laid for a period of 2 weeks. Albumen were assayed for the presence of LLV's by the PM test and for the presence of gs antigen by the direct complement fixation test.

Viremia Titers in Mibolerone-Fed Hens Infected with Lymphoid Leukosis Viruses

The plasmas of viremic hens infected with RAV-1, RAV-2 and field LLV's were titrated in the PM test by making ten-fold dilutions of the infectious plasmas in F10-199 medium and infecting CEF of the C/O phenotype with 0.1 ml of these dilutions.

Statistical Analysis

The significance of differences in the humoral antibody responses to SE and Brucella abortus, PHA stimulation of leukocytes and number of antibody-producing cells between treatments was assessed by analysis of variance based on 1% and 5 % probability levels. The significance of differences between treatment in the shedding studies was calculated by the Chi-square test based on 5 % probability. A method described by Lush et al. (1948) was used to assess the statistical significance of the differences observed among hens within treatments in the shedding studies.

RESULTS

Humoral Immune Response Trials

a. Regression of the bursa of Fabricius

Mibolerone fed to chickens in two of the regimens tested induced a significant regression of the bursa of Fabricius. The 4 µg/3 weeks dose was not as effective in inducing bursa regression as the 1 µg/7 weeks dose (Table 4). In trial 1, the bursae from chickens fed mibolerone at a 1 µg level were not recognizable as such at 10 and 12 weeks of age and had atrophied so far (except for one bursa) that remnants could not be found (Table 4).

b. Humoral antibody responses

The above mibolerone-fed chickens in which the bursa of Fabricius had undergone partial or total atrophy maintained their capacity to produce humoral antibody after primary and secondary antigenic stimulation with SE. The levels of serum antibody were comparable in all mibolerone-treated and in the untreated chickens in two trials (Table 5). No statistically significant differences were detected at the 5% probability when tested by analysis of variance (Table 5). The antibody response to *Brucella abortus* in the 1 µg/7 weeks group appeared to be less than that in both the 4 µg/3 weeks and the control groups.

Table 4. Effect of orally administered mibolerone on the weight of the bursa of Fabricius.

Mibolerone ug/gm feed	Trial 1			Trial 2	
	No. chickens	Mean bursa weight[a] 10 weeks	12 weeks	No. chickens	Mean bursa weight[a] 12 weeks
1 µg/7 wk[b]	10	cr[c]	cr[c]	8	0.218±0.047
4 µg/3 wk[d]	10	0.979±0.085	1.215±0.097	8	0.966±0.120
None	10	5.078±0.160	5.927±0.208	8	4.265±0.359

[a]Weights in gm ± standard error.

[b]1 µg of mibolerone/gm of feed administered from 1-49 days of age.

[c]Bursae had completely regressed (cr) except for one (0.235 gm).

[d]4 µg of mibolerone/gm of feed administered from 28-49 days of age.

Table 5. Antibody response to sheep erythrocytes[a] and Brucella[b] in mibolerone-fed chickens.

Mibolerone µg/gm feed	Responders/ total	Sheep erythrocytes Primary response log_2 titer ± SE	Sheep erythrocytes Secondary response log_2 titer ± SE	Brucella Primary response Responders/ total	Brucella Primary response Mean ± SE log_2 titer
		Trial 1			
1 µg/7 wk	10/10	9.4±0.46	9.8±0.50		
4 µg/3 wk	10/10	10.5±0.42	10.7±0.49		
None	10/10	10.5±0.30	10.7±0.73		
		Trial 2			
1 µg/7 wk	8/8	10.8±0.49	12.3±0.53	7/8	5.0±0.78
4 µg/3 wk	8/8	10.9±0.29	12.0±0.33	8/8	8.5±1.05
None	8/8	10.6±0.32	11.8±0.25	8/8	8.25±0.37

[a]Primary and secondary stimuli consisted of $5x10^8$ SE injected intravenously.

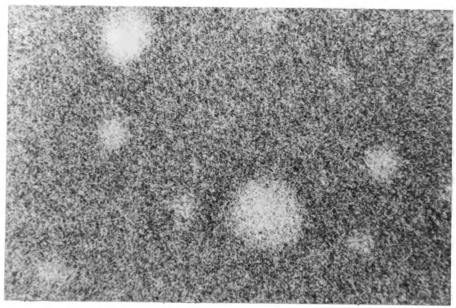
[b]1 ml of 1:50 dilution of the standard Brucella abortus tube antigen, USDA.

However, this difference was significant at the 5% level but not at the 1% probability level when tested by analysis of variance (Table 5).

c. Quantitation of antibody producing cells

The spleens of mibolerone-fed chickens contained many antibody-producing cells as detected by the hemolytic plaque assay (Figure 8). In trial 1, the spleens of chickens fed both the 1 µg/7 weeks and the 4 µg/3 weeks doses contained greater number of antibody-producing cells than did spleens from untreated chickens (significant at the 1 % level) (Table 6). The proportion of IgG- and IgM-producing splenocytes of chickens fed mibolerone also deviated from those of the untreated chickens (significant at the 1% level). More IgM-producing cells were detected in the spleens of mibolerone-fed chickens than in the spleens of untreated chickens. In trial 2, differences between the three treatment groups were not significant in the number of hemolytic plaques for the primary response (Table 6). However, when spleens were taken after the secondary response, those of chickens fed mibolerone at either the 1 µg/7 weeks of 4 µg/3 weeks levels contained higher numbers of antibody-producing cells than those of untreated chickens (Table 6). However, the differences were not significant at the 5% probability level.

Figure 8. Hemolytic plaques induced by antibody-producing splenocytes from a chicken fed mibolerone.



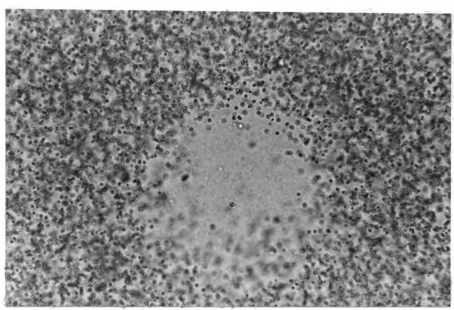


Table 6. Number of antibody producing splenocytes in spleens of mibolerone-fed chickens.

Mibolerone ug/gm feed	Trial 1[a] Primary Response No. chickens	IgPFCx10^3/ spleen Mean ± SE	IgG PFCx10^3/ spleen Mean ± SE	IgM PFCx10^3/ spleen Mean ± SE	Trial 2[b] Primary Response No. chickens	IgPFCx10^3/ spleen Mean ± SE	Secondary Response No. chickens	IgPFCx10^3 spleen Mean ± SE
1 µg/7 wk	9	88.7±5.6	44.2±6.7	44.4±5.4	6	14.8±3.6	8	37.6±7.5
4 µg/3 wk	9	109.1±11.8	91.9±9.2	17.2±4.0	6	22.6±4.2	8	39.7±9.0
none	9	65.0±8.8	60.9±8.1	4.1±0.97	7	17.5±4.2	8	18.6±2.7

[a]Assays were for Ig plaque forming cells (PFC) and IgM PFC. No. of PFC are the grand means of the means of duplicate assays per spleen x 10^3. IgG PFC = IgPFC - IgM PFC.

[b]Assays were made for IgPFC only.

PHA Stimulation of Leukocytes

Peripheral blood leukocytes of mibolerone-fed chickens were successfully stimulated by PHA, a known mitogen for thymic lymphocytes (Alm and Peterson, 1970) as evidenced by increased incorporation of tritiated thymidine (Table 7). The mean stimulation indexes (SI) for lymphocytes derived from the 1 µg/7 weeks mibolerone group and the control group were higher than the SI of lymphocytes from the 4 µg/7 weeks mibolerone group. However, statistical differences were not significant at the 5% level when both the PHA counts and the SI were subjected to analysis of variance.

HVT Vaccination in Mibolerone-Fed Chickens

In this trial, the oral administration of mibolerone did not affect the protection conferred by HVT in chickens challenged with MDV. Mibolerone-fed chickens either vaccinated or vaccinated and challenged did not develop either gross or microscopic evidence of MD (Table 8). Moreover, mibolerone did not alter the course of disease in unvaccinated MDV-infected chickens. The microscopic lesions observed and the percentage of MD mortality were comparable with those observed in the untreated unvaccinated challenged group. One chicken from the untreated unvaccinated unchallenged group showed microscopic non-proliferative lesions similar to those of MD. Lesions of this type have been previously described (Payne and Biggs,

Table 7. PHA stimulation of peripheral leukocytes from mibolerone-fed chickens.

Mibolerone μg/gm feed	No. chickens	Background counts[a] ± SE	PHA counts[b] ± SE	Stimulation index[c]
1 μg/7 wk	7	1120 ± 245	37820±3867	34
4 μg/3 wk	7	1300 ± 198	19590±6283	15
None	7	834 ± 245	31842±4960	38

[a,b] Values are the grand means of the means of duplicate[a] or triplicate[b] ± standard error 1-ml cultures per chicken pulsed for 16 hours (48-64 hours of culture) with 1 μC of ^{3}H thymidine.

[c] Stimulation index = $\frac{\text{Average PHA counts}}{\text{Average background counts}}$

Table 8. HVT prevention of Marek's disease in mibolerone-fed chickens.

Group	Treatment			MD precipitating antibody[a] at			Marek's disease			
	Vaccine[b]	Challenge[c]	Mibolerone µg/gm feed	9wk	11wk	13wk	No. chickens	Gross lesions	Microscopic lesions	Total %
1	+	+	1 µg/7 wk	7/10	9/10	9/10	14	0	0	0
2	+	-	1 µg/7 wk	2/10	3/10	1/10	15	0	0	0
3	-	+	1 µg/7 wk	7/8	5/5	4/4	12	9	11	92
4	-	-	1 µg/7 wk	0/10	0/10	0/10	15	0	0	0
5	+	+	None	7/10	8/10	5/8	15	0	0	0
6	+	-	None	0/10	0/10	0/9	12	0	0	0
7	-	+	None	9/10	6/6	3/3	14	11	13	93
8	-	-	None	0/10	0/10	0/10	15	0	1	7

[a]Number positive/number tested.

[b]Herpes virus of turkeys.

[c]Marek's disease herpes virus.

1967; Sharma et al., 1973) but are of uncertain etiology. HVT was reisolated from the spleens of mibolerone-fed chickens at the same rate as from the spleens of their controls (Table 9). Antibodies to HVT were detected in the sera of all the HVT-vaccinated chickens by the use of the indirect FA technique (Table 9). Antibodies to MDV were also detected in the sera of all chickens challenged with MDV by the use of the AGP technique (Table 8).

B1-LaSota Vaccination in Mibolerone-Fed Chickens

In this trial, reduction in the bursa size was significant in both groups of chickens fed mibolerone (Table 10). No effect of oral administration of mibolerone on protection conferred by the B1-LaSota strain on chickens challenged with the virulent Texas-GB strain of NDV could be detected (Table 10). Moreover, mibolerone did not alter the course of disease in unvaccinated Texas-GB strain challenged chickens. The clinical signs and the percentage of ND mortality were comparable with those obtained in the untreated unvaccinated challenged group. One control unvaccinated chicken survived the Texas-GB challenge without showing clinical signs. HI antibody was detected in the sera of nearly all vaccinated and vaccinated challenged chickens (Table 10). No antibody was detected in any of the unvaccinated or unvaccinated challenged chickens tested (Table 10).

