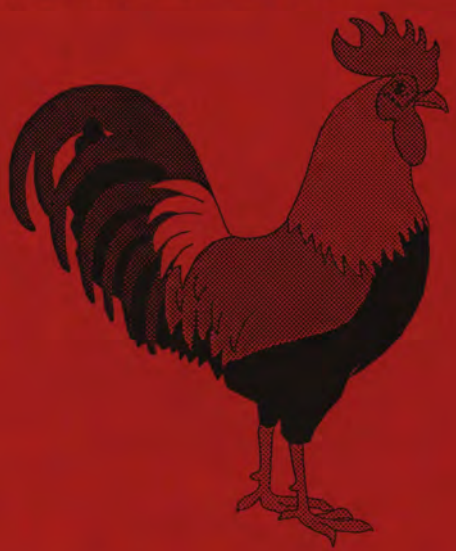
Avian Tumor Viruses Symposium

Diagnosis and Control of Neoplastic Diseases of Poultry



Symposium Program

American Association of Avian Pathologists

40th Annual Meeting
Reno,Nevada
July 21, 1997

DIAGNOSIS AND CONTROL OF NEOPLASTIC DISEASES OF POULTRY

~SYMPOSIUM PROGRAM~

8:00 - 8:05 a.m.	Aly Fadly	Welcome

Session I. Differential Diagnosis of Avian Lymphomas

(Moderator: Patricia Wakenell)

8:05 - 8:20 a.m.	Patricia Wakenell	An overview of problems in diagnosis of neoplastic diseases of poultry
8:20 - 8:40 a.m.	Aly Fadly	Criteria for the differential diagnosis of viral lymphomas of chickens: a review
8:40 - 9:00 a.m.	Donald Ewert	Molecular approaches for the diagnosis of avian lymphomas
9:00 - 9:20 a.m.	Robert Silva	PCR as a tool for differential diagnosis of avian tumor viruses and tumors
9:20 - 9:40 a.m.		Open discussion with all speakers of Session I.
9:45 - 10:00 a.m.		**Break**

Session II. Marek's Disease

(Moderator: Bruce Calnek)

10:00 - 10:20 a.m.	Kenton Kreager	Marek's disease: clinical aspects and current field problems in layer chickens
10:20 - 10:40 a.m.	G. Thomas Holder	Marek's disease: clinical aspects and current field problems in broilers
10:40 - 11:00 a.m.	John Rosenberger	Epizootiology and adult transmission of Marek's disease
11:00 - 11:20 a.m.	Karel A. Schat	Vaccinal immunity to Marek's disease
11:20 - 11:40 a.m.	Richard Witter	Current and future strategies for control of Marek's disease
11:45 - 12:10 p.m.		Open discussion with all speakers of Session II
12:10 - 1:30 p.m.		**Lunch**

SYMPOSIUM PROGRAM - CONTINUED

Session III. Avian Leukosis

(Moderator: William Chase)

1:30 - 1:50 p.m.	J. Lloyd Spencer	An overview of progress and problems in the control of avian leukosis
1:50 - 2:10 p.m.	Aly Fadly	An overview of subgroup J-like ALV infection in broiler breeder flocks in the U.S.
2:10 - 2:40 p.m.	L.N. (Jim) Payne	Current status of diagnosis, epidemiology and control of ALV-J
2:45 - 3:00 p.m.		Open discussion with all speakers of Session III.
3:00 - 3:15 p.m.		**Break**

Session IV. Reticuloendotheliosis and other Neoplastic Conditions

(Moderator: Richard Witter)

3:15 - 3:30 p.m.	Richard Witter	Reticuloendotheliosis virus: an overview of current issues
3:30 - 3:45 p.m.	Hugo Medina	Reticuloendotheliosis virus infection in turkeys: industry perspective
3:45 - 4:15 p.m.	Irit Davidson	Epidemiology and control of reticuloendotheliosis virus in chickens and turkeys in Israel
4:15 - 4:30 p.m.	Scott Taylor	Methods for detection of reticuloendotheliosis virus contamination in poultry vaccines
4:30 - 4:45 p.m.	Mark Goodwin	Dermal squamous cell carcinoma and multicentric histiocytosis in chickens
4:45 - 5:00 p.m.		Open discussion with all speakers of Session IV
5:00 - 5:05 p.m.	Jagdev Sharma	**Closing remarks**

ACKNOWLEDGMENTS

The 1997 Avian Tumor Viruses Symposium follows and builds upon the last symposium held in Seattle in 1991. The theme for this year's symposium is diagnosis and control of neoplastic diseases of poultry: current status and perspectives for the future. The symposium is focused on the use of current information on the molecular and antigenic structure of avian tumor viruses to effectively develop highly specific reagents for differentiating between the most common virus-induced neoplastic diseases of poultry. In this symposium, current efforts to improve existing strategies for better control of Marek's disease, avian leukosis and reticuloendotheliosis are also discussed.

The Organizing Committee is grateful to the speakers who generously agreed to share their expertise with the rest of AAAP membership. The Organizing Committee would also like to thank all of those who have made this symposium possible; particularly members of the AAAP Avian Tumor Viruses Committee for their advise on topics and speakers, the AAAP Board and the AVMA for their financial support, Drs. Donald Waldrip and Robert Eckroade for logistical assistance, past Symposium Chair Dr. T. J. Myers for his advise on planning and scheduling of the symposium, Dora Westbrook of the USDA-Agricultural Research Service, Avian Disease and Oncology Laboratory for assistance in formatting and processing the symposium program, and Rebecca Dodson of Omnipress for her assistance in printing the program.

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AN OVERVIEW OF PROBLEMS IN DIAGNOSIS OF NEOPLASTIC DISEASES OF POULTRY

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INTRODUCTION

The intent of this paper is to discuss the problems encountered in the differential diagnosis of neoplastic conditions of chickens and turkeys that are of major importance to the commercial poultry industry. These include Marek's disease, the avian leukosis/sarcoma complex and reticuloendotheliosis. Because of their potential for complicating diagnosis, multicentric histiocytosis and lymphoproliferative disease (LPD) will also be included.

CHICKENS

In the past, age was used as one of the major criteria for differentiating between tumors caused by Marek's disease virus (MDV) and those caused by avian leukosis virus (ALV) in both the broiler and laying industry. Before 14 weeks of age, it is unlikely to encounter lymphoid tumors caused by ALV (15). Since the majority of tumors in this age range are caused by MDV, gross evidence of visceral and/or peripheral nerve tumors has generally been considered sufficient for a presumptive diagnosis of MDV (4). However, it has been recently apparent that multicentric histiocytosis and tumors caused by reticuloendotheliosis virus (REV) may have been erroneously attributed to MDV, particularly at processing where only gross diagnosis is used. Currently, only limited information is available concerning the epidemiology of REV in the US. A previous report showed a 21-23% seroconversion in layer and broiler and broiler breeder flocks (20,21). Research conducted in Israel suggests that the prevalence of REV is similar and may be increasing despite controls placed on importation of chicks used for breeding flocks. Although REV tumors are more frequent in older birds, it is possible to find tumors in young birds that affect some of the same organs (including peripheral nerves) as those affected by MDV (22,23). An additional complicating factor is that REV nerve infiltrates can be pleomorphic in appearance on histopathologic examination, similar to MDV (22). However, visceral tumors may be composed of a monomorphic cell population in contrast to MDV (23). Anecdotally, organ preference may be suggestive, REV has a higher incidence of tumors in the pancreas than do the current pathotypes of MDV. However, differences in MDV pathotypes are associated with a wide variation in organ preference further complicating diagnosis. Serology may be useful for flocks that have single infections with REV but is less practical for the ubiquitous MDV (17,21). Polymerase chain reaction (PCR) conducted on the tumor tissue or blood may aid in diagnosis and is faster and less labor intensive than virus isolation (1,5). Attributing tumors to REV or MDV in the presence of dual infection can be difficult. Generally, REV tumors lack the tumor-associated surface antigen found in MDV tumors which

can also aid in diagnosis (18). Histiocytosis complex has not been described in the peripheral nerves, but does occur in the spleen and other organs in a similar manner to MDV and REV (10,11). This complex has not as yet been proven to be a neoplastic condition (11). Microscopic examination of the mass will readily distinguish histiocytic cells versus the lymphoid cells found with both REV and MDV (11). No agent has been identified for multicentric histiocytosis and diagnosis is based upon splenomegaly and microscopic examination of the masses.

With older chickens, differential diagnosis becomes less clear. Although lymphoid tumors are still predominantly caused by ALV, MDV tumors are increasing in incidence particularly with the emergence of the highly pathogenic vv+ strains. With backyard poultry, MDV in aged chickens is more common than ALV caused tumors. For older chickens, however, the standard "rules of thumb" still apply: rarely does MDV cause tumors of the bursa whereas ALV does (7,15). In addition, upon microscopic examination, ALV tumors are composed of a monomorphic population of cells whereas MDV tumor cells are pleomorphic (7,15). Again, REV can be a factor and multiple (double or triple) infections do occur (8,9). Chronic bursal tumors caused by REV can be indistinguishable from ALV tumors - both cause monomorphic B-cell tumors with cell surface IgM (6,8,15,18). Using PCR to distinguish between MDV and REV and virus isolation for ALV is useful in single virus infections (1,5).

An additional frustrating problem with MDV is the inability to ascertain the different pathotypes of the serotype 1 virus *in vitro*. Although PCR can distinguish between an attenuated serotype 1 virus and a non-attenuated, live virus; it cannot differentiate between a virulent MDV and a vv+ MDV (16). Currently, pathotyping *in vivo* against known standard pathotypes is the only way to determine the strain of serotype 1 MDV. In addition to being expensive, time consuming and labor intensive, the pathotyping of MDV strains may not distinguish between two strains of the same pathotype, i.e. RB1B and MD5. Before the pathotyping can be done, the MDV must be isolated and purified from the vaccine virus(es). Isolation of MDV's is not available as a routine diagnostic laboratory service.

There is recent evidence that multiple infections with the retroviruses and MDV may be synergistic for one or more of the involved viruses (2,12). Recombination between REV and MDV has been demonstrated in the laboratory and may have significant implications for the epidemiology of both diseases, if recombination occurs under natural conditions. At the very least, multiple infections do change the characteristics of the individual diseases which further complicates differential diagnosis.

TURKEYS

In the U.S., pathogenic MDV and LPD have not yet been found in turkeys leaving REV as the primary tumorigenic agent (12). In Israel where both MDV and LPD are found in the commercial industry, these can greatly complicate tumor diagnosis (12). Tumors caused by REV in turkeys are made up of a monomorphic population of cells (18,19). Pleomorphic cell populations are still observed with MDV as well as with LPD (3,12,13,24). Unlike in the

chicken, bursal involvement with REV is uncommon (18). PCR can be used for diagnosis of all three agents and can be quite accurate when used in combination with serology for REV and histopathology (12). An indirect immunofluorescence test and an indirect ELISA are also available for LPD (14). Again, ascribing a tumor to a single agent in face of multiple infections can be difficult.

SUMMARY

Currently, there are more options than gross and microscopic examination for identification of the causative agent(s) for neoplasms is chickens and turkeys. Unfortunately, many of these are unavailable through either state or private diagnostic laboratories and still are considered primarily as research tools. Even with the advent of new techniques, pathologic examination of the tissues is still important is defining the diagnosis. In the case of multiple infections, synergism between or amongst the agents can significantly change the "normal" pictures of the diseases. Much work still needs to be done on the epidemiology of REV and multicentric histiocytosis in order to determine the extent of their roles in the commercial poultry industry.

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CRITERIA FOR THE DIFFERENTIAL DIAGNOSIS OF VIRAL LYMPHOMAS OF CHICKENS: A REVIEW

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INTRODUCTION

Lymphomas caused by avian retroviruses and herpesviruses are the most common viral neoplastic diseases of poultry (2,11,17). Retroviral lymphomas are caused by avian leukosis virus (ALV) or by reticuloendotheliosis (RE) virus (REV), whereas Marek's disease (MD) lymphomas are caused by a herpesvirus (MDV). Differential diagnosis of viral lymphomas of poultry has been based on pathological criteria (7,10,11,14). Further, virologic and serologic criteria, such as assays for virus, antigens or antibodies (8,13) are not particularly helpful in tumor diagnosis because ALV, REV and MDV are all widespread and infection in the absence of tumor formation is common. Current technology such as immunocytochemistry and molecular hybridization is being used to develop more sensitive and specific procedures for a more definitive diagnosis of avian viral lymphomas. This review describes the criteria currently available for the diagnosis of viral neoplastic diseases of chickens, namely avian leukosis, Marek's disease and reticuloendotheliosis.

PATHOLOGIC CRITERIA

The development of ALV-induced lymphomas (LL) involves a multistage process that takes from 14-25 weeks (6,11). The target cell for ALV transformation is a B-cell that is present in the bursa of Fabricius during late embryonic development and approximately the first 2 weeks after hatching. It is believed that only 1 or 2 lymphomas develop in an individual bursa and by 14-25 weeks of age, the neoplastic cells spread to visceral organs such as liver and spleen. Because the pathogenesis and pathology of RE bursal lymphoma is similar to that of LL (17), the presence of gross and microscopic bursal lesions, considered to be pathognomonic for LL, can not be used to differentiate between LL and RE bursal lymphomas. However, the presence of such bursal lesions and the absence of gross and microscopic nerve lesions, usually associated with MD can be used to rule out MD and RE nonbursal lymphomas. Also, homogeneity of the tumor cells is considered a characteristic of LL, but not MD. In most cases, age at the onset of disease may be used to rule out LL and RE bursal lymphomas only if tumors occur in chickens younger than 14 weeks of age. Clearly, the pathological criteria currently used in the diagnosis of viral lymphomas of chickens (Table 1) are subjective and rarely provide a definitive diagnosis.

VIROLOGICAL AND SEROLOGICAL CRITERIA

The widespread incidence of avian tumor viruses limits the usefulness of virological and serological assays as a tool for the differential diagnosis of viral lymphomas of chickens, as multiple infection with more than one virus is likely to be common under field conditions. However, such serological and virological assays may be useful in the diagnosis of viral lymphomas in pathogen-free and other commercial chicken flocks that are known to be free from multiple viral infections.

TABLE 1. Pathological, virological and serological criteria used in the diagnosis of viral lymphomas of chickens.

	Lymphoma			
Criterion	LL	RE/bursal	RE/nonbursal	MD
Bursa lesions	+(-)	+(-)	-	-
homogeneity of tumor cells	+	+(-)	+(-)	-
Nerve lesions	-	-(+)	+(-)	+(-)
Age at onset (older than 14 weeks)	+	+	-(+)	+(-)
Virus	±	±	±	±
Antibody	±	±	±	±

+ = present; - = absent; () = occasionally; ± = present or absent

IMMUNOCYTOLOGICAL CRITERIA

Retroviral lymphomas caused by ALV or by REV originate from either B-cells (LL, RE bursal) or T-cells (RE nonbursal), whereas MD lymphomas are of T-cell origin (2,11,17). Such characteristics provided the basis for tests that distinguish among B- and T-cell lymphomas (10). Monoclonal antibodies specific for cell surface antigens of B- and T-lymphocytes and those that

are directed towards viral antigen (3,5,9) can be used in histochemical staining of affected tissues to determine type of tumor cell or viral antigen. This procedure appears especially well suited for use in diagnostic laboratories (Table 2).

MOLECULAR CRITERIA

Retroviral lymphomas of chickens are clonal and are characterized by specific insertion of proviral DNA at one or few sites in the cellular genome adjacent to cellular oncogenes (11,17). These characteristics represent opportunities for definitive diagnoses based on hybridization with DNA probes. Southern blotting and hybridization analysis of tumor DNA can be used to identify clonal insertions of exogenous ALV or REV and for detection of displacement of cellular oncogene (c-*myc*) (Table 3). On the other hand, clonality of MD lymphomas has not been confirmed, but probably they contain a higher frequency of infected cells and more copies of viral DNA per infected cell than are typically found in tissues of chickens that are not bearing tumors (2).

TABLE 2. Predicted reactions of monoclonal antibodies that can be used for diagnosis of viral lymphomas of chickens by immunocytochemistry.

Monoclonal antibody	Lymphoma			
	LL	RE/bursal	RE/nonbursal	MD
B-cell markers	+	+	-	-
T-cell markers	-	-	+	+
REV	-	+(-)	+(-)	-
ALV	+(-)	-	-	-
MDV (pp38)	-	-	-	-(+)

+ = present; - = absent; () = rarely.

Furthermore, the polymerase chain reaction (PCR) is a sensitive assay that through repetitive cycles of DNA denaturation, primer annealing and polymerase extension can amplify DNA sequences by a factor of 10^6 or more. The specific amplification of targeted DNA can be easily detected on agarose gels. Use of the PCR technique allowed the detection of two copies of the 132-base-pair repeat in the DNA extracted from MD, but not from LL or RE lymphomas (15).

TABLE 3. Use of Southern blotting and hybridization in the differential diagnosis of viral lymphomas of chickens.

	Lymphoma			
Probe	LL	RE/bursal	RE/nonbursal	MD
c-*myc*	+(-)	+(-)	+(-)	-
pRAV-2	+	-	-	-
pSNV	-	+	+	-

c-*myc* = a probe used to detect cellular myc oncogene alteration; pRAV-2 = a probe used to detect clonal insertion of ALV; pSNV = a probe used to detect clonal insertion of REV; + = positive; - = negative; (-) = occasionally, 10 to 20% of tumors may be negative.

PCR assays have also been shown to detect REV sequences in DNA from RE, but not from LL or MD lymphomas (1,4,12). Further, using PCR proviral DNA and viral RNA were detected in various tissues early after infection with ALV (16).

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MOLECULAR APPROACHES FOR DIAGNOSIS OF AVIAN LYMPHOMAS

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INTRODUCTION

An understanding of the molecular mechanisms associated with neoplastic transformation in avian lymphomas provides a basis for developing strategies for their diagnosis. Most lymphomas in *aves* are induced by viruses; a herpesvirus or a retrovirus, which either carry genes that are oncogenic or, which when integrated in cellular DNA near proto-oncogenes cause their dysregulation. In the latter case, the insertion of strong-viral promoter-enhancer sequences in the long terminal repeat (LTR) of the retrovirus results in enhanced expression of the cellular proto-oncogene. These two basic mechanisms of transformation, therefore, provide a means for distinguishing between different viral etiologies. First, those viruses that have been shown to induce lymphomas that contain genes encoding oncoproteins, i.e. Marek's herpesvirus (*meq*) (1), reticuloendotheliosis virus, REV-T (*rel*) (2,3,4) and avain myelocytomatosis virus, MC29 (*myc*) (5) mark of their presence in all tissues they infect, normal and transformed, in the form of viral message (DNA or RNA), cDNA, or protein. By contrast dysregulation of the cellular proto-oncogene and elevated expression of the respective oncoprotein will only be detected in cells where the virus is integrated in the cellular gene.

Second, a growth or survival advantage resulting from deregulation of a cellular proto-oncogene as a result of proviral insertion will cause a clonal expansion of cells carrying viral insertion. Thus, in the case of the transforming retroviruses all cells within a tumor will carry random integrations of proviral DNA, but viruses that require integration in a cellular proto-oncogene to transform cells will have, in addition, a proviral integration in the cellular proto-oncogene at a particular site in the DNA that is the same in all cells within a single tumor. Using restriction enzymes that cut in the proviral and cellular proto-oncogene DNA fragments can be generated that contain both viral and oncogene information. Since these fragments are clonally expanded their copy number is sufficient to be easily detected by Southern analysis of tumor DNA.

VIRUSES THAT INDUCED LYMPHOMAS BY INSERTIONAL MUTAGENESIS OF CELLULAR ONCOGENES

Two groups of nondefective avian retroviruses, the avian leukosis viruses (ALV) (6) and the reticuloendotheliosis viruses (REV) (7) utilize the insertional mutagenesis mechanism to dysregulate the c-*myc* gene. Although the primary target cells of transformation for both ALV and REV are immature cells of the B lymphoid lineage found in the bursa of Fabricius, at least one report has identified insertion of a nondefective REV in the c-*myc* locus of a T cell lymphoma resembling a Marek's disease lymphoma (8).

ALV is the etiological agent of one of the most common naturally occurring lymphoid neoplasia in chickens, lymphoid leukosis (LL). In LL, dysregulation of the c-*myc* gene occurs in B cell lymphomas by the integration of ALV upstream of coding exons in the c-*myc* locus. The ALV provirus is arranged in the same transcriptional direction as c-*myc* placing the *myc* gene under control of the viral LTR which contains the promoters and enhancers for transcription. Approximately 90% of the lymphomas that develop in ALV infected chickens have proviral-*myc* junction fragments, detectable by restriction fragment analysis. The dysregulation of c-*myc* by insertional mutagenesis is believed to maintain cells in the mitotic cycle, preventing differentiation of the B cell, and is evidenced by cellular hypertrophy consisting of cells arrested at an early stage of B cell development.

A nondefective strain of REV, the chicken syncytial virus (CSV), has also been shown to induce B cell lymphomas indistinguishable from those induced by ALV by experimental infection of chickens as embryos or at hatch (7,9). Both the pattern and orientation of integration of the CSV provius in the c-*myc* locus is similar to that of ALV proviruses involved in LL (10).

Although the c-*myc* locus is involved in most naturally occurring lymphomas, insertional mutagenesis of the c-*myb* locus has been demonstrated in B cell lymphomas in chickens infected as embryos with either the recombinant ALV, EU-8 (11) or a subclone of RAV-1 (12). The target cell of these viruses appears to be a transient population in the embryo which, when infected, gives rise to liver tumors 4-6 weeks post hatch without gross involvement of the bursa of Fabricius.

METHODS FOR DETECTION OF PROVIRAL-ONCOGENE INSERTIONS

In a study of over 200 ALV induced lymphomas we found the ALV provirus to be integrated in the first intron of the c-*myc* locus near the 5' end of Exon II and in the same orientation as the *myc* gene in about 95% of the tumors. DNA is typically digested with Eco-RI enzyme and analyzed by Southern transfer and hybridization using probes for the exon III of *myc* and the U5 region of ALV. The c-*myc* locus has only one Eco-I site, at the 3' end of exon III. Therefore, integration of the ALV provirus results in the insertion of an Eco-RI site found in the LTR in the c-*myc* locus upstream of exon II. Digestion with the Eco-RI enzyme therefore creates a DNA fragment that is clonally expanded in tumor DNA that contains the U5 region of the provirus, all of Exon II and most of exon III of c-*myc*. These ALV-*myc* junction fragments can be detected by Southern analysis and hybridization with probes for either c-*myc* or the ALV U5 region (fig. 2A). The size of this restriction fragment is dependent of the site of integration within the c-*myc* locus relative to the Eco-RI site in exon III.