Table 9. Influence of mibolerone feeding on HVT infection and immunity.

Mibolerone µg/gm feed	HVT isolation from spleen			No. positive HVT antibodies[b]		
	No. spleens[a]	Mean plaque no. per $2x10^7$ spleen cells	No. tested	9wk	11wk	13wk
1 µg/7 wk	11	19.4	10	10	10	10
None	9	21.1	10	10	10	10

[a]At 13 weeks after vaccination.

[b]By indirect fluorescent antibody method.

Table 10. B-1 LaSota vaccine prevention of Newcastle disease in mibolerone-fed chickens.

Group	Vaccine[a]	Challenge[b]	Mibolerone ug/gm feed	Bursa wt[c]/gm 7 weeks	HI antibody[d] 7wk	HI antibody[d] 9wk	Newcastle disease No. chickens	Clinical signs	Dead	Total %
1	+	+	1 µg/7 wk	0.9	5/6	6/6	30	0	0	0
4	-	+	1 µg/7 wk	0.45	0/6	ND[e]	30	30	29	100
7	+	-	1 µg/7 wk	0.2	6/6	5/6	20	0	0	0
10	-	-	1 µg/7 wk	0.26	0/6	0/6	20	0	0	0
2	+	+	2 µg/7 wk	0.0	5/6	6/6	30	0	0	0
5	-	+	2 ug/7 wk	0.04	0/6	ND	30	30	30	100
8	+	-	2 ug/7 wk	0.0	4/6	6/6	20	0	0	0
11	-	-	2 ug/7 wk	0.09	0/6	0/6	20	0	0	0
3	+	+	None	4.1	5/6	6/6	30	0	0	0
6	-	+	None	3.8	0/6	ND	30	29	29	97
9	+	-	None	3.89	5/6	6/6	20	0	0	0
12	-	-	None	4.45	0/6	0/6	20	0	0	0

[a] $1x10$ EID_{50}/chick of B-1 LaSota intranasally at 4 wk of age.

[b] $5x10^{5.3}$ EID_{50}/chick of Texas-GB intramuscularly at 7 wk of age.

[c] Mean weight of 5 bursae/group.

[d] Number of positive/number tested.

[e] ND = not done.

Infectious Laryngotracheitis Vaccination in Mibolerone-Fed Chickens

In this trial, the oral administration of mibolerone did not interfere with the protection afforded by vaccination against a challenge with the virulent Boudreau strain of ILT (Table 11). Moreover, mibolerone did not alter the course of disease in unvaccinated challenged chickens.

Infectious Bronchitis Vaccination in Mibolerone-Fed Chickens

Administration of mibolerone did not affect the vaccination immunity of chickens challenged with the virulent strain of IBV Massachusetts 41, as judged by the virus recovery test results. Chickens that had been vaccinated and then challenged with virulent virus did not yield IBV in the recovery test in either mibolerone-treated or untreated groups (Table 12). On the other hand, unvaccinated challenged chickens yielded IBV in the recovery test while no virus was isolated from the swabs of vaccinated unchallenged chickens of either the mibolerone-treated or untreated groups.

Pigeon Pox Vaccination in Mibolerone-Fed Chickens

Chickens that had been fed mibolerone and were vaccinated with pigeon pox vaccine were fully protected against challenge with a virulent fowl pox virus. Vaccinated chickens in both mibolerone-treated and untreated groups did not develop fowl pox lesions at the site of challenge (Table 13), while unvaccinated challenged

Table 11. Prevention of avian infectious laryngotracheitis by vaccination in mibolerone-fed chickens.

Group	ILT[a] vaccination	ILT[b] challenge	Mibolerone ug/gm feed	Infectious laryngotracheitis[c] No. chickens	Clinical signs	Total %
1	+	+	1.5 µg/7wk	15	0	0
2	+	+	none	14	0	0
3	-	+	1.5 µg/7wk	12	12	100
4	-	+	none	12	12	100
5	+	-	1.5 µg/7wk	10	0	0
6	+	-	none	10	0	0

[a]Vaccinated with modified live virus by eye-drop method at 4 weeks of age.

[b]Challenged with the virulent Boudreau strain of ILT virus into the infraorbital sinus at 7 weeks of age.

[c]Observation period was 5 days.

Table 12. Prevention of avian infectious bronchitis by vaccination in mibolerone-fed chickens.

Group	IBV[a] vaccination	IBV[b] challenge	Mibolerone ug/mg feed	Infectious bronchitis		
				No. chickens	Positive by virus recovery test[c]	Total %
1	+	+	1.5 µg/7wk	15	0/10	0
2	+	+	none	15	1/10	10
3	-	+	1.5 µg/7wk	12	10/10	100
4	-	+	none	12	10/10	100
5	+	-	1.5 µg/7wk	10	0/5	0
6	+	-	none	10	0/5	0

[a]Vaccinated with the Massachusetts and Connecticut strains of IBV by the eye-drop method at 3 weeks of age.

[b]Challenged with the virulent 41 strain of the Massachusetts IBV (5×10^6 EID_{50}/ml) by the eye-drop method at 6 weeks of age.

[c]Number of swabs positive/no. tested.

Table 13. Prevention of fowl pox by vaccination in mibolerone-fed chickens.

Group	Pigeon-pox[a] vaccination	Fowl pox[b] challenge	Mibolerone µg/gm feed	Fowl Pox[c] No. chickens	Lesions	Total %
1	+	+	1.5 µg/7wk	15	0	0
2	+	+	none	15	0	0
3	-	+	1.5 µg/7wk	12	12	100
4	-	+	none	12	11	92
5	+	-	1.5 µg/7wk	10	0	0
6	+	-	none	10	0	0

[a]Vaccinated with pigeon pox vaccine by stabbing left wing at 4 weeks of age.

[b]Challenged with virulent fowl pox virus (1 X $10^{4.5}$ EID_{50}/ml) by stabbing right wing at 6 weeks of age.

[c]Observation period was 10 days.

chickens of both groups developed areas of induration, and crusty scabs at the site of challenge.

Fowl Cholera Vaccination in Mibolerone-Fed Chickens

Chickens fed mibolerone and vaccinated twice with a fowl cholera bacterin were protected against a challenge with the virulent strain X-73 of Pasteurella multocida as evidenced by survival during the post-challenge observation period (Table 14). All unvaccinated challenged chickens died of fowl cholera in both mibolerone-treated and untreated groups.

Reconstitution of the Immune Response in Mibolerone-Fed Chickens

a. Restoration of the immune response to Brucella abortus

Spleen cell suspensions from donors that had been fed mibolerone did not restore the immunocompetence to Brucella abortus (Figures 9, 10). Only 2 chickens transplanted with spleen cells from 13 week-old mibolerone-fed donors responded with humoral antibodies after a secondary stimulation with Brucella abortus (Figure 10). Restoration of the primary and secondary humoral immune responses was obtained with spleen cells from 2, 4, 8, 13, 15, 17, and 20 week-old normal donors (Figures 9, 10). No restoration was obtained with spleen cells from 6 and 11 week-old normal donors. Long term restoration was demonstrated in 50 percent of the recipients transplanted with spleen cells from 15 week-old normal donors and in 100 percent of the recipients

Table 14. Prevention of fowl cholera by vaccination in mibolerone-fed chickens.

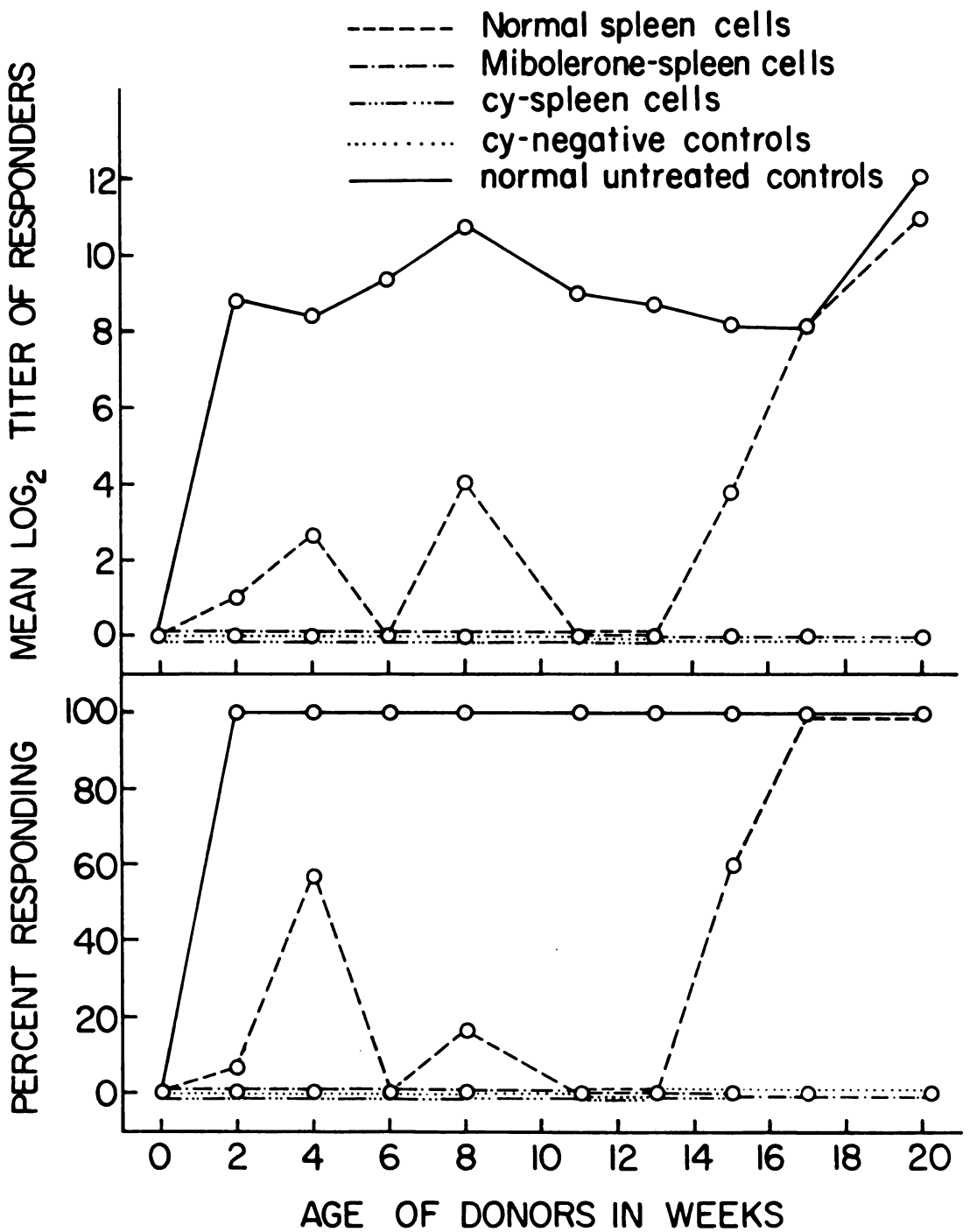
Group	Fowl cholera[a] vaccination	Fowl cholera[b] challenge	Mibolerone µg/gm feed	Fowl cholera[c] No. chickens	Dead	Total %
1	+	+	1.5 µg/7wk	25	1	4
2	+	+	none	25	6	24
3	-	+	1.5 µg/7wk	20	20	100
4	-	+	none	20	20	100

[a]Vaccinated with Pasteurella multocida bacterin (strains 1059, 1662 and X-73) by subcutaneous injection at 14 and 17 weeks of age.