We have recently developed a second approach to analyze these ALV-*myc* junction fragments using the polymerase chain reaction (PCR). Primer sets consisting of sequences in the ALV U5 LTR and in exon II of c-*myc* were tested for ability to expand the intervening sequences found in the junction fragments of LL tumors. This combination of primers was selected to mediate amplification only of DNA fragments containing sequences from the LTR of ALV and sequences

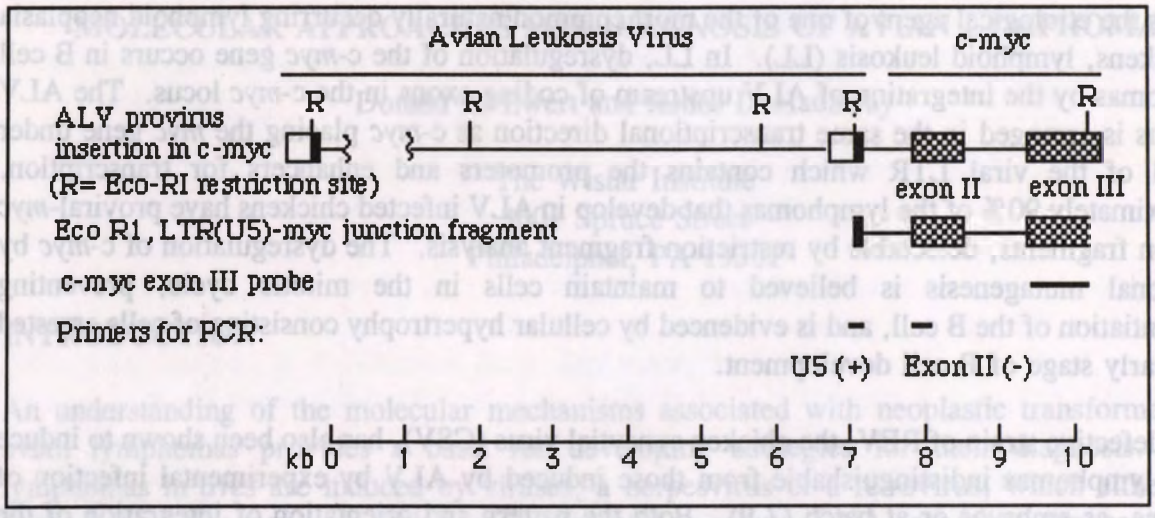


FIGURE 1. ALV insertion in the c-*myc* proto-oncogene

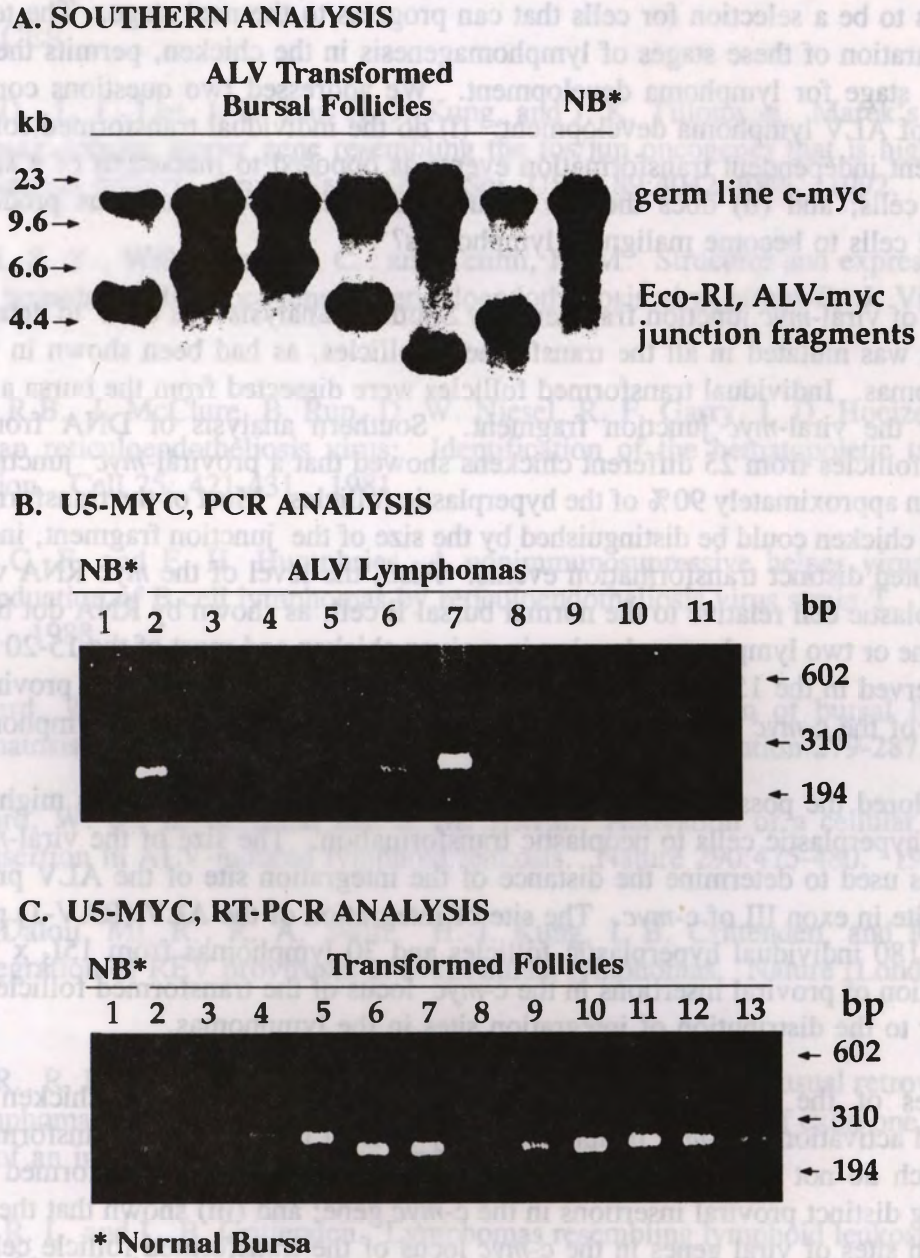
of exon II of c-*myc*. We found that after optimizing the PCR conditions only a few, less than 10%, of the tumor DNA gave detectable PCR bands. We rationalized that structures within the intervening sequences between the U5 region of the provirus and exon II may interfere with synthesis of DNA. To minimize the influence of intervening intron sequences on the PCR reaction lymphomas in which the proviral integrations had been mapped to within o.5 kb of exon II were selected for analysis. Nevertheless, about 50% of these tumors failed to gave detectable PCR product (fig. 2B).

Another approach was tested based on the fact that RNA transcribed by the viral promoter contains both viral and *myc* message without the intron sequences. RNA isolated from tumors and transformed follicles was first reverse transcribed to create a cDNA copy which was then used as a template with the same primers to expand any ALV-*myc* junction fragments in the sample. This method worked consistently for detection of the viral-*myc* junction fragments in LL tumors and transformed follicles in the bursa (fig. 2C). However, because the PCR products are only several hundred base pares in size and do not contain the sequences intervening between the provirus and exon II of c-*myc* there was less variability in size of fragment obtained from different lymphomas. Therefore, this method while useful for detection of insertional mutagenesis of the *myc* locus, it does not yield qualitative information to distinguish one insertional event from another in the same chicken.

APPLICATION OF PROVIRAL-MYC JUNCTION FRAGMENT ANALYSIS TO THE STUDY OF LYMPHOID LEUKOSIS

We have taken advantage of the fact that independent transformation events are marked by distinct sized ALV-*myc* junction fragments to analyze the process of lymphoma development in ALV infected chickens. The natural history of avian lymphoid leukosis is a multistage process having at least three identifiable stages of lymphoma development. Beginning at about 4-8 weeks after neonatal infection, a variable number of enlarged follicles can be detected

Figure 2.


A. SOUTHERN ANALYSIS
ALV Transformed Bursal Follicles
NB*
kb
23
9.6
6.6
4.4
germ line c-myc
Eco-RI, ALV-myc junction fragments
B. U5-MYC, PCR ANALYSIS
NB*
ALV Lymphomas
1 2 3 4 5 6 7 8 9 10 11
bp
602
310
194
C. U5-MYC, RT-PCR ANALYSIS
NB*
Transformed Follicles
1 2 3 4 5 6 7 8 9 10 11 12 13
bp
602
310
194
* Normal Bursa

microscopically in the bursa of Fabricius (13). At about 12-14 weeks post-infection, most of the hyperplastic follicles regress, as do normal bursal follicles, with the onset of sexual maturity, and one or two macroscopic lymphomas become detectable in the bursa. A third stage is the metastasis of cells to peripheral organs, principally the liver and spleen, which occurs after a variable interval of days to weeks following the detection of palpable lymphomas in the bursa. These metastases can be shown to be derived from a bursal lymphoma based on the size of the DNA fragment containing the c-*myc* gene (14). Thus, at each stage of LL tumor development, there appears to be a selection for cells that can progress to the next stage. The temporal and physical separation of these stages of lymphomagenesis in the chicken, permits the analysis of cells at each stage for lymphoma development. We addressed two questions concerning the early stages of ALV lymphoma development: (i) do the individual transformed follicles in the bursa represent independent transformation events as opposed to metastasis of a single or few transformed cells; and (ii) does the site of integration in the c-*myc* locus predispose some preneoplastic cells to become malignant lymphomas?

The analysis of viral-*myc* junction fragments by Southern analysis was used to determine if the c-*myc* locus was mutated in all the transformed follicles, as had been shown in the terminal ALV lymphomas. Individual transformed follicles were dissected from the bursa and the DNA analyzed for the viral-*myc* junction fragment. Southern analysis of DNA from over 200 transformed follicles from 25 different chickens showed that a proviral-*myc* junction fragment was present in approximately 90% of the hyperplastic follicles. Most of the transformed follicles from a given chicken could be distinguished by the size of the junction fragment, indicating that they represented distinct transformation events. Also, the level of the *myc* RNA was elevated in the hyperplastic cell relative to the normal bursal B cell, as shown by RNA dot blot analysis. Since only one or two lymphomas develop in a given chicken and most of the 15-20 transformed follicles observed in the $15I_5 \times 7_1$ chickens regress, our findings suggest that proviral-mediated deregulation of the c-*myc* gene is not sufficient to cause development of a lymphoma.

We also explored the possibility that the site of integration in the *myc* locus might predispose some of the hyperplastic cells to neoplastic transformation. The size of the viral-*myc* junction fragment was used to determine the distance of the integration site of the ALV provirus from the Eco-RI site in exon III of c-*myc*. The site of integration of the ALV (RAV-1) provirus was mapped for 180 individual hyperplastic follicles and 30 lymphomas from $15I_5 \times 7_1$ chickens. The distribution of proviral insertions in the c-*myc* locus of the transformed follicles was found to be similar to the distribution of integration sites in the lymphomas.

These studies of the early stages of lymphoma development in the chicken have: (i) demonstrated activation of c-*myc* in approximately 95% of pre-neoplastic transformed follicles, most of which do not give rise to lymphomas; (ii) shown that each transformed follicle is a clone, having distinct proviral insertions in the c-*myc* gene; and (iii) shown that the distribution of integration sites of viral genes in the c-*myc* locus of the transformed follicle cells is similar to that of lymphomas. We conclude that transcriptional activation of c-*myc* is necessary for the induction of B cell hyperplasia, but it is not sufficient to achieve neoplastic transformation of the bursal B cells.

SUMMARY

The presence of viral-oncogene junction fragments in tumors is a powerful tool for the detection of transformation events and for the analysis of the multistage process of lymhomagensis. Sufficient information is available to develop appropriate combinations of restriction enzymes and nucleic acid probe for Southern analysis or sets of primers for PCR analysis for the differential diagnosis of most known avian lymphomas.

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PCR AS A TOOL FOR THE DIAGNOSIS OF AVIAN TUMOR VIRUSES AND TUMORS

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INTRODUCTION

Criteria such as lesion distribution and age are the usual means to differentially diagnose avian lymphomas in the field. Enlarged peripheral nerves, and/or visceral tumors typically characterize Marek's disease virus (MDV)-induced tumors in young chickens. Lymphoid leukosis caused by avian leukosis viruses (ALV), generally occurs in birds older than 16 weeks and in most cases involves gross bursal lesions. Reticuloendotheliosis virus (REV) induces lymphomas in chickens and can also be accompanied by nerve lesions and bursal lymphomas. The fact that none of these three viral agents induce pathognomonic gross lesions, can make the differential field diagnosis of avian lymphomas somewhat problematical (2,13).

ELISA, immunoperoxidase- and immunofluorescent-based assays are relatively simple biochemical assays that have been used to differentiate between ALV, REV and MDV. However, these procedures usually require the viruses to be isolated and subsequently replicated in tissue culture cells (3,5,9,12).

The polymerase chain reaction (PCR) is a relatively simple technique that can amplify rare DNA sequences by a factor of 10^6 or more. Because of its sensitivity and specificity, PCR is becoming a standard procedure used in human clinical diagnosis. PCR has also been used in an attempt to diagnose avian tumors. This presentation describes the current status of using PCR to detect REV, ALV, and MDV in avian tumors.

PCR of MDV

In addition to specifically differentiating MDV sequences from ALV or REV sequences, an MDV-based PCR assay must differentiate between the pathogenic MDV strains and all the closely related, but non-pathogenic vaccine MDVs. Our approach has been to choose PCR primers that anneal specifically to DNA unique to serotype 1 MDV DNA. In addition, the primers were chosen to anneal to regions that flank the 132 base pair tandem direct repeats. We chose to PCR expand DNA across the repeats because it has been shown that MDV attenuation is strongly linked to expansion of DNA sequences within this region (10). The two PCR primers are shown below.

Forward Primer 5' TGCGATGAAAGTGCTATGGAGG 3'
Reverse Primer 5' GAGAATCCCTATGAGAAAGCGC 3'

PCR amplification with these primers specifically amplified DNA only from serotype 1 MDV. In addition, oncogenic MDVs with one, two, or three copies of the repeats generated PCR amplified fragments of 185, 317, or 449 base pairs. PCR amplification of attenuated serotype 1 MDVs generated a population of high molecular weight DNAs visible as a smear on an ethidium bromide stained agarose gel and is easily differentiated from oncogenic MDVs (8).

PCR OF ALV

Avian leukosis viruses are relatively small enveloped retroviruses containing an RNA genome. During infection, the RNA is reverse transcribed into DNA and subsequently integrated into the host chromosomal DNA. The integrated proviral DNA is flanked by viral regulatory sequences designated long terminal repeats (LTR). Each LTR contains three regions: an unique 3' end, designated U3; R, a highly conserved 21 base pair region; and unique 5' end, designated U5.

To detect exogenous ALV infections, the forward PCR primer (#26) was designed to anneal to a CArG box (5'-CC(A/T)$_6$GG-3' motif found upstream from some cellular genes and required for tissue-specific expression) in the U3 region of the Rous sarcoma virus (RSV) LTR (16). This region is missing in endogenous ALVs. The reverse primer (#30) was designed to bind to conserved sequences in the RSV U5 region (4). Using these primers, we PCR amplified a 220 base pair fragment from 10 different exogenous ALV containing samples. However, no PCR amplified product was seen in DNA from RAV-0 and ALV-J infected cells.

Exogenous ALV	Forward Primer #26	5' AAGTAAGGTTACGATCGTGCCTTA 3'
	Reverse Primer #30	5' CTGCTTCATTCAGGTGTTCGCAAT 3'
Endogenous ALV	Forward Primer #7	5' ATAGAGCCAGAGGCACCTGAATAG 3'
	Reverse Primer #8	5' CATAGCTTCGTCTACGCCCATA 3'

For the detection of endogenous viruses, both primers (#7 and #8) were derived from the U3 region of RAV-0. The primers amplified a 125 base pair product from endogenously infected CEFs. No product was amplified from exogenously infected Line 0 CEFs.

PCR OF REV

REVs comprise a group of avian retroviruses that includes defective transforming viruses (REV-T), replication competent viruses (REV-A), spleen necrosis viruses (SNV), and chick syncytial viruses (CSV). In susceptible chickens, different strains of REV can induce either ALV-like bursal lymphomas or non-bursal lymphomas that resemble Marek's disease (14,15).

Primers were derived from the LTR of SNV and amplified a 291 base pair product from SNV, CSV, REV-T, or duck infectious anemia virus infected cells (1). Initial REV reconstruction

assays using serial dilutions of plasmid bearing SNV indicated PCR detected about 600 copies of SNV in the presence of 250 ng of DNA from uninfected cells. This limit of detection is equivalent to one proviral copy of REV in about 170 noninfected cells.

Forward Primer 5' CATACTGGAGCCAATGGTT 3'
Reverse Primer 5' AATGTTGTACCGAAGTACT 3'

CONCLUSIONS

The sensitivity and specificity of PCR suggests it can be a valuable aide in the differential diagnosis of lymphomas induced by MDV, ALV, and REV. However, cautious interpretation of any results is still necessary. The PCR assay is not always 100% accurate (6,11). PCR is a highly sensitive viral assay, capable of detecting viral genomes in animals that do not contain tumors (8). Finally, in the case of ALV tumors, so far it has not been possible to design a single set of primers that will unambiguously detect all exogenous ALVs.

Despite the problems, PCR can still be useful in the differential diagnosis of avian lymphomas. Since it is possible to have viruses in tumor tissue samples that were not responsible for the tumor, a negative PCR result can be especially useful in differential diagnosis. Recently PCR was used to determine that the outbreak of lymphomas in two broiler breeder flocks was due to an REV contamination of a commercial fowlpox vaccine (7).

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MAREK'S DISEASE: CLINICAL ASPECTS AND CURRENT FIELD PROBLEMS IN LAYER CHICKENS

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INTRODUCTION

Experiences the layer industry has had with Marek's disease (MD) the last few years serve to remind us it is still a major potential threat. The MD virus will take advantage of opportunities we give it to spread between flocks, to become more virulent, and adapt to evade the vaccines we have been using. The legacy workshop at the 5th International Symposium on Marek's Disease spoke of the disastrous losses to MD in the early days before licensed vaccines were developed. But, just three to four years ago, we were still seeing sporadic cases of commercial layer flocks losing 20-50% to MD - flocks that were vaccinated with bivalent or trivalent vaccines. Although we have gained a lot in our knowledge and control methods, we may be just barely staying ahead of the problem.

In this presentation, I would like to briefly review some history of recent MD problems in layers, the lessons we have learned, our current state of control, and our vision of the future.

RECENT MAREK'S DISEASE PROBLEMS IN LAYER CHICKENS

The years of 1993-94 were probably the peak for MD in layers in the last 10-15 years. What led up to this problem? I believe it was a combination of long-term HVT/SB-1 vaccine use and changes in common pullet growing management. Consider that HVT, as a monovalent vaccine, had a lifespan of about ten years - from the early 70's to the early 80's. It was then that very virulent (vvMD) viruses emerged that were not well protected against by HVT alone (1). The serotype 2's were introduced at that point and protected well as bivalent vaccines for about the next 10 years. Viruses we now term vv+ MD were emerging in the early 90's that were not well protected against by HVT and SB-1. This pattern suggests that 10 years is about the natural lifespan of a MD vaccine, or the time it takes for genetic resistance of the field MD virus to emerge and become noticeably prevalent.

The other factor that has been changing in the last 10 years is the trend toward consolidation in the layer industry. The small independent producers are disappearing and the large ones are getting bigger. In the desire for more efficiency, multiple age layer farms are now the norm and, unfortunately, multiple age pullet growing farms are becoming more common. Some farms have as many as 8 or 10 houses of different age growing flocks side-by-side at one location.

In some cases, the growing houses are located very close to the layer houses. These dense housing arrangements provide plenty of opportunity for airborne transmission between older flocks and newly housed chicks.

The severe MD losses we experienced several years ago mostly seemed to originate from these large multi-age growing farms. Losses were quite heavy, with mortality in the range of 20-50% in some of the worst cases. Typically, the mortality would begin the last few weeks in the growing house and then continue in the layer house for a total of 10-15 weeks. Coincidentally, many times the MD mortality would begin just after the flock had recovered from a period of laryngotracheitis (LT) mortality. Generally, no association could be found with other immunosuppressive agents, such as CAV, REO, REV, or unusually severe bursal damage from IBD. Most of these flocks had normal vaccination programs for IBD and had received the standard subcutaneous MD vaccination at hatch with bivalent HVT and SB-1. A few even received trivalent combinations including CR6 or Md 11/75.

On these large multi-age growing farms, houses were cleaned and disinfected as well as commercially possible before chicks were housed, but with continuing bad results. Apparently, the exposure was primarily airborne - going out the exhaust fans of a house of older birds and into the air intake of a neighboring house with new chicks. Of course, there is also potential for personnel to track contamination between nearby houses, although efforts were made to prevent this from occurring.

The viruses that evolved on some of these farms have now been characterized and are felt to represent a new pathotype, termed very virulent plus (vv+) (2). They have demonstrated some new characteristics which probably partly explain our difficulty in controlling them. They apparently are more invasive and rapidly cause immunosuppression and transformation of affected tissues. They demonstrate a high degree of resistance to HVT/SB-1 vaccine. They have also demonstrated the ability to be transmitted from adult to adult and initiate an outbreak at advanced ages - even after molt.

SOLUTIONS USED TO ALLEVIATE RECENT MAREK'S DISEASE PROBLEMS IN LAYERS

Many things were attempted to alleviate these problems, but in the end, there were two major keys. One was that the original-type Rispens vaccine (CVI-988) became available just in time. Two products were licensed in the fall of 1994 and were immediately adopted by these companies having the most serious problems. In vaccine trials at Hy-Line International, the Rispens vaccine has consistently proved to be the most effective product against these vv+ MD viruses, whether used in a monovalent, bivalent, or trivalent combination. Any products or combinations with Rispens have shown better protection than any product or combination without Rispens. Perhaps that is simply due to its being a new vaccine type in the U.S.

However, we have also learned that vaccination alone cannot provide the entire answer. A few flocks that received Rispens have still experienced excessive MD losses when there was early

exposure from neighboring flocks of older pullets. So, the other key has been changes in management to avoid this early house-to-house exposure. Several of these companies wisely decided they had to do something to break the ongoing cycle of transmission on these farms. The only way was to change the placement schedule so that the farm could be operated in an all-in/all-out manner. This provides a period of time with no birds on the farm when all houses can be cleaned and disinfected at once. Although we undoubtedly do not eliminate all the MD virus, we are eliminating the more significant house to house transmission between age groups. The combination of Rispens use and the pullet farm placement changes have seemed to eliminate the serious MD problems for now.

My company's current philosophy is to use only the vaccine necessary to control the current MD on a farm. That means that most average customers that have not had a serious MD problem still receive the standard HVT and SB-1 vaccine. We like to reserve Rispens use for only those farms that have had problems, for a couple of reasons. One is biological; since the field viruses seem to be able to adapt over time to our vaccine immunity, we do not want to give them the chance to start adapting to Rispens until it is really necessary. We would rather save our best defense until it is needed. The other is simple economics. Rispens vaccine costs about an extra cent per chick. If we assume the maximum value of pullets at the beginning of lay is about $2.50 each, then it would require saving at least 0.4% of them from MD at that age to break even with the additional vaccine cost. At earlier or later ages when their value is less, it would require proportionately higher MD losses to justify Rispens use. Despite these reasons, some customers may request Rispens vaccine anyway. Currently about 2/3 of the commercial chicks we produce receive only HVT and SB-1 and about 1/3 have Rispens vaccine added.

FUTURE STRATEGIES TO CONTROL MAREK'S DISEASE IN LAYERS

Despite our prudent use of Rispens vaccine and lessons we have learned in management, I am concerned how long this current state of calm will last. We have already seen that Rispens is not a cure-all when heavy, early challenge is present. The current experience in Europe is somewhat parallel to ours in that they now have field viruses emerging that are apparently becoming resistant to Rispens vaccine. If vaccines seem to have effective lifespans of about 10 years, how long can we count on Rispens? How long will it take for these pullet farm managers to get complacent and revert to their old multi-age placement schedules? In one case, it has already happened. I am concerned that we could quickly find ourselves back in a serious MD situation; this time with no new vaccine ready to save us.