[b]Challenged with the virulent X-73 strain of P. multocida (250 CFU/chicken) by intramuscular injection at 20 weeks of age.

[c]Observation period was 14 days.

Figure 9. Long term restoration of the primary immune response to Brucella abortus by 2 X 10^7 spleen cells injected into cy-treated 8-9 day-old recipient chicks. Brucella abortus injected together with sheep erythrocytes intravenously 5 weeks after the transfer and sera were obtained 1 week later. Each point represents 5-18 chickens tested.


Normal spleen cells
Mibolerone-spleen cells
cy-spleen cells
cy-negative controls
normal untreated controls
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PERCENT RESPONDING
AGE OF DONORS IN WEEKS
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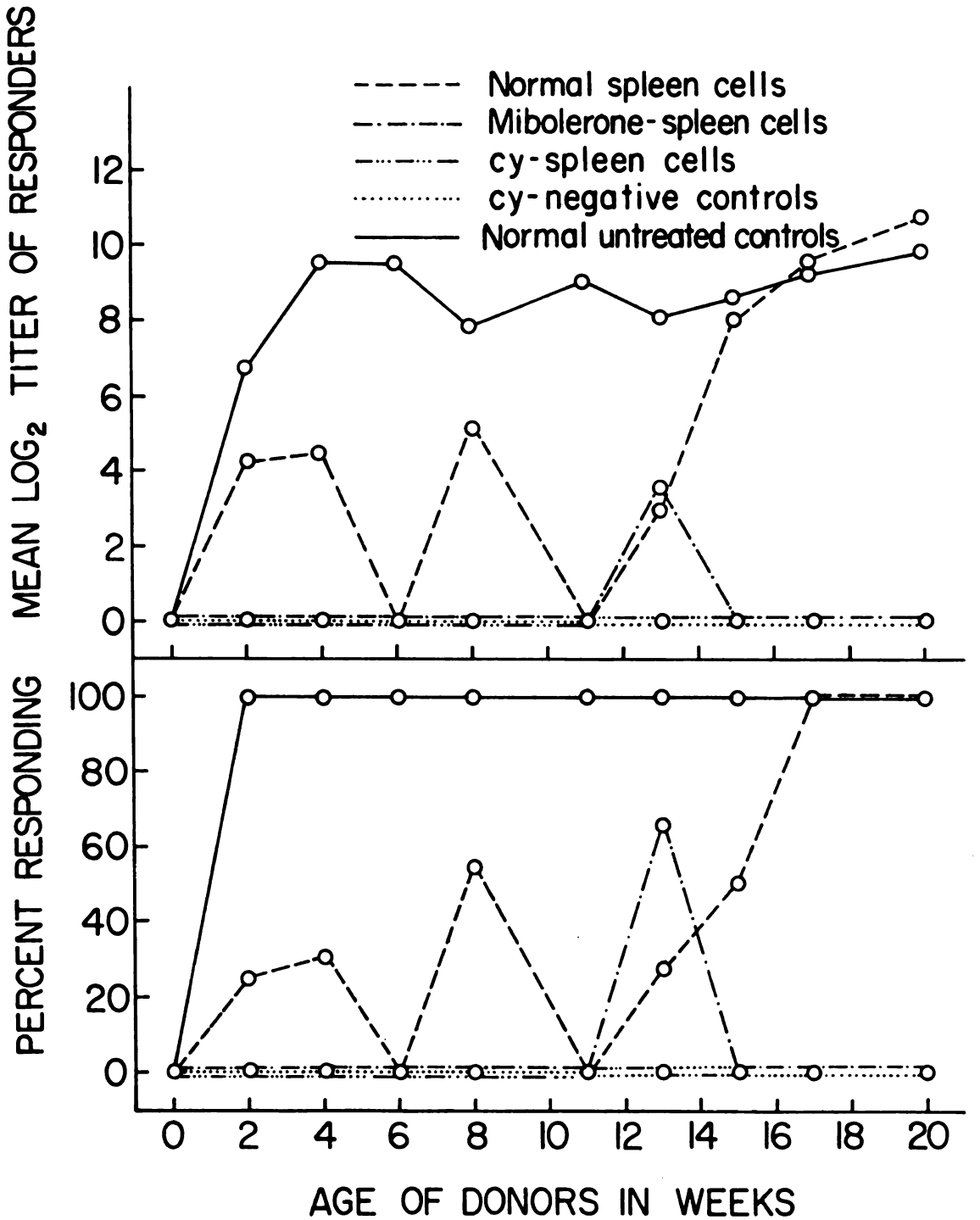
60
80
100

Figure 10. Long term restoration of the secondary immune response to Brucella abortus by 2 X 10^7 spleen cells injected into cy-treated 8-9 day-old recipient chicks. Brucella abortus injected together with sheep erythrocytes intravenously 7 weeks after the transfer (2 weeks after primary stimulation) and sera were obtained 5 days later. Each point represents 5-18 chickens.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PERCENT RESPONDING
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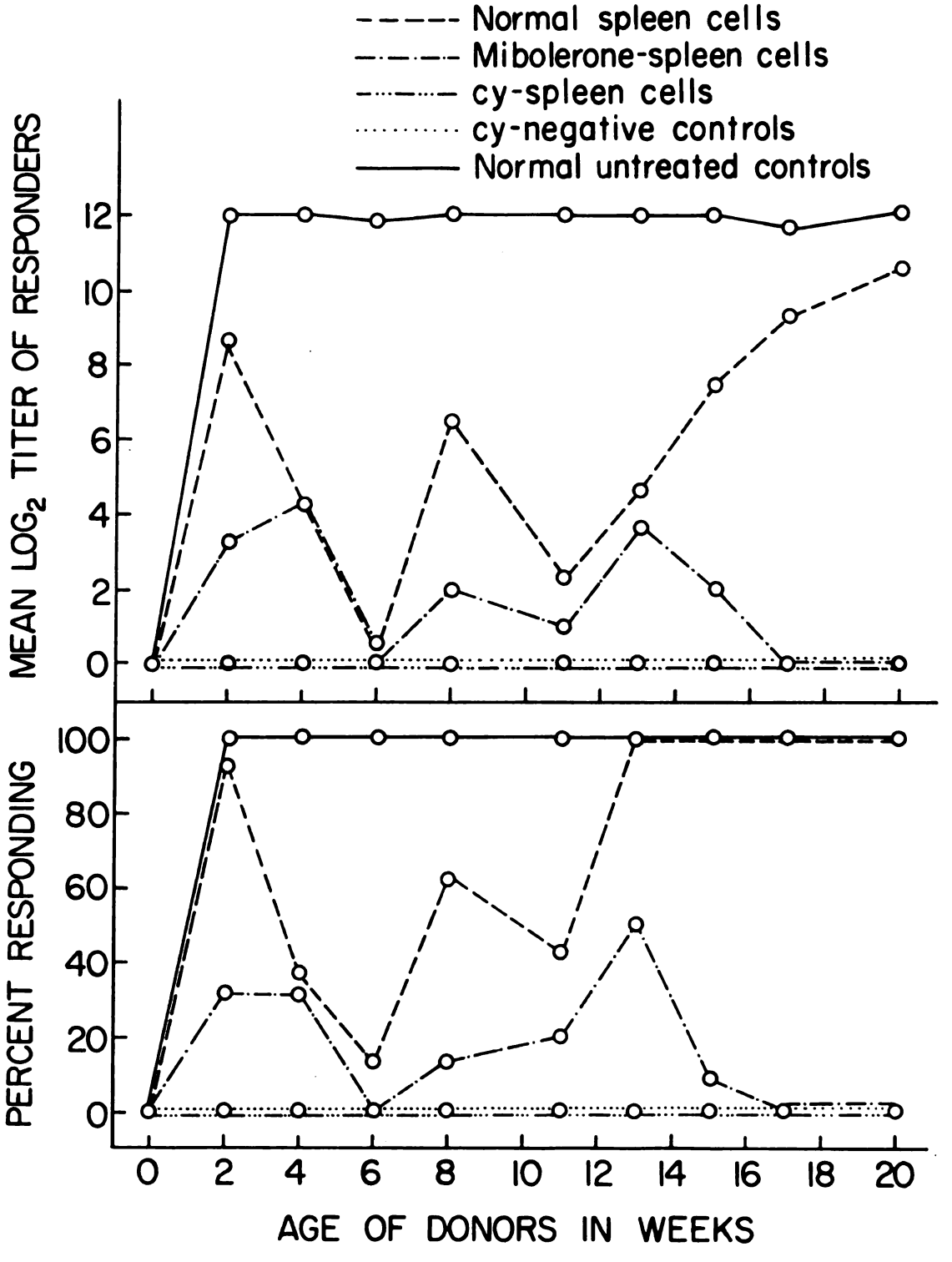
transplanted with spleen cells from 17 and 20 week-old normal donors for the primary antibody response (Figure 9) and in 29, 50, 100, and 100 percent in recipients transplanted with spleen cells from 13, 15, 17, and 20 week-old normal donors respectively for the secondary humoral antibody response (Figure 10). The humoral antibody titers achieved by the latter recipients were of the same magnitude as those titers found in the normal untreated chickens (Figure 10). Spleen cell suspensions from CY-treated donors did not restore the humoral immunocompetence of CY-treated recipients at any ages tested. Normal untreated chickens were always immunologically responsive to Brucella abortus in all 9 restoration trials, while CY-treated chickens did not respond with detectable humoral antibodies either after a primary or a secondary stimulation with Brucella abortus (Figures 9, 10).

b. Restoration of the immune response to SE

Spleen cell suspensions from 2, 4, 8, 11, 13, and 15 week-old mibolerone-fed donors restored the immunocompetence to SE for both the primary and secondary humoral antibody responses (Figures 11, 12). However, no restoration was obtained by transferring spleen cells from 6, 17, and 20 week-old mibolerone-fed donors (Figures 11, 12). Restoration of the primary and secondary humoral antibody responses was obtained with spleen cells from normal donors of 2 to 20 weeks of age in all 9 restoration trials