Hy-Line believes that one answer needs to be improved genetic resistance to MD in the layer varieties. This work, using blood typing, was pioneered in the 60's before vaccines were available and was proving to be quite successful. However, too much reliance was probably placed on vaccines solving the problem in the 70's and 80's. Now it should be clear that vaccines are not the only answer, and having a good genetic base of resistance should be very desirable. In this regard, Hy-Line operates a 10,000 bird capacity growing house strictly for MD challenge and genetic evaluation. We find there are significant genetic differences that can be used in the breeding program.

It is my hope that researchers worldwide will continue to strive for new improved vaccines, administration methods, or other intervention strategies to use in our war against MD. We have learned it is a continually changing disease and what works today probably will not work five or ten years from now. Everyone shares part of the responsibility; producers to use management that reduces the challenge and controls other immunosuppressive diseases; breeders to improve genetic resistance of their varieties; vaccine companies to develop new products that will protect against the newly emerging field strains; and hatcheries to administer the vaccine properly. If we all do our parts, hopefully we can stay ahead of the Marek's problem.

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MAREK'S DISEASE: CLINICAL ASPECTS AND CURRENT FIELD PROBLEMS IN BROILERS

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INTRODUCTION

Marek's disease (MD) as a potential economically disastrous disease continues to exist. Even after the development of new strains of vaccine to protect against virulent viruses, more are sure to develop. It seems that every ten or so years, a new strain of virulent virus is isolated. These new isolates are most likely to occur in high density poultry growing areas. New vaccines are needed to control these new strains. With developmental time by researchers and vaccine companies along with the slowness of USDA in clearing new vaccines, how long can the poultry industry continue to depend on vaccines to solve the Marek's problem?

FACTORS THAT INFLUENCE THE INCIDENCE OF MAREK'S DISEASE CONDEMNATIONS

Control of MD by vaccination is a complex interaction between a competent immune system and various factors. A proper breeder vaccination program for infectious bursal disease and prevention of early exposure to chick anemia virus in the breeder pullets are necessary to ensure an intact and competent immune system in the young broiler chick.

The following factors are known to influence the incidence of MD condemnations in broiler chickens:

a. genetics of host
b. field virus challenge
c. layout time between flocks
d. vaccination techniques
e. brand and type of vaccine
f. dosage of vaccine
g. age of birds at slaughter

Genetics of host

Recently, most genetic selection by the primary breeders has been on increased breast yield and faster growth rate. The customers have requested these and the breeders have obliged. What has this done to Marek's susceptibility? How far can these birds be pushed without damaging

the immune system or increasing Marek's disease susceptibility beyond the economically acceptable limits? Genetics will be more and more important in the control of Marek's disease. We cannot rely on vaccines to solve the MD problem forever.

Field virus challenge

With continuing pressure from live production, new more virulent MD field viruses will develop. This area of the poultry industry must assume some responsibility for increased Marek's condemnations. Some areas to consider are:

a. more frequent cleanouts
b. longer layouts between flocks
c. increased floor space per bird
d. improved chick management
e. reduced stress

The above will not be considered seriously until the cost of MD condemnations is greater than the changes suggested.

Layout time between flocks

The less time poultry houses are empty between flocks, the more severe the challenge is from MD. However, economics determines the amount of down time between flocks. An empty house does not make money. Demand for chicken, grower pressure, housing availability, and expansion of the industry determine layout time.

Vaccination techniques

Most companies use the Inovoject to vaccinate for MD. Injecting 18-19 day embryos has proven to be an effective vaccination technique. This allows more time for building immunity prior to exposure to the field virus. Proper quality assurance programs must be established to keep MD condemnations low.

Brand and type of vaccines

Certain brands of vaccines seem to work for some companies and not for others. Sufficient field testing is required to determine the proper vaccines to use in your operations. Certain areas of the country need to use multiple types of vaccine to control MD. The ultimate is to use HVT, SB1, and Rispens. Another consideration in determining which vaccines to use are monovalent, bivalent, or trivalent vaccines. Differences do exist between these types of vaccines.

Dosage of vaccine

Many companies will use a higher PFU dosage of vaccine in the winter months and a lower dosage in the summer months. Does this strategy allow for the development of virulent field strains?

Age of birds at slaughter

Field observations suggest that MD condemnations may be influenced by age of birds at slaughter, however, the reasons for such influence are not clearly understood.

EPIZOOTIOLOGY AND ADULT TRANSMISSION OF MAREK'S DISEASE

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Beginning in the early 1990s the incidence and severity of Marek's disease (MD) increased in broilers, broiler breeders and commercial layers located in different parts of the United States. Marek's disease virus isolates were obtained from buffy coat samples collected from several different clinically-affected flocks that were produced in the Midwest, Northeast and mid-Atlantic areas (Table 1). The flocks ranged in age from 4 to 64 weeks and all had been vaccinated with HVT and SB-1 via the subcutaneous route at 1 day. Both meat-type and commercial layers were included in the sampling.

TABLE 1. Source of Marek's Disease Virus (MDV) isolates.

MDV Identification	Source of Isolates	Date Isolate Collected	Age[A]	Location of Chickens
T.K.	Broilers	8/92	4 wk	DE/MD
L	Broiler Breeders	1/95	32 wk	DE/MD
N	Commercial Egg Layers	1/95	37 wk	NH/ME
U	Commercial Egg Layers	5/94	64 wk	PA
X	Broilers	5/95	8 wk	NC

[A] Age of birds from which samples were collected for MDV isolations.

The viruses were isolated by inoculation of buffy coat suspensions into susceptible 1-day-old SPF chickens which were then held in isolation for 2 weeks. At 2 weeks of age a second group of 1-day-old SPF chickens were placed in the isolators to serve as contacts. Buffy coats were collected at 2 to 4 weeks post-exposure from the contacts and used to prepare MDV stock for subsequent evaluations. They were assayed for adventitious agents and then titrated in SPF chickens. Using approximately equivalent doses of each virus a comparative pathogenesis and age susceptibility trial were completed for 5 selected MDV isolates and the very virulent MDV prototype RB1B. Pathogenesis was evaluated by assessing the onset of viremia and the development of typical MD gross and histologic lesions in susceptible chickens.

The comparative pathogenesis study of selected MD field isolates revealed that these isolates can be classified as very virulent MDV strains. The development of clinical signs following inoculation with these isolates was typically observed by 10 days post-inoculation (PI). In general, the most common clinical signs included depression, emaciation, anorexia, uneven growth, ruffled feathers, transient paralysis and early death in the absence of tumors or nerve enlargement by 12 days PI. In contrast, birds inoculated with RB1B challenge strain were not clinically affected until 4 weeks PI.

The development of gross and microscopic lesions in the spleen associated with MD were identified as early as 5 days PI. Subsequently, gross lesions were observed in visceral organs, eye, lymphoid system and skin. The most severely affected organs were the spleen, liver, thymus and bursa. Pathologic changes were characterized by focal to multifocal tumor formation, enlargement of the organs, necrosis of the spleen and atrophy of the lymphoid tissues.

Based on microscopic evaluation of the collected tissues, lesions were characterized by infiltration of pleomorphic populations of small to medium size lymphocytes, lymphoblasts and accumulation of mononuclear phagocytic cells. Microscopic lesions in the spleen were characterized by massive lymphoproliferation with mitotic figures within the periarteriolar sheath which gave rise to lymphoma formation. In severe cases visceral and spleen tumors appeared to undergo central necrosis. The proventriculi were also severely affected and characterized by thickening of the mucosal and muscularis layers. In severe cases, the proventricular glands were infiltrated by polymorphic lymphocytes, heterophils and macrophages, resulting in reduction in digestive gland size and columnar metaplasia. The thickening and destruction of the proventricular tissue may lead to malnutrition and/or improper digestion of food. As a result, uneven growth, cachexia and starvation occurred. The lymphoid tissues including the thymus and the bursa of Fabricius from birds inoculated with these MD field isolates showed marked atrophy and tissue destruction as early as 5 days post-inoculation. This may play a role in the failure of the immune system to mount an adequate vaccine response leading to increased MDV infection and condemnations. These results also suggest an early onset of immunosuppression which may lead to an increase in opportunistic infections. Furthermore, the onset of viremia was observed as early as 12 hours PI with four of the characterized MD field isolates. In contrast, the onset of RB1B viremia required more than 12 hours PI. This may account for the more rapid development of lesions. Based on gross and microscopic lesions and the onset of viremia the selected MD field isolates have the ability to rapidly replicate within the lymphoid tissues leading to clinical MD infection in vaccinated birds shortly after exposure.

Age susceptibility was studied by evaluating groups of seventy 19-, 34- and 49-week-old, female, SPF chickens following inoculation or contact exposure with the MD field isolates compared to RB1B. RB1B and each of the MDV field isolates caused morbidity in the inoculated birds and were transmitted to contact birds regardless of their age at inoculation or contact exposure. However differences in the onset of morbidity between the MDV field isolates and RB1B were apparent.

Following inoculation or contact exposure of the 19-week-old SPF chickens, several chickens inoculated with isolates T.K. and L developed central nervous system (CNS) involvement within 2 weeks. By 5 to 6 weeks post-exposure, birds inoculated with each MDV field isolate were affected while RB1B inoculates and contacts were unaffected until 7 weeks post-inoculation. Central nervous system signs were also observed early (2 to 3 weeks post-exposure) in the 34-week-old birds inoculated with MDV field isolates T.K. and U. Clinical signs were evident by week 5 in all treatment groups but the number of chickens affected was greater in the chickens exposed to MDV field isolates N, U and X than in RB1B at 6 weeks post-exposure. No early CNS signs were observed in the 49-week-old birds but morbidity was noted in groups inoculated with 4 of the 5 MDV field isolates by week 5 post-exposure.

Egg production dropped quickly in the chickens inoculated with the MD field isolates and RB1B between the fourth and fifth week post-infection. These determinations were based on number of eggs per surviving hen per week in each house as compared to control hens.

It is apparent that the recently-isolated MD viruses can infect and induce tumors in birds 19 weeks of age or older. This finding is important in developing MDV control strategies in layers and broiler breeders and in differentiating MDV isolates. Further experiments will be performed using these and other recently-isolated MDVs to challenge vaccinated birds raised in a production environment. Results could provide a better understanding of how these viruses evolve and how well the commonly-used vaccination programs are performing in a commercial setting.

From an epizootiological perspective, it appears that many of the MD isolates found to date in broilers and breeders share many common features with each other and with isolates obtained from commercial layers. This suggests common origins which perhaps should be expected in view of the close proximity of various poultry and egg production activities in many parts of the United States.

VACCINAL IMMUNITY TO MAREK'S DISEASE

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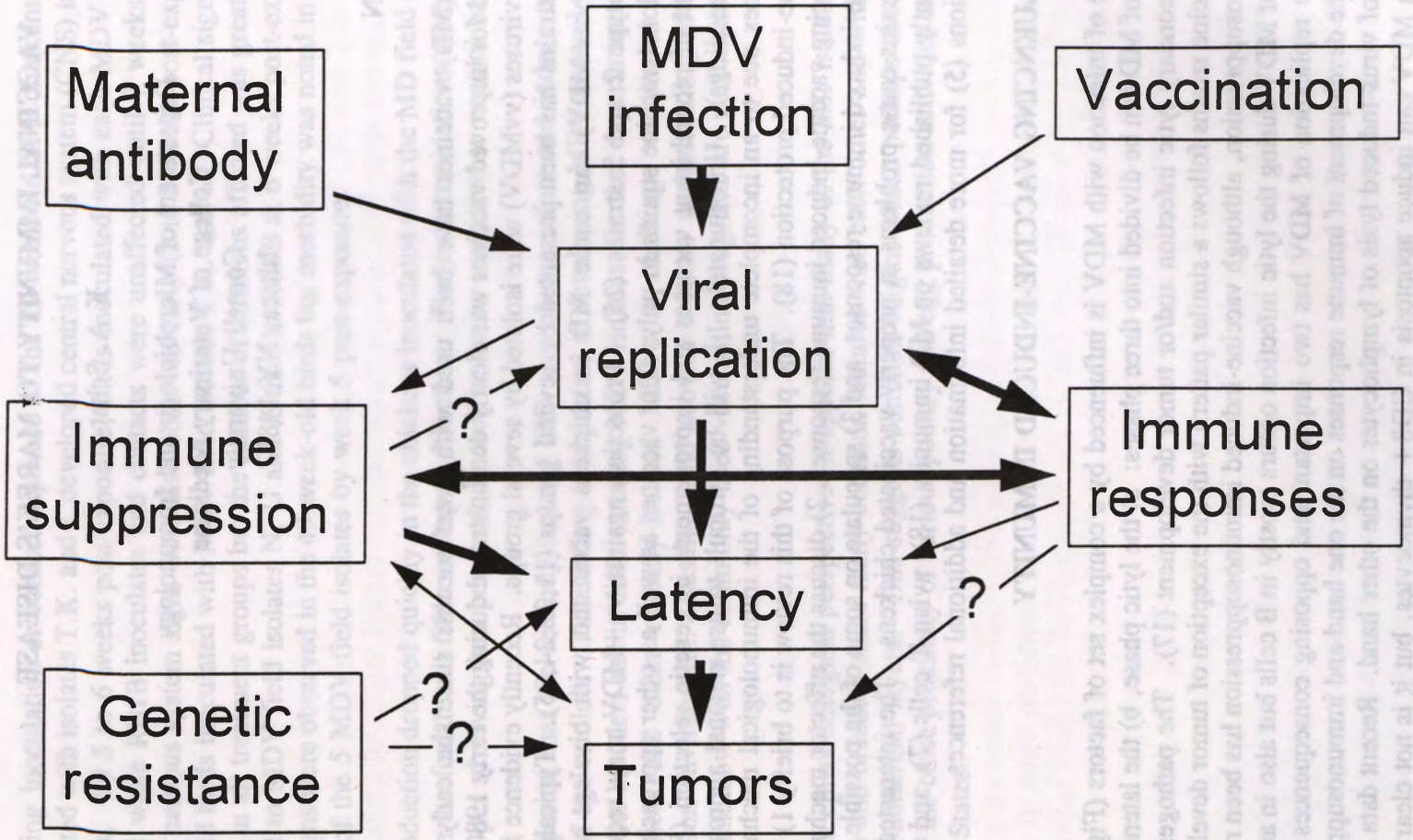
INTRODUCTION

Marek's disease (MD) vaccines have been used with great success since the early 1970s. However, the need for improved vaccines was clearly demonstrated during the early 1980s when very virulent MD viruses (vvMDV) were isolated by several groups. Recently evidence for even more pathogenic strains has been presented by several groups (11,22,24,25). Typically these new pathotypes (vv+MDV) can cause MD in chickens vaccinated with bivalent vaccines consisting of serotype 2 and 3 strains (25). Protection against vv+MDV may require the inclusion of additional vaccine strains in polyvalent vaccines as well as other approaches such as the development of recombinant vaccines and improvements in genetic selection (See also Witter, these proceedings). Unfortunately, it will be difficult to use rational approaches to design new vaccines due to an incomplete understanding of the immunological mechanism(s) involved in vaccine-induced protection (18). The purpose of this review is to briefly 1) analyze the factors influencing vaccine-induced immune responses, 2) discuss the effector mechanism(s) involved in vaccine-induced immune responses, and 3) speculate on some of the possible reasons why vv+MDV strains cause problems in properly vaccinated chickens. The interested reader is referred to recently published reviews on MD immunity (18), avian T cells (7), and avian T cell effector functions (5) for more detailed information and additional references.

FACTORS INFLUENCING VACCINE-INDUCED IMMUNITY

The final outcome of infection with MDV is influenced by a complex set of factors (Figure 1). The pathogenesis of MD can be divided into three phases: a) the lytic phase, b) the latent phase, followed by c) secondary lytic infection and/or tumor development (17). The pathogenesis of infection with vaccine strains follows a similar pattern with the exception of tumor development and severe immunosuppression, although vaccine-induced immunosuppression has been reported (6). Replication of MDV during the lytic infection occurs mostly in B cells but also in activated T cells (17). The replication of MDV has two important and opposing consequences for the immune system: the development of immune responses on the one hand and immunosuppression as a consequence of virus-induced lysis of lymphocytes on the other hand. Recent data suggest that infection with MDV can induce apoptosis in CD8+ thymocytes, but it is not clear if cells have to become virus-infected in order to undergo apoptosis (12). The extent of the lytic infection is influenced by at least three host-dependent factors (age at infection, the presence of

Figure 1. Interactions between Marek's disease virus and immune responses.



maternal antibodies and vaccination) as well as the virulence of the challenge virus. Apparently, the recently isolated vv+MDV strains are more cytolytic than earlier isolates (Calnek, unpublished data, 11, 22). Maternal antibodies and vaccinal immunity reduce the level of virus replication, but do not prevent the establishment of infection with the challenge virus.

It is not known which viral proteins are important for the development of virus-neutralizing maternal antibodies. Glycoprotein B (gB) is likely to be one of the proteins based on its ability to induce VN antibodies (13). Vaccination reduces the level of lytic infection after challenge, but the level of reduction may depend on the challenge strain. Schat et al. (19) reported that HVT- or SB-1-vaccinated, genetically susceptible P2a ($B^{19}B^{19}$) chickens had higher levels of virus-positive lymphocytes at 19 days post challenge with the vvMDV strain RB-1B than with the JM strain. These data suggest that vvMDV strains replicate more effectively than less virulent MDV strains in vaccinated chickens.

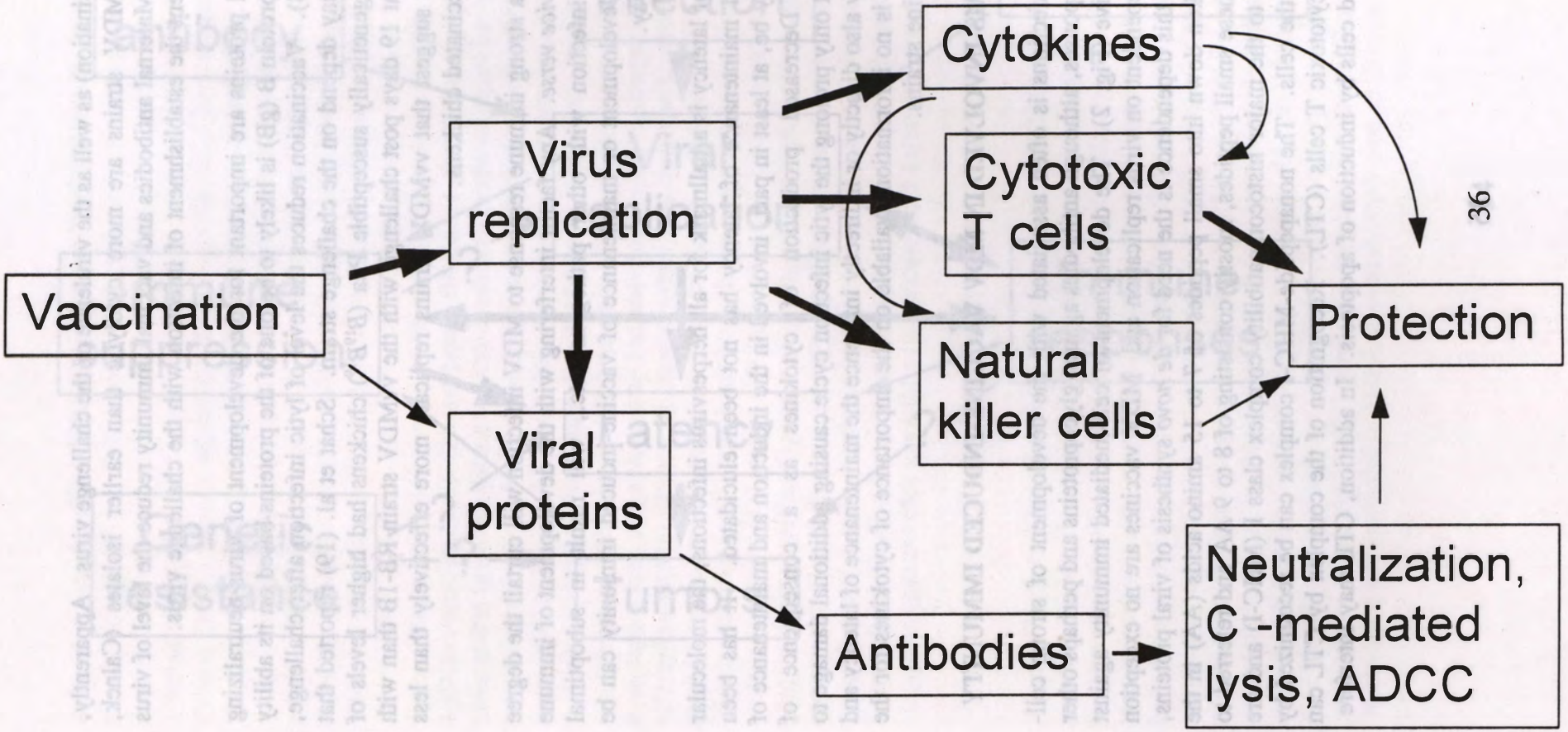
The timely development of a strong immune response to MDV infection will curtail the degree of immunosuppression and *vice verse*. Any factor interfering with the development of immune responses such as stress, infection with other pathogens, etc., will result in suboptimal immunity. Likewise, the development or maintenance of vaccine-induced immunity can be compromised in a similar way.

Although the development of latency is a hallmark for all herpesvirus infections, the molecular basis for the induction and maintenance of latency has not been elucidated. It has been suggested that cytokines may be, at least in part, involved in the induction and maintenance of MDV latency (2,23). Decreased production of cytokines as a consequence of immunosuppression may not only prolong the lytic infection cycle causing additional damage to the immune system, but may also directly or indirectly influence the maintenance of latency and tumor development. There is no information available on the importance of cytokines for the induction of latency of vaccine strains.

EFFECTOR MECHANISMS INVOLVED IN MDV VACCINE-INDUCED IMMUNITY

Immunity to herpesvirus infections is often associated with the development of strong cell-mediated immune (CMI) responses, although antibodies against glycoproteins and perhaps other proteins can also be protective (Fig. 2). The development of cell-mediated immunity against viral infections is largely dependent on virus replication and MDV vaccines are no exception to this rule. The reason for this dependence is the need for *de novo* synthesis of viral proteins, which are subsequently broken down into small peptides of 7 to 15 amino acids (AA) in the cytosol of infected cells. These small peptides, mostly consisting of 8 to 9 AA and referred to as nonapeptides, are bound to the major histocompatibility complex class I (MHC-I) and are presented on the surface of the cells. The nonapeptide-MHC-I complex can be recognized by T cell receptors (TCR) on cytotoxic T cells (CTL). Recognition of the complex by CTL can lead to killing of the infected cells by induction of apoptosis. In addition, CTL may interfere

Figure 2. Vaccine induced immune responses.



with virus replication after recognition of the peptide-MHC-I complex by releasing specific cytokines such as interferon-γ.

Although it is assumed that MDV vaccine immunity has a strong CMI component formal proof using passive transfer of MDV-specific CTL in the absence of virus has not yet been provided. Likewise, the contribution of individual vaccine proteins to the generation of MDV-specific CTL has not been elucidated. An *in vitro* assay system has been developed in my laboratory, which will allow the systemic examination of the contribution of individual viral proteins to cell-mediated immunity (14,15). Thus far we have identified a few proteins that can be recognized by CTL, but additional studies are clearly needed. The MDV immediate early (IE) protein ICP4 is one of the proteins that is recognized, but only by CTL from the genetically resistant N2a line (MHC: $B^{21}B^{21}$) but not by CTL from the susceptible P2a line (MHC: $B^{19}B^{19}$). This finding, if extended to other genetically resistant strains, may have important implications, because ICP4 is the key protein initiating MDV replication. Thus far, there are no published data available on the composition of MDV nonapeptides binding to the MHC which can be recognized by CTL.