Figure 11. Long term restoration of the primary immune response to sheep erythrocytes by 2 X 10^7 spleen cells injected into cy-treated 8-9 day-old recipient chicks. Sheep erythrocytes injected together with Brucella abortus intravenously 5 weeks after the transfer and sera were obtained 1 week later. Each point represents 5-18 chickens.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Normal untreated controls
MEAN LOG2 TITER OF RESPONDERS
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PERCENT RESPONDING
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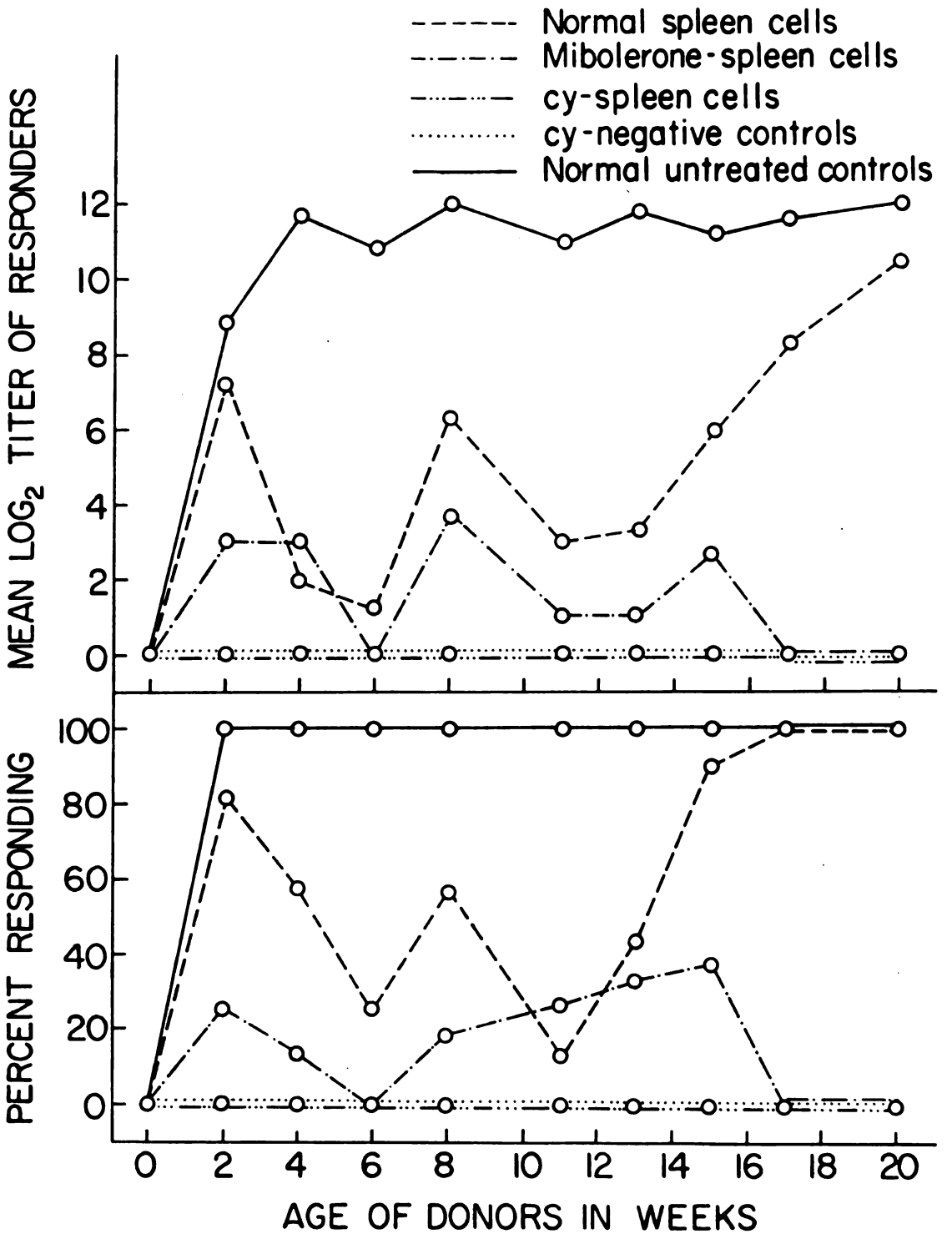
AGE OF DONORS IN WEEKS

Figure 12. Long term restoration of the secondary immune response to sheep erythrocytes by 2 X 10^7 spleen cells injected into cy-treated 8-9 day-old recipient chicks. Sheep erythrocytes injected together with Brucella abortus intravenously 7 weeks after the transfer (2 weeks after primary stimulation) and sera were obtained 5 days later. Each point represents 5-18 chickens.


Normal spleen cells
Mibolerone-spleen cells
cy-spleen cells
cy-negative controls
Normal untreated controls
MEAN LOG2 TITER OF RESPONDERS
PERCENT RESPONDING
AGE OF DONORS IN WEEKS
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(Figures 11, 12). Long term restoration was obtained in 100 percent of the recipients of spleen cells from 17 and 20 week-old normal donors for the primary response (Figure 11) and in 100 percent of the recipients of spleen cells from 13, 15, 17, and 20 week-old normal donors for the secondary humoral antibody response (Figure 12). The humoral antibody titers of recipients transplanted with spleen cells from mibolerone-fed donors were generally lower than the titers of recipients transplanted with spleen cells from normal donors. Spleen cell suspensions from CY-treated donors did not restore the humoral immunocompetence of CY-treated recipients at any ages tested. Normal untreated chickens were always immunologically responsive to SE in all 9 restoration trials, while CY-treated chickens did not respond with detectable humoral antibodies either after a primary or a secondary stimulation with SE (Figures 11, 12).

RAV-1 Induced Lymphoid Leukosis in Mibolerone-Fed Chickens

a. Effect of mibolerone on LL tumors induced by RAV-1

Mibolerone administered in the feed at the 1.5 µg/7 weeks dose completely prevented the development of LL tumors in males regardless of the route or time of infection and, with exception of 2 chickens infected at 1 day intracardially, it also prevented LL tumors in females (Table 15). Infection with RAV-1 produced high LL mortatliy in untreated male and female chickens regardless of the route

Table 15. Prevention of RAV-1 induced lymphoid leukosis tumors in chickens fed mibolerone.

Mibolerone	Sex	Age and Route of Infection[b]	RAV-1 Lymphoid Leukosis[a]						
			Viremia[c]	Antibody[d]	No. at risk	Survivors	Other neoplasms	No. with LL	% LL tumors
1.5µg/7wks	M	2wk IP	0/14	16/16	16	16	0	0	0.0
none	M	2wk IP	0/14	13/14	14	4	0	10	71.3
1.5µg/7wks	F	2wk IP	0/16	10/11	18	18	0	0	0.0
none	F	2wk IP	0/13	14/14	19	10	0	9	47.4
1.5µg/7wks	M	1day IC	8/12	11/13	15	14	1[e]	0	0.0
none	M	1day IC	4/10	8/9	15	2	2[e]	11	84.6
1.5µg/7wks	F	1day IC	7/17	14/15	23	20	1[f]	2	9.1
none	F	1day IC	8/16	14/14	23	1	1[e]	21	95.4

[a]Trial terminated at 150 days.

[b]1 x 10^5 TCIU of RAV-1 per chick intraperitoneally (IP) or intracardially (IC) at 2 weeks or 1 day of age, respectively.

[c]Assayed by PM of plasmas at 18 weeks of age (number positive/number tested).

[d]Assayed by neutralization of virus by plasmas at 18 weeks of age (number positive/number tested).

[e]Erythroblastosis.

[f]Liver and spleen hemangiomas.

or time of infection (Table 15). Erthroblastosis and hemangiomas were also induced by RAV-1 in a few chickens injected by the intracardial route at 1 day of age regardless of the treatment (Table 15).

b. Effect of mibolerone on RAV-1 neutralizing antibody and viremia

Chickens fed mibolerone developed neutralizing antibodies and viremia at a rate comparable to those seen in untreated chickens (Table 15). Chickens infected at 1 day of age were all viremic at 7 weeks regardless of the treatment. Approximately half these chickens remained viremic at 18 weeks of age. However, most of the latter also had antibodies in their plasma (Table 15).

c. Effect of mibolerone on the development of microscopic lesions in the bursae of RAV-1 infected chickens

Histological sections of 7 week-old bursae of RAV-1 infected mibolerone-fed chickens never showed LL lesions. Early histological lesions of LL bursal transformation were seen in 3 out of 6 of the bursae of the untreated 7 week-old chickens infected at 1 day of age (Table 16). These lesions were characterizec by the presence of lymphoblast-type cells throughout a single bursal follicle (Figure 4). No evidence of transformation was seen in sections of bursae of untreated chickens infected at 2 weeks of age.

Table 16. Effect of mibolerone on bursa weight, number of bursal follicles, number of germinal centers and early LL transformation in 7-week-old chickens infected with RAV-1.

Mibolerone	Sex	RAV-1 Trial			
		Mean Bursa wt. in gms	Avg. No. Bursal follicles[a]	Avg. No. Germinal Centers/cecum	No. of bursae with LL lesions[b]
1.5 µg/7 wk	M	0.124±0.006(6)[c]	40(6)	12.3(4)	0/6
None	M	3.579±0.24(6)	2250(5)	14.6(5)	1/6
1.5 µg/7 wk	F	0.163±0.01(6)	122(6)	16.4(5)	0/6
None	F	3.246±0.18(6)	1950(6)	9.6(5)	2/6

[a]Mean of the total number of bursal follicles remaining per cross-section of the regressed bursae in mibolerone-fed chickens. In the untreated chickens, figures are the mean number of bursal follicles of a cross-section of one plica seen in one microscopic field (3.5 X) multiplied by the number of plicae (15) found in normal bursae (no direct comparison is implied).

[b]Number positive/number checked for lesions.

[c]Number in parenthesis indicate number of chickens for which the mean was calculated ± standard error.

d. Effect of mibolerone on the weight and morphology of the bursa and on germinal centers of RAV-1 infected chickens

Mibolerone administered at the 1.5 µg/7 weeks regimen induced a significant regression of the bursa of Fabricius. The number of lymphoid follicles in the bursae of mibolerone-fed males ranged from 25-59 per cross section with a mean of 40 (Table 16). In mibolerone-fed females, the number ranged from 91-168 per cross section with a mean of 122. In the untreated chickens, because of the large size of the bursa, an accurate figure was difficult to obtain. However, an estimation of the number of bursal follicles for comparison purposes against the total numbers obtained in the bursae of mibolerone-fed chickens was made by multiplying the average number of bursal follicles seen in one microscopic field (3.5 X) by the number 15, which was the average number of plicae seen in each untreated bursa. Untreated males contained an average of 2250 bursal follicles per microscopic field while females had an average of 1950 bursal follicles per microscopic field (Table 16). Thickening of the connective tissue around remnant follicles was observed in the bursae of mibolerone-treated chickens. Some follicles showed various degrees of ballooning and often there was no clear demarcation between the cortical and medullary portions of the bursal follicles as seen in the untreated bursae (Figures 13, 14). Histological sectioning of cecal tonsils revealed the presence of germinal centers in mibolerone-fed chickens. The numbers

Figure 13. Histological section of a regressed bursa of Fabricius from a 7 week-old male chicken that had been fed the 1.5 µg/7 weeks mibolerone regimen and had been infected with RAV-1 at one day of age intracardially. There is abundant proliferation of connective tissue, very few bursal follicles and ballooning and shrinkage of the latter with total loss of the cortical medullary junction.

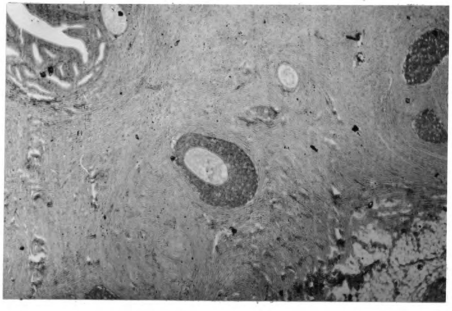
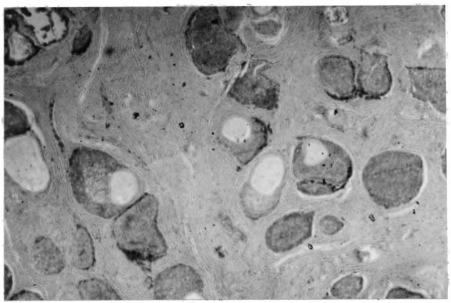


Figure 14. Histological section of a regressed bursa of Fabricius from a 7 week-old female chicken that had been fed the 1.5 μg/7 weeks mibolerone regimen and had been infected with RAV-1 at one day of age intracardially. There is proliferation of connective tissue and the bursal follicles remaining show some degree of ballooning, atresia and loss of the cortical medullary junction.



were comparable to those in the untreated chickens (Table 16). Germinal centers of mibolerone-treated and untreated chickens infected with RAV-1 are shown in Figures 15 and 16.

RAV-2 Induced Lymphoid Leukosis in Mibolerone-Fed Chickens

a. Effect of mibolerone on LL tumors induced by RAV-2

Mibolerone fed to both male and female chickens completely prevented the development of LL tumors induced by RAV-2 (Table 17). RAV-2 infection of susceptible untreated chickens at 2 weeks of age by the intraabdominal route resulted in the production of LL tumors in both male and female chickens (Table 17). Hemangiomas were seen in a male treated with mibolerone.

b. Effect of mibolerone on RAV-2 neutralizing antibody and viremia

Mibolerone-fed chickens infected at 2 weeks of age with RAV-2 developed neutralizing antibodies. Most chickens were also non-viremic at 18 weeks of age regardless of the treatment (Table 17).

c. Effect of mibolerone on the weight and morphology of the bursa and on the germinal centers of RAV-2 infected chickens

Mibolerone induced a significant regression of the bursa of Fabricius. Although no microscopic lesions were found in sections of the bursae at 7 weeks of age, the regression achieved was not considered great enough to

Figure 15. Histological section of germinal centers from the cecal tonsils of a 7 week-old chicken infected with RAV-1 at one day of age intracardially that had been fed the 1.5 µg/7 weeks mibolerone regimen.

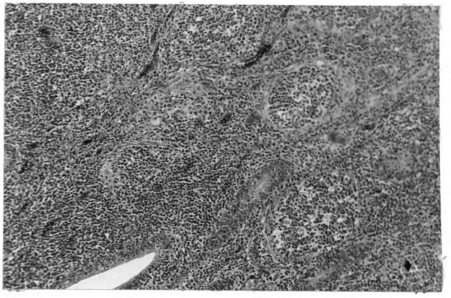


Figure 16. Histological section of germinal centers from the cecal tonsils of a 7 week-old chicken infected with RAV-1 at one day of age intracardially that had been fed the standard diet.

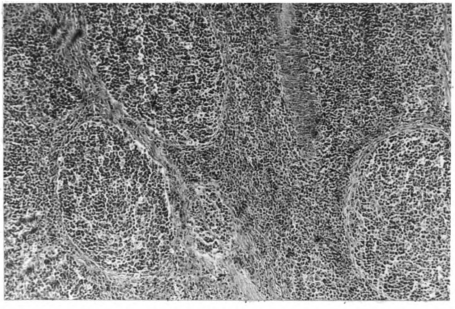


Table 17. Prevention of RAV-2 induced lymphoid leukosis tumors in chickens fed mibolerone.

Mibolerone	Sex	Age and Route of Infection[b]	RAV-2 Lymphoid Leukosis[a]						
			Viremia[c]	Antibody[d]	No. at risk	Survivors	Other neoplasms	No. with LL	% with LL tumors
1.5µg/7 wk	M	2wks IP	1/16	16/16	16	15	1[e]	0	0.0
none	M	2wks IP	2/16	15/16	16	10	0	6	37.5
1.5µg/7 wk	F	2wks IP	0/12	11/12	12	12	0	0	0.0
none	F	2wks IP	0/8	8/8	10	5	0	5	50.0

[a]Trial terminated at 180 days.

[b]1 x 10^5 TCIU of RAV-2 per chick intraperitoneally (IP) at 2 weeks of age.

[c]Assayed by PM test of plasmas at 18 weeks of age (number positive/number tested).

[d]Assayed by neutralization of virus by plasmas at 18 weeks of age (number positive/number tested).

[e]Liver and spleen hemangiomas.

prevent the development of LL tumors. Mibolerone was then administered for an extra week. At the end of the 8th week, one female was killed in both the mibolerone-treated and untreated groups and the bursae were removed and weighed. The bursa of the mibolerone-treated female weighed 0.125 gms with a total of 88 bursal follicles per bursa cross section, while the bursa of the untreated female weighed 3.650 gms with 2100 bursal follicles per microscopic field. Germinal centers were also found in the cecal tonsils of chickens fed mibolerone and infected with RAV-2 (Table 18).

Naturally Occurring Lymphoid Leukosis in Mibolerone-Fed Chickens

a. Effect of mibolerone on naturally occurring LL tumors

Mibolerone fed at the 3 regimens completely prevented the development of LL tumors, while LL tumors developed in 4 out of 16 chickens at risk in the untreated lots (Table 19). LL mortality in chickens that had become infected by horizontal spread of the natural LLV's did not occur. All 4 affected chickens in the untreated lots were females of the V+Ab- (viremia positive-antibody negative) type. Two males in the 1.0 µg/7 weeks regimen had nephroblastomas at termination. Non-specific early mortality was observed in all lots. However, higher mortality was experienced in congenitally infected (13.8 percent) than in non-infected chickens (7.5 percent).

Table 18. Effect of mibolerone on bursa weight, number of germinal centers and early LL transformation in 7-week-old chickens infected with RAV-2.

Mibolerone[a]	Sex	RAV-2 Trial		
		Mean bursa wt. in gms	Avg. No. germinal centers/cecum	No. of bursae with LL lesions[c]
1.5 µg/7 wk	M	0.232±0.005(3)[b]	17.5(2)	0/3
none	M	3.440±0.20(3)	13.7(3)	0/3
1.5 µg/7 wk	F	0.422±0.13(3)	28.3(3)	0/3
none	F	2.767±0.43(3)	19.5(2)	0/3

[a]Since the level of bursa regression obtained at 7 weeks was not the expected, chickens in this trial were fed mibolerone for 8 weeks. At the the end of the 8th week a bursa from a mibolerone-treated female weighed 0.125 gms, with 88 bursal follicles per cross-section, while the bursa from an untreated female weighed 3.650 gms with 2100 bursal follicles per microscopic field.

[b]Number in parenthesis indicate number of chickens for which the mean was calculated ± standard error.

[c]Number positive/number checked for lesions.

Table 19. Prevention of naturally occurring lymphoid leukosis tumors in chickens fed mibolerone.

Mibolerone	Natural lymphoid leukosis[b]					Viremia[c] & antibody[c]			
	No. chickens at risk	Survivors	Other neoplasms[a]	No. with LL	% LL tumors	V+Ab-	V-Ab+	V+Ab+	V-Ab-
1.0 µg/7wk	12	12	2	0	0.0	4	7	0	1
1.5 µg/7wk	13	13	0	0	0.0	7	2	3	1
2.0 µg/7wk	12	12	0	0	0.0	4	4	3	1
none	16	12	0	4(V+Ab-)	25.0	7	8	1	0
Total	53 (100%)	49 (92.5%)	2 (3.8%)	4 (7.5%)		22 (41.5%)	21 (39.6%)	7 (13.2%)	3 (5.7%)

[a]Nephrosblastomas occurred in 2 males.

[b]Trial terminated at 220 days.

[c]Assayed at 18 weeks of age.

b. Effect of mibolerone on the development of antibody and viremia

Virus assay by the PM test of the naturally infected progeny at 3 weeks of age showed that 44 percent of the chickens were viremic, and since these chickens remained viremic for 18 weeks and did not develop neutralizing antibodies they were considered to have been congenitally infected and consequently, to be immunologically tolerant to the infecting LLV. Thus, 8 out of 17 in the standard diet lot (47.1 percent), 6 out of 18 in the 1.0 µg/7 weeks lot (33.3 percent), 8 out of 18 in the 1.5 µg/7 weeks lot (44.4 percent), and 10 out of 19 in the 2.0 µg/7 weeks lot (52.6 percent) were identified as congenitally infected by assaying heparinized blood by the PM test. Naturally occurring LLV's spread horizontally from congenitally infected to contact chickens in all mibolerone treated and untreated lots as evidenced by increased rate of viremia (Table 20). Evidence of horizontal transmission as exemplified by transient viremia could be detected at 6 weeks of age, with the highest peak being detected at 10 weeks. At 18 weeks there were basically 4 types of chickens as classified by their viremia and antibody status. Chickens of the V+Ab- and V-Ab+ types were the most numerous in this highly infected flock.

Table 20. Horizontal transmission of natural LL viruses in chickens fed mibolerone.

Mibolerone	Proportion of chickens with transient viremia[a]			
	3 wks	6 wks	10 wks	18 wks
1.0 µg/7wk	0/12[b] (6)[c]	1/11 (3)	5/9 (3)	1/9 (3)
1.5 µg/7wk	0/10 (8)	2/8 (8)	5/8 (7)	2/8 (5)
2.0 µg/7wk	0/9 (10)	2/10 (6)	5/6 (7)	2/7 (5)
none	0/9 (8)	0/9 (8)	8/9 (8)	1/9 (7)
Total	0/40 (32)	5/38 (25)	23/32 (25)	6/25 (20)

[a]Sequential testing of heparinized blood from individual chickens by PM test.

[b]Number with transient viremia/number of contact exposed.

[c]Numbers in parenthesis are the number of congenitally infected chickens that served as spreaders of horizontally transmitted virus to contact exposed chickens.