Natural killer cells (NK) cells can also play an important role in protection against viral infections. MDV vaccination increases the level of NK cell activation as early as 3 to 4 days post infection (8). However, the importance of this observation for vaccine-induced protection is not clear. NK cells need two signals in order to lyse target cells. The first signal is provided by the target antigen, but these have not yet been identified for NK cell recognition in general. The second signal discriminates the target cell as self or foreign based on the presence of the MHC-I-selfpeptide complex. If a target cell is recognized as foreign the NK cell will be able to lyse this cell, but if the cell is recognized as self there will be no killing. Low levels of MHC-I expression may prevent the recognition of the target cell as self and lead to some degree of NK cell lysis. This may be relevant to genetic resistance in MDV, because at least some of the resistant chicken lines have lower levels of MHC-I expression than susceptible lines. Kaufman (9) speculated that these chicken lines are more resistant because they are more efficient in lysing MDV-infected target cells by NK cells. On the other hand, Chausse et al. (3) reported that MDV infection increased the level of MHC-I expression, which could protect against NK cell-mediated lysis.

Vaccination of chickens with MDV vaccines results in the production of antibodies against MDV-specific proteins. However, there is a paucity of information concerning the relative contribution of antibodies against individual proteins for the protection against challenge (reviewed in 18). In addition, it is unknown how these antibodies protect especially once the challenge virus has established a cell-associated infection. Presumably, antibody-dependent cell-mediated cytotoxicity and antibody complement-mediated lysis play a role.

POSSIBLE CAUSES OF VACCINATION BREAKS

Vaccination breaks can be caused by many "extrinsic" factors such as excessive stress, poor

vaccination techniques, infection with other pathogens, etc. However, the emergence of vvMDV strains and, more recently, vv+MDV strains suggests that "intrinsic" factors may play a role as well. These putative factors can be divided into two categories: 1) changes in genes responsible for increased virus replication or transformation, and 2) ability to evade or interfere with immune responses. The latter explanation for increased pathogenicity is relevant for the discussion on vaccine immunity. Especially members of the poxvirus and herpesvirus families have developed strategies to interfere with the immune responses (20). Certain strains of vaccinia virus express functional receptors for cytokines such as interleukin-1, interferon-γ, interferon-α/β, and tumor necrosis factor. Herpesviruses can interfere directly with antigen processing for MHC-I presentation and MHC-I expression. Thus far it is unknown if vv or vv+MDV strains are more pathogenic because they are able to interfere with immune responses. A recent report suggests that pp38 can suppress antibody responses to certain antigens (4). Similarly, we have noted that expression of pp38 in reticuloendotheliosis virus (REV)-transformed cell lines often resulted in a decrease in killing of the target cells using REV-sensitized CTL. The decreased killing was apparently not caused by changes in MHC-I expression, because the level of MHC-I expression was not influenced by pp38 expression (16,21). It will be important to determine if vvMDV or vv+MDV strains can indeed interfere with immune responses.

PRACTICAL CONSIDERATIONS

Existing vaccines are performing extremely well from an immunological point of view. In general very few, if any, vaccines are able to protect animals against such an intensive challenge especially when vaccinated at an immunological immature age as is the case with MDV vaccinations. It may be possible to increase the level of protection by using specific types of vaccines based on the MHC-I antigens of the chickens. The specific binding of antigenic nonapeptides to the MHC-I molecules is probably the basis for the observation by Bacon and Witter (1) that certain haplotypes perform better with specific serotypes of vaccines. An understanding of the precise interactions between nonapetides of antigenic MDV proteins and different MHC-I antigens will be essential to fully exploit this possibility. It may also be possible to improve immune responses by using adjuvants to stimulate immune responses (10), but practical applications have been disappointing. Prevention or removal of extrinsic factors interfering with the development of immune responses will remain the most critical factor for protection against challenge with MDV.

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CURRENT AND FUTURE STRATEGIES FOR CONTROL OF MAREK'S DISEASE

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INTRODUCTION

Marek's disease (MD) is a common and important neoplastic disease of chickens. The causative herpesvirus (MDV) belongs to serotype 1, and is related antigenically to nononcogenic herpesviruses of chickens (serotype 2) and turkeys (serotype 3); the latter is also known as turkey herpesvirus (HVT). Within serotype 1 MDV four pathotypes are recognized; mild (m), virulent (v), very virulent (vv), and, recently, very virulent + (vv+). All pathotypes of serotype 1 cause disease, but serotype 1 isolates can be attenuated by serial passage in cell cultures. Vaccination of chickens *in ovo* or at hatch with attenuated serotype 1, serotype 2, or serotype 3 viruses induces protection against subsequent challenge with virulent serotype 1 MDVs (1).

Following the introduction of serotype 3 vaccine about 1971, losses from MD in broiler and layer chickens were dramatically reduced. Based on this early success, the poultry industry has relied on vaccination as the principal means of control. However, control can also be achieved through selection for specific class I MHC and other genes associated with resistance to tumor induction. Because MDV is not transmitted vertically, control may also be achieved through biosecurity procedures sufficient to prevent horizontal infection such as filtered air positive pressure housing, although use of such techniques is presently restricted to specific-pathogen-free flocks. Genetic selection and management have been used more frequently as adjuncts to vaccination rather than as primary control strategies.

CURRENT TECHNOLOGY

In the United States, seven vaccine strains are currently licensed.

Additional strains are licensed in other countries. The vaccine strains may be used singly or in combinations which are of three general types or formulation in ascending order of efficacy: (1) HVT alone, (2) HVT plus a serotype 2 strain, and (3) CVI988 (Rispens) with or without viruses of serotypes 2 or 3. The CVI988 (Rispens) strain appears to be the most efficacious of the serotype 1 strains and, at present, is widely used in layer and breeder chickens (Table 2).

TABLE 1. Licensed vaccine strains.

Vaccine Strain	Serotype
FC126 (HVT)	3
SB-1	2
301B/1	2
CVI988 clone C	1
CVI988/C/R6	1
CVI988 (Rispens)	1
R2/23 (Md11/75)	1

TABLE 2. Most common vaccine formulations for MD

Most common vaccine formulations	Relative efficacy vs. MDV pathotype			Current use in commercial flocks	
	v	vv	vv+	Broiler	Layer/Breeder
FC126 alone	+++	+	+	+	+
FC126 plus serotype 2	+++	+++	++	+++	++
CVI988 (Rispens) alone or plus serotype 3 or plus serotypes 2 and 3	+++	+++	+++	+	+++

Plus values indicate relative frequency (approximate)

Although much attention has been given to selection of the optimal combination of vaccine strains, a number of other considerations may (or may not) be important. These include synergism, dose, route of administration, multiple vaccinations, adjuvants, and genetic constitution of the host.

• **Synergism:** Protective synergism - the property of two vaccine viruses to induce greater protection in combination than either could induce alone - is easily demonstrated between

serotypes 2 and 3, but is less obvious with other combinations. The common practice of mixing serotypes in virtually all combinations has little support from controlled studies, but is not detrimental and often seems to be advantageous in the field.

• **Route:** *In ovo* vaccination has become the predominant method of administration of MD vaccines to broilers. This technique is efficacious with HVT vaccine but more studies are needed to demonstrate whether this route is equally useful for serotypes 1 and 2.

• **Dose:** Vaccine dose has increased to 5000-10000 PFU per full dose, more due to marketing than to efficacy considerations. Broilers are still given only a partial dose which is adjusted to optimize the return on cost.

ARE IMPROVEMENTS NEEDED?

Current control methods have been demonstrated to provide high levels of protection and are generally cost effective. Why then is such priority given to development of improved methods? There are several reasons. The current level of control is considered fragile at best, probably because significant disease outbreaks continue to occur and are often unpredictable. It is usually impossible to determine the reasons for such outbreaks. Also, because of the tendency of the virus to mutate towards greater virulence with concomitant reduction in efficacy of vaccines (7), many fear that current vaccines will not provide adequate protection indefinitely. Other factors are the increased competitiveness within the industry and the tendency to base management on cost rather than disease control criteria. For these and other reasons, MD has continued to rank high on industry priority lists despite a relatively low level of loss as measured by current industry averages.

TABLE 3. Disease priorities as determined by the Committee on Transmissible Diseases of Poultry, U.S. Animal Health Association

	1992	1993	1994	1995	1996
Layers	SE	SE	SE	AI	SE
	Marek's	AI	**Marek's**	**Marek's**	**Marek's**
	IB	MG/MS	E. coli	SE	fowl pox
Broilers	IB	MG/MS	IB	**Marek's**	IB
	Marek's	**Marek's**	**Marek's**	AI	**Marek's**
	MG/MS	IB	MG/MS	IB	others

AI = avian influenza; E. coli = colibacillosis; fowl pox = variant fowl pox; IB = infectious bronchitis; Marek's = Marek's disease; MG/MS = mycoplasmosis due to M. gallisepticum and M. synoviae; SE = Salmonella enteritidis;

FUTURE TECHNOLOGIES

Recombinant DNA technology has stimulated a variety of sophisticated approaches towards the production of superior vaccines. Vaccines in which MDV immunogenes are expressed in live fowl pox virus or herpesvirus (usually HVT) vectors have been described (3-6), are known to induce protective immunity, and seem the closest to commercial use. The quest for a deletion mutant of serotype 1 MDV is still ongoing, but has been hindered by inadequate technologies to produce mutants and the lack of knowledge of which genes are critical to virulence or oncogenicity. A naked DNA vaccine has also been described (2). For any such novel vaccine to be commercially successful, an advantage over existing products must be demonstrated - and this has proved a formidable challenge.

OBSTACLES AND OPPORTUNITIES

A number of issues need to be considered as one contemplates the future of MD control. Some of these represent opportunities for those seeking improvements.

- **Immune system:** By standards used for other vaccines, MD vaccines appear eminently successful; full protection is induced within 7-10 days of age and normally exceeds 90%. Further improvements may be limited by the capacity of the immune system.

- **Early challenge:** Under current management practices, most broilers and some layer pullets in multiple age facilities are most surely exposed to virulent MDV challenge within the first days or hours of placement, well before the onset of immunity.

- **Evolution of pathotypes:** As previously discussed, there is good evidence that MDV has mutated over time toward greater virulence (7). This trend may continue, depending on the severity of the selection pressure placed by vaccines and genetic resistance.

- **In ovo vaccination:** The recent predominance of in ovo administration of vaccines in broilers may require some reassessment of vaccine strain immunopathogenesis and efficacy, especially by the research institutes.

- **Vaccine efficacy assessments:** Recently, the poultry industry appears to be relying more on field trial data and less on controlled laboratory trials to compare the efficacy of commercial vaccines.

- **Genetics:** As new knowledge and better tools are being developed, breeding companies are showing renewed interest in genetic resistance as an adjunct to MD control.

- **Costs:** Decisions on vaccines and other mechanisms for MD control seem increasingly to be based on cost/benefit analysis rather than on absolute efficacy.

PARADIGMS FOR CONTROL

The current paradigm driving the control of MD seems to be largely based on optimizing the vaccine strain or formulation, making adjustments in dosage as needed, and employing alternate strategies for vaccine administration, including the use of adjuvants. Management improvement to reduce early exposure is rarely implemented, except in extreme cases. MD resistance is normally assigned a relatively low priority in selection programs unless the strain appears more susceptible than the competition. The vision for the future depends heavily on development of an improved "super" vaccine, either from conventional or recombinant DNA technologies, to mitigate the effects of pathogen evolution. There is relatively little joint strategy development among breeders, producers, vaccine manufacturers, and other stakeholders.