Shedding of RAV-1 and RAV-2 in Mibolerone-Fed Hens

Infection of mibolerone-treated and untreated females with RAV-1 resulted in hens of the V+Ab+ type. Hens of this type shed virus in the albumen and in the yolk and also gs antigen in the albumen (Table 21). Although virus was isolated from albumen at a higher frequency than from egg yolk, the pattern of shedding seemed to be erratic in both mibolerone-treated and untreated hens. Although RAV-1 and gs antigen were detected more consistently in the albumen and yolks of mibolerone-treated hens than in the albumen and yolks of untreated hens, the differences were found to be due to individual variation within treatments and not to the treatments (5% probability) when tested by the method of Lush et al. (1948). Infection of mibolerone-treated and untreated females with RAV-2 resulted in hens of the V+Ab+, V+Ab-, and V-Ab+ types. Virus was isolated from the albumen and yolk and gs antigen was detected in the albumen of hens of the V+Ab+ and V+Ab- types from both treatments. Mibolerone-treated and untreated hens of the V-Ab+ type were essentially negative for virus and gs antige (Table 21).

Shedding of Lymphoid Leukosis Viruses in Naturally Infected Mibolerone - Fed Hens

LLV's and gs antigen were detected in the albumen of eggs obtained from mibolerone-treated and untreated hens of the V+Ab+ and V+Ab- types (Table 22). The shedding of

Table 21. Shedding of RAV-1 and RAV-2 in chickens fed mibolerone.

No. of hens	Mibolerone	Virus	Type	Detection of virus[a] & gs antigen[b]		
				Virus in		Gs antigen in
				Albumen	Yolk	Albumen
3	1.5 µg/7wk	RAV-1	V+Ab+	40/54 (74.1)[c]	7/54 (13.0)	52/54 (96.3)
5	none	RAV-1	V+Ab+	31/51 (60.8)	6/51 (11.8)	42/51 (82.4)
1	1.5 µg/7wk	RAV-2	V+Ab+	4/9 (44.4)	0/9 (0.0)	9/9 (100.0)
1	none	RAV-2	V+Ab+	8/8 (100.0)	1/8 (12.5)	8/8 (100.0)
2	1.5 µg/7wk	RAV-2	V+Ab-	25/25(100.0)	13/25 (52.0)	25/25 (100.0)
3	none	RAV-2	V+Ab-	88/89 (99.0)	8/89 (9.0)	85/89 (95.5)
14	1.5 µg/7wk	RAV-2	V-Ab+	1/170 (0.6)	0/38 (0.0)[d]	0/170 (0.0)
5	none	RAV-2	V-Ab+	0/45 (0.0)	0/11 (0.0)[e]	0/45 (0.0)

[a]0.4 ml of egg albumen or 0.2 ml of egg yolk assayed by PM test.

[b]Direct complement fixation on egg albumen.

[c]Number positive/number assayed (percent positive).

[d]Egg yolks from only 2 hens tested.

[e]Egg yolks from only 1 hen tested.

Table 22. Shedding of lymphoid leukosis viruses in naturally infected hens fed mibolerone.

No. of hens	Mibolerone	Type[a]	Detection of virus[b] & gs[c] antigen in albumen	
			Virus	Gs antigen
1	2.0 µg/7wk	V+Ab+	1/3 (33.3)[d]	2/2 (100.0)[d]
3	1.5 µg/7wk	V+Ab-	22/25 (88.0)	25/25 (100.0)
3	none	V+Ab-	61/121 (50.4)	121/121 (100.0)
2	1.0 µg/7wk	V-Ab+	1/39 (2.6)	0/39 (0.0)
1	1.5 µg/7wk	V-Ab+	0/2 (0.0)	0/2 (0.0)
2	none	V-Ab+	2/98 (2.0)	1/98 (1.0)

[a]V+ = persistent viremia; V- = transient viremia; Ab+ = antibody positive; Ab- = antibody negative.

[b]0.4 ml of albumen assayed by PM test.

[c]Direct complement fixation on egg albumen.

[d]Number positive/number assayed (percent positive).

virus was somewhat erratic and albumen from mibolerone-treated hens yielded virus more often than the albumen from untreated hens (Table 22). Albumen from hens of the V-Ab+ type were very rarely positive for virus and gs antigen and mibolerone did not increase the rate of shedding (Table 22).

Combined Results of Shedding of Lymphoid Leukosis Viruses in Mibolerone-Fed Hens

Infection of hens with RAV-1, RAV-2 and natural LLV's resulted in infections characterized by either a sustained viremia with or without concomitant development of antibody (V+Ab+ or V+Ab- types) or a transient viremia with the subsequent development of antibody (V-Ab+). As seen in the previous section, hens of the V+Ab+ and V+Ab- types shed LLV at a different rate than hens of the V-Ab+ type, and since the different LLV's and the various regimens of mibolerone used in these trials did not seem to influence the rate of shedding, the combined results are being presented for viremic (V+Ab+ and V+Ab- types) and non-viremic (V-Ab+) hens in both mibolerone-treated and untreated lots. Again, the pattern of shedding by the viremic hens proved to be erratic in both treated and untreated hens. However, LLV was detected at a high frequency in the albumen of eggs from both mibolerone-treated and untreated hens (Table 23). The differences in virus recovery from albumen of mibolerone-treated and untreated hens were not significant at the 5% probability level. LLV's were also recovered

Table 23. Combined results of shedding of lymphoid leukosis viruses in unincubated eggs.

Miboleroneª	Typeᵇ	No. of hens	Virus in albumenᶜ	Virus in yolkᶜ	Gs antigen in albumenᵈ
+	Viremic	10	92/116 (76.3)ᵉ	20/88 (22.7)	113/115 (98.3)
-	Viremic	12	188/269 (69.9)	15/148 (10.1)	256/269 (95.2)
+	Non-viremic	17	2/211 (0.9)	0/38 (0.0)	0/211 (0.0)
-	Non-viremic	7	2/143 (1.4)	0/11 (0.0)	1/143 (0.7)

ª1.0 - 2.0 μg/7wk regimens.

ᵇViremics were of the V+Ab+ and V+Ab- (permanent viremics) types; non-viremics were of the V-Ab+ (transient viremics) type.

ᶜAssay by PM test.

ᵈAssayed by direct complement fixation test.

ᵉNo. positive/no. tested (percent positive).

from the yolks of viremic hens in both mibolerone-treated and untreated groups although at a much lower rate than the recovery from albumen (Table 23). More yolks in the mibolerone-treated lot were positive for LLV than yolks from the untreated lot and the differences were significant at the 5% level. However, the variation in the rate of shedding was from one extreme to the other in hens within treatments and it was concluded by testing for heterogeneity of variance (Lush et al., 1948) that the differences were due to individuals tested and not to the treatments (5% level). Gs antigen was detected in the albumen of viremic hens but no statistically significant differences could be measured in the rate of shedding of gs antigen in both mibolerone-treated and untreated hens (5% level). In the non-viremic untreated hens, shedding of LLV or gs antigen in the albumen or yolk was of rare occurrence or did not occur at all (Table 23) and feeding mibolerone did not increase the rate of shedding of LLV or gs antigen in hens of the non-viremic type.

Viremia Titers in Mibolerone-Fed Hens Infected with Lymphoid Leukosis Viruses

The plasmas of viremic hens in the RAV-1, RAV-2, and natural LL shedding trials were titrated by the PM test to quantitate the amount of virus. No appreciable differences between mibolerone-treated and untreated hens were observed (Table 24).

Table 24. Viremia titers[a] in hens fed mibolerone and infected with lymphoid leukosis viruses.

Treatment	RAV-1	RAV-2	Natural LL viruses
Standard diet	$10^{4.8}$ (5)[b]	10^{5} (3)	10^{5} (4)
Mibolerone[c]	$10^{4.8}$ (5)	$10^{4.3}$ (3)	10^{5} (7)

[a]TCIU/ml of plasma.

[b]No. in parenthesis represents no. of chickens assayed by PM test.

[c]1.5 µg/7wks in RAV-1 and RAV-2 trials; 1.0, 1.5, 2.0 µg/7wks in natural LL trial.

DISCUSSION

The most important finding in these studies is that the androgen analog mibolerone when fed during the first 7 weeks of life prevents the development of natural and experimental LL tumors without interfering with the biological cycle of infection of LLV's. The finding is significant because mibolerone induces a slow but progressive involution of the bursa of Fabricius that results in practically bursaless chickens at the end of the feeding period without interfering with the immune capability of the chicken to produce antibodies and be protected by vaccination against avian pathogens of economic importance.

Chickens that had been fed mibolerone by the regimens used, had remnant lymphoid follicles in the bursa, but these follicles were atresic, with few recognizable lymphoid cells and there was not a clear demarcation between the cortical and the medullary areas. Moreover, there was abundant proliferation of connective tissue that compressed the remnant follicles. Mibolerone-fed chickens also possessed germinal centers in the cecal tonsils, a finding indicating that bursa-dependent peripheral lymphoid tissue remained intact in spite of bursa regression.

The experimental results also showed that mibolerone does not adversely affect the immunocompetence of the chicken. The humoral antibody response to SE known to be thymus dependent (McArthur et al., 1973) was similar in mibolerone-fed and control chickens; this similarity indicates that B and T cells interact normally in mibolerone-fed chickens. On the other hand, the humoral antibody response to Brucella abortus, a known T cell independent but B cell dependent antigen (Gilmour et al., 1970), was slightly reduced in the 1 µg/7 weeks regimen (significant at the 5% probability level but not at the 1% level when tested by analysis of variance).

Two trials were run to test for the presence of antibody-producing cells in the spleens of chickens that had been fed mibolerone. In the first trial, the number of antibody-producing cells was generally greater in mibolerone-fed chickens than in untreated controls. Also, relatively greater numbers of IgM-producing cells were found in the spleens of mibolerone-fed chickens than in the untreated controls. Kinkade and Cooper (1971) believe that the intraclonal switch in expression of immunoglobulin classes takes place in the bursa micro-environment before migration of bursa cells to the spleen and other organs. There is also evidence that the earliest B cells to leave the bursa are committed to IgM synthesis only (Kinkade et al., 1973). One possible explanation for our findings

is that mibolerone induces an early migration of bursal lymphocytes expressing surface IgM to the periphery. The bursa cells would migrate before the intraclonal switch in expression of immunoglobulin classes and increased numbers of IgM-producing cells in the peripheral lymphoid organs would result. Another explanation for the increased number of IgM-producing cells in the spleen is that mibolerone affects the intrinsic switch mechanism from IgM to IgG expression. Differences in the number of antibody-producing cells were not significant in the second trial. However, the assays were conducted for only the total number of Ig-producing cells.

The stimulation of peripheral lymphocytes and spleen cells by PHA is considered to be an in vitro correlate of cellular immunity, which in turn is an expression of thymus function (Weber, 1967). Lymphocytes from chickens fed mibolerone at the 1 µg/7 weeks regimen were induced into blastogenesis as measured by the incorporation of ^{3}H thymidine to the same level as lymphocytes from untreated chickens. This result reflected the integrity of the thymus function in chickens fed mibolerone at this level. Lymphocytes from chickens fed mibolerone at the 4 µg/3 weeks regimen did not react to the same extent as those from chickens fed the 1 µg/7 weeks regimen or the untreated control groups. However, the differences were not statistically significant. Moreover, the group fed the 4 µg/3 weeks regimen always reacted to an antigenic

stimulation with SE, a known T cell dependent antigen (McArthur et al., 1973).

Once it became evident that chickens that had been fed mibolerone remained immunologically competent in their response to inert antigens, one needed to know whether mibolerone-induced bursa regression could in any way interfere with the protection afforded by vaccination against the most economically important avian pathogens.

Although the role of the various specific humoral antibodies in the pathogenesis of MD is not fully known, mibolerone-fed HVT vaccinated chickens developed antibodies to both HVT and MDV that were detected up to 13 weeks after vaccination, the longest period tested. The mechanism of immunity or resistance to tumor induction provided by HVT on MD is not fully understood (Biggs et al., 1972; Purchase et al., 1971; Rouse et al., 1973) but it was important to know whether chickens treated with mibolerone could be successfully protected against MD with HVT. In recent experiments, chickens treated with large doses of cyclophosphamide were not protected against MD by vaccination with HVT (Purchase and Sharma, 1974), and infection with the IBA, a bursatrophic virus, interfered with HVT immunity to MD tumor development (Giambrone et al., 1976) and enhanced the severity of the nerve involvement in chickens infected with MD virus (Cho, 1970). In the present studies, however, mibolerone-fed HVT-vaccinated chickens were immune to MD, indicating that they had normal

immune functions. Ablation of the bursa by mibolerone did not prevent MD tumors agreeing with previous studies which showed that the course of MD was not affected by bursectomy (Payne and Rennie, 1970). Attempts to reisolate HVT from vaccinated chickens have shown that HVT can be recovered from the spleens of mibolerone-fed chickens as easily as from the spleen of vaccinated controls. Thus, mibolerone treatment had no effect on the development of MD or the protection from this disease by HVT vaccination.

In the Newcastle disease trial, mibolerone-fed chickens vaccinated with the B-1 LaSota strain developed HI antibody that was detected up to 5 weeks after vaccination and 2 weeks after challenge. The HI antibody is a good indicator of the immune status of a flock when serum from individual chickens are tested shortly after vaccination (Beard and Brugh, 1975). Perey et al. (1975) have postulated that although an intact humoral immune system is important in recovery from ND, the ability to produce neutralizing or HI antibody does not preclude mortality by NDV. Moreover, if the HI titer of random serum samples taken 3 weeks after vaccination is less than 1:10 with 10 hemagglutination units most of the birds will die upon exposure of the respiratory system with a virulent NDV (Beard and Brugh, 1975). In our experiment, the average HI antibody titer after vaccination was never higher than 1:10. Nevertheless, all chickens survived a challenge with the virulent Texas-GB strain. Treatment

with mibolerone did not prevent the development of ND in unvaccinated challenged chickens. Previous studies had shown that neonatally bursectomized agammablobulinemic chickens could not be protected by vaccination against a challenge with the Texas-GB strain (Cheville and Beard, 1972). Moreover, infection with the bursatrophic IBA produces immunosuppression as exemplified by, poor serological response to ND vaccination (Allan et al., 1972; Faragher et al., 1974; Biambrone et al., 1976; Hirai et al., 1974), lack of protection to challenge with virulent NDV (Allan et al., 1972; Faragher et al., 1974; Hirai et al., 1974), and increase in the carrier state of NDV (Pattison and Allan, 1974). In the present studies, however, mibolerone-fed chickens vaccinated with the B-1 LaSota strain were immune to ND, indicating that they had a normal immune function and mibolerone treatment did not alter the normal pathogenesis of ND.

In the infectious laryngotracheitis trial, mibolerone-fed chickens that had been vaccinated were immune to ILT challenge, also indicating that they had a normal immune function. However, the role of the bursa on immunity to ILT is uncertain, and the antibody titers in flocks exposed to ILT are generally low and give a poor correlation of a bird's resistance to virus challenge (Benton et al., 1958).

In the IB trial, persistance of the challenging virus in the trachea compared with the controls was used to

assess immunity (Hofstad, 1967; Winterfield and Fadly, 1971). The administration of mibolerone did not interfere with the ability of vaccinated challenged chickens to abort the challenge virus infection, as judged by the inability to recover virus from tracheal swabs. Although virus neutralizing antibodies, which are dependent on normal bursa function have not correlated well with immunity (Raggi and Lee, 1965), mibolerone-fed chickens were immune to IBV, indicating that they had a normal immune function.

In the fowl pox trial, mibolerone-fed vaccinated chickens were fully protected against challenge with virulent FPV. Although the role of the bursa in immunity to FP has not been evaluated with certainty, it is possible that resistance to challenge or infection results from the combined effect of both bursa and thymus (Tripathy and Hanson, 1975). Whatever the role of the bursa, vaccinated mibolerone-fed chickens were immune to FP, indicating that they had a normal immune function.

In the fowl cholera trial, vaccinated mibolerone-fed chickens were immune to a challenge with Pasteurella multocida. Little is known about the role of the bursa on immunity to FC. However, by analogy, it has been shown that destruction of the lymphoid elements of the bursa by the bursatrophic IBA increases the susceptibility and is responsible for the poor antibody response to bacterial infections caused by Salmonella typhimurium and Escherichia coli (Wyeth, 1975) and Hemophilus gallinarum (Hirai et al.,

1974). Similarly, bursectomized chickens are more susceptible to disease induced by Salmonella typhimurium than unbursectomized controls and are also poorly protected by vaccination (Chang, 1957; Chang et al., 1959). In the present studies, however, mibolerone-fed chickens vaccinated against FC were immune to challenge with Pasteurella multocida, indicating that they had a normal bursa-dependent function.

The experiments on immune responses, enumeration of antibody-producing cells, blastogenesis assay and vaccination immunity demonstrated that chickens that have been fed mibolerone remain immunologically competent in both their bursa and thymus functions. Also, it was found that the spleens of mibolerone-fed chickens contained higher numbers of antibody-producing cells than the spleens of hatch mates fed a standard diet. Questions, then, were raised on the mechanism of action of mibolerone and whether mibolerone by inducing a progressive involution of the bursa of Fabricius, also induced a more rapid maturation of the postembryonic stem cells, which in turn were also induced to migrate from the bursa microenvironment much sooner than under normal circumstances. If the hypothesis were correct, these migratory cells should have been found in the spleen earlier in the ontogeny of the plasma cell line in mibolerone-fed chickens than in chickens that were fed the standard diet. The results obtained in the restoration studies do not support the hypothesis of earlier peripherilization of stem cells from the bursa to peripheral lymphoid organs such as

the spleen, bone marrow and thymus. Some explanations for the failure of the restoration experiments are: a) mibolerone not only induces peripheralization of bursal cells but it also induces the bursal stem cells to differentiate beyond the stem cell stage. Since the present experiments were designed to test for stem cell restoration and not for immediate immunocompetence of the transferred lymphoid cells, a different experimental design would be required to test this alternative in which both the transferred cells and antigens are injected at the same time (Toivanen et al., 1972c). If mibolerone did, indeed, induce maturation of bursal stem cells to a point where they became immunocompetent, the immunocompetent cells would circulate for about 4 weeks (Toivanen et al., 1972a) then disappear from the body before they had the chance to be exposed to the antigens injected 5 weeks after the cell transfer (Toivanen et al., 1972b); b) stem cells in the spleen of mibolerone-fed chickens lost their homing ability because of changes induced in the stem cells by the hormonal action of mibolerone. These cells, then, would have circulated for a short time and died out as they became senile; c) stem cells of spleens of mibolerone-fed chickens were so modified by the action of mibolerone that they no longer fulfilled the criteria of being syngeneic with the recipient hosts. Consequently, the cells were rejected and no restoration was the outcome. However, this explanation seems unlikely since partial restoration of antibody activity to SE was

obtained; d) there was antigen competition between SE and Brucella abortus with consequent underimmunization. If the threshold for antibody response to SE is lower than the threshold for antibody response to Brucella abortus, then a response was obtained to SE but not to Brucella abortus; e) it is possible that treatment with mibolerone reduced the number of bursa-derived stem cells. Since the immune response to SE is known to be thymus-dependent (McArthur et al., 1973), then a synergistic cooperation between T and B cells was obtained resulting in amplification of the immune response due to the helper activity of T cells and possibly because the immune response to SE has a lower threshold than the immune response to Brucella abortus. On the other hand, with Brucella abortus there is little if any amplification because the immune response is largely thymus independent (Gilmour et al., 1970; Rouse and Warner, 1972) and if the number of B cells was the limiting factor, then, little or no antibody response would be the outcome. Of all these possibilities, explanation a) is the most compatible with the finding that mibolerone-fed chickens remain immunocompetent. While some restoration of the primary and secondary antibody response to SE was obtained at all times by the transfer of spleen cells from normal-donors, only partial restoration with lower antibody titers was achieved with spleen cells from mibolerone-donors. No antibody activity could be detected with spleen cells from 6, 17, and 20 week-old mibolerone-donors.

However, antibody activity against SE was detected at all other times. Unexpectedly, no restoration of the primary or the secondary humoral antibody responses against Brucella abortus was obtained (except for 2 chickens transplanted with spleen cells from 13 week-old donors) with spleen cells transferred from mibolerone-fed donors 2 weeks of age to 20 weeks of age. Then, partial restoration of the immune response was obtained against SE but not against Brucella abortus by transferring spleen cells from mibolerone-fed donors. This finding indicated a certain degree of selective responsiveness.

The findings on the restoration of the immune response with spleen cells from normal-donors support the concept that there exists migration of stem cells from the bursa of Fabricius to the spleen (Toivanen et al., 1974) at least during the first 20 weeks of life of the chicken. Previous studies on surgically bursectomized irradiated chickens showed that spleen cell transfers from sygeneic donors immediately restored the antibody response to Brucella abortus and to Salmonella pullorum, only if the donors were over 18 days of age. Moreover, spleen cell transfers from syngeneic bursectomized chickens failed to restore the antibody response to the same antigens (Matsuda et al., 1975) indicating the bursa-dependence of spleen cell immunocompetence. The cell transfer results confirm these findings, since spleen cells from CY-treated donors failed to restore the humoral immune response at anytime.

The work of Toivanen et al. (1972b) showed that spleen cells from 3 1/2-week-old syngeneic donors did not restore the immunocompetence of CY-treated chickens, while spleen cells from 14-week-old donors contained bursa-derived stem cells capable of long term immunological restoration. More recently, transfer studies performed with increasing numbers of spleen cells from donors of different ages (Toivanen et al., 1974) have shown that spleen cells from 1 1/2-week-old donors were inactive, while spleen cells from donors over 3 1/2-week-old were capable of restoring the primary immune response to both SE and Brucella abortus. The response was dependent on the number of spleen cells transferred and attained peaked levels at 7 1/2-weeks of age and was low at 16 weeks. Similarly, the secondary antibody response was spleen cell dose dependent, with higher titers obtained with spleen cells from 3 1/2 to 5 1/2-week-old donors. However, cell migration studies of bursa cells labeled in situ with ^{3}H thymidine showed that, by the time of hatching the traffic of lymphoid cells from the bursa to the spleen is well underway. This migration was also detected at 9 days and peaked at 6 weeks of age (Hemmingsson and Linna, 1972) while labeling at 14 weeks of age did not show any significant cell traffic. Since the in situ labeling technique only detects cells that are actively synthesizing DNA (Linna et al., 1969) and may not actually detect migration of stem cells (Toivanen and Toivanen, 1973), the migration patterns described by Hemmingsson and Linna (1972)

may in all probability not be representative of the migration of stem cells but of immunologically mature lymphoid cells. This assertion seems more plausible since colonization of germinal centers is a bursal stem cell-dependent function (Toivanen et al., 1972a) and in the Hemmingsson and Linna studies, no evidence of colonization of germinal centers by bursa-derived cells was shown. Of interest was the finding that spleen cells from 6 and 11 week-old normal donors had the capability of restoring the humoral immune response to SE but not to Brucella abortus. No explanation was found for this selective response. Since the degree of reactivity to SE by spleen cells from 6 and 11 week-old normal donors was consistently low, lack of reactivity to Brucella abortus might have been an indication of low antibody titers and the poor sensitivity of the agglutination test to detect antibodies. However, this seems unlikely, since good restoration was obtained with spleen cells from normal donors at all other ages. Examples of selective responsiveness to antigenic stimuli by transplanted lymphoid cells have been described by Matsuda et al. (1975) who found that bone marrow transplants restored the immunocompetence against Brucella abortus but not against Salmonella pullorum when the transplanted cells were from 18 day old donors. These workers believed that this selective reactivity was possibly associated with the relative delay in immune maturation to Salmonella pullorum. In the present experiments, the restorative ability of spleen

cells is not, then, a parameter of immunocompetence but of cell traffic from the bursa to the peripheral lymphoid organs, i.e., spleen.

The present studies have conclusively shown that the androgen analog mibolerone can prevent the development of LL tumors when administered in the feed during the first 7 weeks of life of the chicken. These studies confirmed previous knowledge (Kakuk et al., 1977) that mibolerone significantly prevented the development of LL tumors induced by RAV-1, a subgroup A virus, in highly inbred-LL susceptible chickens. Furthermore, it was shown that mibolerone is also effective in preventing LL tumors induced by RAV-2, a subgroup B virus, in highly inbred-LL susceptible chickens, and in preventing naturally occurring LL tumors in congenitally infected commercial White Leghorn chickens. LLV's belonging to subgroups A and B are most prevalent in field flocks (Calnek, 1968; Churchill, 1968) and are thought to be responsible for most of the LL losses. In the naturally occurring LL trial, although the LL tumor mortality was only associated with chickens of the V+Ab- type in the untreated lots, mibolerone also completely prevented the development of the disease in chickens of that type. It is not known whether treatment with mibolerone removed the target cells from the bursa. Histological sections of bursas removed at 7 weeks of age have shown that LL tumors can be prevented in chickens fed mibolerone even though some bursal follicles still remained

as judged by the absence of transformed follicles at this age or by the absence of gross tumors at terminationl Mibolerone may act (a) by inducing a rapid maturation and migration of bursal cells to the peripheral lymphoid organs or (b) by eliminating the target cell from the bursal follicles or (c) simply because the number of foìlicles remaining is not large enough for transformation and conseuqent disease to take place. In this respect, the chicken immune system may be able to eliminate the smaller number of transformed lymphoid cells from the bursas of mibolerone-treated chickens. However, no early lesions indicative of LL transformation could be observed in any of the bursal follicles of mibolerone-treated chickens at a time when 50 percent of the bursas from untreated chickens infected with RAV-1 at one day of age showed microscopic lesions of LL.

Germinal centers were always found in histological sections of the cecal tonsils of mibolerone-fed chickens that had regressed their bursae. This is in contrast to chickens bursectomized with CY, which lack germinal centers and are unable to mount an immune response (Toivanen et al., 1972a; Toivanen et al., 1972b; Toivanen et al., 1972c). Moreover, chickens that have been fed mibolerone are immunocompetent in both their bursa and thymus functions.

Mibolerone did not affect the development of viremia and neutralizing antibody in chickens that had been experimentally infected with RAV-1 and RAV-2. Generally, chickens

inoculated at one day of age had both neutralizing antibody and viremia as measured at seven and 18 weeks of age. Chickens inoculated at two weeks of age were non-viremic and already had neutralizing antibody at seven weeks of age. Previous work showed that only ten percent of chickens infected at hatching had neutralizing antibody at eight weeks and 50 percent at 16-24 weeks of age (Dent et al., 1968). Chickens fed the standard diet, infected with RAV-1 or RAV-2, and with neutralizing antibody were not protected against the development of tumors. These findings are compatible with the knowledge that leukotic and non-leukotic chickens possess similar levels of neutralizing antibody (Burmester et al., 1963). Thus, antibody is only an indication of infection and does not protect against the early local bursal transformation or the dissemination of the lymphoid tumor (Burmester et al., 1963; Dent et al., 1968). In the naturally occurring LL trial, chickens that were hatched viremic and thus were tolerant to LLV were identified at three weeks of age, the earliest time tested, because they remained viremic up to 18 weeks of age, the longest time tested and would have probably remained viremic for life (Rubin et al., 1961). The peak of horizontally transmitted viremia was detected at ten weeks, and by the 18th week of age, most of the contact exposed infected chickens in both mibolerone-treated and untreated lots had neutralizing antibody. These findings are consistent with previous findings that the sharpest increase in the

proportion of chickens with antibody occurred between 14 and 18 weeks (Rubin et al., 1962). The chickens used in the natural LL experiment were a selected group from known shedders. The highest proportion of chickens were of the V+Ab- type while birds of the V-Ab- type constituted the minority. Thus, horizontal transmission was probably facilitated by the high density of congenitally infected chickens (Burmester and Gentry, 1954).

LLV's could be isolated from egg albumen of viremic hens at a very high frequency in hens that had been fed mibolerone as well as in hens that had been fed the standard diet. Moreover, gs antigen was nearly always detected in the egg albumen of these hens and proved to be a most reliable indicator of infection in viremic hens. It had previously been shown that large numbers of LL viral particles and viral "buds" were present in the magnum of mature-egg laying-LLV congenitally infected hens (Distefano and Dougherty, 1965) and LLV and gs antigen had been detected in unincubated eggs of LLV infected hens (Spencer et al., 1976). More yolks in the mibolerone-treated lot were positive for LLV than yolks from the untreated lot and differences were significant when an overall contingency chi square was conducted. However, the variation in the rate of shedding among hens within the treatment groups was very large. When a method was used to take into account this variation (Lush et al., 1948), the difference was not significant at the 5% probability level, indicating that

the overall difference was probably due to chance selection of hens. Hens that had been infected with either RAV-1, RAV-2, or by contact exposure to tolerant chickens and had experienced a transient viremia with development of antibody very rarely if at all excreted virus or gs antigen in the eggs, regardless of whether they had been fed mibolerone or not. This finding indicated that treatment with mibolerone of non-viremic hens did not increase the rate of shedding.

The data presented in this work are of considerable significance since none of the existing methods for the control of LL can yet be extensively used on a large scale basis. It has been suggested that eradication is probably the method of choice for the control of LL (Calnek et al., 1967). However, the technology presently available to achieve a virus-free flock is time consuming, complicated, expensive, and is not yet applicable for large scale use (Purchase and Burmester, 1977). Other methods for the control of LL such as breeding for resistance to infection (Crittenden, 1975), breeding for resistance to tumor development (Crittenden, 1975), vaccination (Burmester, 1955), and control by elimination of target cells (Peterson et al., 1964; Purchase and Gilmour, 1975; Burmester, 1969; Purchase and Cheville, 1975) have not yet been extensively developed so that they may be used in the field without introducing the hazard of lowering productivity, aggravating the occurrence of LL tumors or interfering with immunity

against other important avian pathogens.

The androgen analog mibolerone can be conveniently fed at g levels to growing chickens and can be used to prevent the development of LL tumors induced by viruses of subgroups A and B. There is no reason to believe that mibolerone could not be used in the prevention of LL tumors induced by viruses of subgroups C and D, reported to occur in European field flocks (Sandelin and Estola, 1974). Mibolerone does not interfere with the biological cycle of infection of LLV's. Both vertical and horizontal spread seem to take place in the usual manner and consequently LLV's are not eliminated from infected flocks. The merit of mibolerone, thus, is that it prevents economic losses due to LL mortality in chickens infected with LLV's.

SUMMARY

This dissertation deals with studies performed with the androgen analog mibolerone (17 -hydroxy-7α, 17dimethy-lestr-4-en-3-one) in order to assess its effects on the general immunocompetence of the chicken and on the prevention of lymphoid leukosis tumors when fed at μg levels during the first seven weeks of life. The findings of these studies are summarized below.

1. Feeding mibolerone in the diet during the first seven weeks of life induces a progressive regression of the bursa of Fabricius that results in practically bursa-less chickens.

2. Females seem to be more resistant to mibolerone regression of their bursae than males as evidenced by the finding of greater number of atrophic lymphoid follicles in their regressed bursae.

3. Chickens fed mibolerone produce humoral antibodies to sheep erythrocytes of similar titers to those fed a standard diet, and humoral antibodies to Brucella abortus of lower titers than those fed a standard diet.

4. The spleens of chickens fed mibolerone contained greater number of antibody-producing cells to sheep erythrocytes than the spleens of chickens fed the standard diet.

5. The peripheral blood leukocytes of mibolerone-fed chickens are stimulated into blastogenesis to the same degree as those leukocytes from chickens fed a standard diet when cultured in the presence of PHA.

6. Chickens fed mibolerone are protected against Marek's disease after vaccination with HVT, and develop humoral antibodies against HVT and MDV.

7. Chickens fed mibolerone are protected against Newcastle disease after vaccination with B1-LaSota strain, and develop antibodies to NDV.

8. Chickens fed mibolerone are successfully immunized by vaccination against avian infectious laryngotracheitis.

9. Chickens fed mibolerone are successfully immunized by vaccination against avian infectious bronchitis.

10. Chickens fed mibolerone are successfully immunized by vaccination against fowl pox.

11. Chickens fed mibolerone are successfully immunized by vaccination against fowl cholera.

12. The spleens of chickens fed mibolerone could not be shown to contain post-bursal stem cells that will differentiate into antibody-producing cells to Brucella abortus.

13. The spleens of chickens fed mibolerone contain low levels of post-bursal stem cells that will differentiate into antibody-producing cells to sheep erythrocytes.

14. Mibolerone prevents the development of LL tumors induced by RAV-1, a subgroup A virus.

15. Mibolerone prevents the development of LL tumors induced by RAV-2, a subgroup B virus.

16. Mibolerone prevents the development of naturally occurring LL tumors.

17. Mibolerone-fed chickens develop neutralizing antibodies and viremia in a similar pattern to those observed in chickens fed the standard diet.

18. Hens viremic with LLV's and fed mibolerone shed virus and gs antigen in a pattern similar to that of hens fed the standard diet.

19. Non-viremic mibolerone-fed hens and hens fed the standard diet shed virus and gs antigen in the albumen very rarely or not at all.

20. Hens viremic with LLV's and fed mibolerone carry the same load of virus in their plasmas as those hens that are fed the standard diet.

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