An alternate paradigm is proposed. This is based on the thesis that vaccines alone will be unsuccessful in the long run and that a more integrated strategy will be needed. The basis for such a strategy is multiple levels of control, all working together. Early exposure would be minimized, genetic resistance improved, and novel vaccines developed. Genetic resistance could be of multiple types based on MHC genes, nonMHC genes and transfer of foreign, pathogen-derived genes, and could be directed to reduce viral replication, reduce susceptibility to neoplasia and increase immune response. Vaccines would be improved and increasingly tailored to host chicken genotypes. Host genetic resistance that would specifically inhibit replication of virulent, serotype 1 MDVs with minimal effect on other serotypes would be especially desirable. Breeders, producers, vaccine manufacturers, and other stakeholders, including public sector research institutes, would share the cost for development and implementation of such a control strategy.

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AN OVERVIEW OF PROBLEMS AND PROGRESS IN CONTROL OF AVIAN LEUKOSIS

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INTRODUCTION

Prior to initiation of disease control programs in the late 1970s, it is likely that all commercial White Leghorn flocks in North America were infected with exogenous avian leukosis virus (ALV) (4). While ALV of both subgroups A and B were isolated from the field, subgroup B virus was comparatively rare. Differences in the distribution of the two virus subgroups was attributed to the fact that a much higher percentage of commercial White Leghorns were susceptible to infection with subgroup A than with subgroup B virus (4,6).

In recent years a number of commercial poultry breeding companies have successfully eradicated exogenous avian leukosis virus (ALV) from their stocks. It is estimated that 75% of the commercial White Leghorns in North America are from breeding stock that is free of ALV. It is less clear what percentage of today's broilers come from ALV-free breeding stock.

Based on *in ovo* studies, Crittenden and Motta (6) suggested that meat birds would be more resistant than White Leghorns to infection with ALVs. Gilka et al. (10) found that all meat birds and White Leghorns were infected following intraabdominal challenge with a subgroup A virus but, in accord with Crittenden and Motta (6), virus replication was usually more extensive in egg-type than in meat-type birds. In that study, lymphoid leukosis was the most common tumor disease caused by ALV in White Leghorns whereas nephroblastomas were the most common tumor in meat birds.

The emergence of subgroup J as a pathogen is of current concern to broiler breeders. Meat birds have been found to be more susceptible to the virus than White Leghorns (14). More information on subgroup J virus is given elsewhere in these proceedings. There have been recent reports of myelosarcomas in broiler breeders (3) and it would be of value to characterize the viruses responsible for these losses.

The ALVs are retroviruses and control has depended on development of *in vitro* methods for detecting infection in chickens. This paper reviews the development of these test procedures and their application in eradication of ALV.

TEST DEVELOPMENT

A serendipitous discovery led to development of the first *in vitro* test procedure for avian leukosis. Rubin in California noted that cells from some chicken embryos were resistant to challenge with Rous sarcoma virus. He determined that the resistance inducing factor (RIF) present in embryos was an ALV and the test to detect the virus was referred to as the RIF-test (15). Through application of the RIF-test Rubin and his associates showed that congenital transmission of ALV was an erratic phenomenon and that congenitally infected chicks were permanently viremic (16,17). The conclusion of Rubin and his colleagues that males do not contribute to congenital transmission of the virus may not always hold true (7).

Hughes et al. (12) used the RIF-test to develop the first ALV-free flock of chickens. While other workers were also successful in producing ALV-free flocks (28), the techniques were considered too costly and cumbersome to be used on a large scale by industry. However, experience with laboratory flocks showed that only minimal isolation was required to keep them free of ALV.

Outbreaks of what we now know as Marek's disease occurred in ALV-free flocks and this confirmed earlier claims that two separate diseases were responsible for the so called avian leukosis complex. As the Marek's problem grew in the 1960s, a number of research laboratories shifted their efforts to control that disease and then returned to avian leukosis research in the 1970s. Several new tests were developed for detection of ALV that were improvements over the RIF-test, but still required the use of tissue culture (21).

The discovery that egg albumen from ALV-infected hens contained high levels of group specific (gs) antigen led to development of rapid tests and large scale testing of commercial flocks (26,27). The complement fixation test was first used to test for the antigen in eggs but this was soon replaced by the more efficient enzyme linked immunosorbent assay (ELISA) (19). ELISA kits for detecting antibody to ALV of subgroups A and B are useful for monitoring flocks and are commercially available.

PROCEDURES AND PROBLEMS RELATED TO CONTROL

Procedures for prevention and control of ALV have been reviewed (7,13,20). One of the problems in initiating an eradication program is that most of the adult stock might be infected with ALV and the breeder can only afford to eliminate those hens most likely to congenitally transmit infection. To this end, good results have been achieved by testing for gs antigen in albumen and vaginal swabs. The sensitivity has been improved by increasing the number of eggs tested per hen (7). Inoculating buffy coat cells or other specimens onto cell cultures and then testing culture fluids by ELISA for viral antigen is another option (5,20). Congenitally infected chicks can be detected at hatch by testing for gs antigen in feather pulp and cloacal swabs (20).

The ELISA for ALV is complicated by the fact that the gs antigen may be of exogenous or of endogenous origin. Virtually all chickens carry endogenous viral (ev) genes and some of these express gs antigen. Meat birds typically carry more of the ev genes and have higher levels of gs antigen in their eggs than do White Leghorns (23). Infection with exogenous ALV increases the level of the gs antigen above that produced by the ev genes but the cut off point for considering birds positive is arbitrary.

EXPERIENCE AT AGRICULTURE AND AGRI-FOOD CANADA

In 1983 a leukosis control program was initiated at the Centre for Food and Animal Research (CFAR) in Ottawa where long term studies were underway to breed for increased production. White Leghorns were kept in cages and the plan was to test one egg per hen per generation for gs antigen and to eliminate hens as breeders that tested positive for antigen. Details on the program have been reported (25). Ten strains of chickens were tested and after two generations there had been an overall reduction in the prevalence of infection. The poorest response was in Strain 5. In that strain the percent of hens test-positive in 1983, 84, 85, 87, 89 and 1990 was 22, 24, 15, 0, 1 and 0, respectively. The sudden drop in prevalence of infection from 15% to 0 coincided with a program in 1987 to test all chicks for gs antigen in feather pulp and to remove high positives from the flock. The cut off point for eliminating chicks was arbitrary but this procedure seemed to have been a key factor in eliminating the virus from Strain 5 chickens.

An eradication program was also undertaken on meat-type breeding stock at CFAR (23). Again, the plan was to test one egg per hen per generation for gs antigen and to select test-negative hens as breeders. After four generations of testing there was virtually no change in the percentage of chickens that were considered positive for antigen and it was concluded that most of the antigen had been produced by ev genes. Despite the persistence of antigen positive birds, the testing and culling program appeared to have eliminated exogenous ALV from the population.

Subsequent studies have been conducted on a White Leghorn stock, in which a low percentage of birds had high levels of gs antigen in egg albumen but tested negative for ALV. For control purposes, ALV-infected chickens were obtained from other flocks. To isolate virus, cultures of chicken embryo fibroblasts were inoculated with egg albumen, buffy coat, or extracts of heart, liver, spleen and oviduct. Again, interpretation of the ELISA was complicated by the presence of gs antigen in the original inoculum. This problem was avoided by using the indirect immunofluorescence test. This test detected antigen in cells that were infected with ALV but did not detect endogenous gs antigen (22). This was probably because the endogenous gs antigen was not present in sufficient concentration in the cell cultures to fluoresce.

The above observation is consistent with what has been observed in sections of tissues from infected chickens. For example, in sections of spleen the immunofluorescent antigen appeared to be intimately associated with ALV particles that were largely confined to the sheathed capillaries (24). Although there was likely endogenous antigen throughout the splenic tissue, this did not fluoresce.

INCENTIVES FOR ERADICATION

Infection with ALV has been found to negatively affect hen day production, egg weight, shell thickness, egg specific gravity, fertility, hatchability from fertile eggs, mortality from all causes and body weight (9).

The ev21 gene, which seems to be tightly linked to the slow feathering gene, renders White Leghorn chickens more susceptible than other stock to infection with ALV (1,8,11,18). In this regard, vaccination with serotype 2 Marek's disease vaccine was more likely to precipitate losses from avian leukosis in slow feathering stocks than in rapid feathering birds (2,5).

The economic benefits from eradication to a commercial White Leghorn breeding company were reported in the 1991 AAAP symposium in Seattle, Washington (5). To quote from that report, "if you don't have the virus, you don't have the disease."

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AN OVERVIEW OF SUBGROUP J-LIKE AVIAN LEUKOSIS VIRUS INFECTION IN BROILER BREEDER FLOCKS IN THE UNITED STATES

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INTRODUCTION

Avian leukosis virus (ALV) is the most common naturally occurring avian retrovirus associated with neoplastic diseases and other production problems in chickens (1,5). It is now established that the economic loss in productivity due to ALV infection is greater than that from tumors. Although ALV is capable of inducing a variable incidence of a variety of neoplasms, lymphoid leukosis (LL) is the most common naturally occurring B-cell lymphoma of chickens caused by ALV (5). However, a novel subgroup (J) ALV was found to be associated with myelocytomatosis in meat-type chickens in the United Kingdom (4,6). This presentation describes the isolation, incidence and some characteristics of a subgroup J-like ALV (ALV-J) isolated from broiler breeder flocks experiencing a relatively high incidence of myelocytomastosis in the United States.

ISOLATION OF SUBGROUP J-LIKE ALV FROM BROILER BREEDER FLOCKS IN THE USA

Table 1 shows the protocol used for the isolation of ALV from samples of blood obtained from broiler breeder flocks experiencing myeloid leukosis mortality. Using chicken embryo fibroblasts (CEF) that are susceptible or resistant to exogenous and endogenous ALV (1), virological assays (3,7) of blood samples from several broiler breeder flocks revealed the isolation of a non-subgroup A ALV.

Table 2 shows the association between isolation of a subgroup J-like ALV and myeloid leukosis in chickens from 4 different broiler breeders in the USA. The lack of antibodies to subgroup A ALV (data not shown) confirmed that the myeloid leukosis lesions noted in these flocks were probably induced by ALV-J.

Antigenic characterization of the new isolate by cross neutralization tests using strain HPRS-103, the prototype of ALV-J and its antibody revealed that it is related (one way cross) to the prototype of ALV-J (Table 3). Furthermore, these virological and serological data were confirmed by nucleotide sequence comparisons between the prototype HPRS-103 strain and one of the subgroup J-like ALV isolates termed ADOL-Hc1 which revealed only two base substitutions in the E element.

TABLE 1. Protocol used for the isolation of ALV from broiler breeder flocks.

Sample ID#	Inoculated on CEF of line					
	alv6		15B1		0	
	Plasma	PBM	Plasma	PBM	Plasma	PBM
1	+	+*	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+

alv6 = C/A and C/E CEF (resistant to infection with subgroup A and E ALV); 15B1 = C/O CEF (susceptible to infection with all subgroups of ALV including endogenous virus); 0 = C/E CEF (resistant to infection with endogenous ALV); PBM = Peripheral blood monocytes; * = Isolate used for further propagation and characterization.

TABLE 2. Association of subgroup J-like ALV with myeloid leukosis in flocks from four different broiler breeders in the USA.

Breeder	Tumor problem	Subgroup J-like ALV isolated	Designation
1	Myelocytomatosis	+	ADOL-Hc1*
2	Myelocytomatosis	+	ADOL-R6.1
3	Myelocytomatosis	+	ADOL-A20
4	Myelocytomatosis	+	ADOL-F1

* Isolate designated as the US prototype of ALV-J.

INCIDENCE OF SUBGROUP J-LIKE ALV INFECTION BROILER BREEDERS FLOCKS

Table 4 shows incidence of ALV and antibody to strain ADOL-Hc1, the US prototype of ALV-J in flocks of two different broiler breeders. The incidence of virus isolation varied from 16% to 48%, whereas the incidence of antibody to ADOL-Hc1 varied from 72% to 87%. The results clearly suggest that subgroup J-like ALV is widespread, at least in flocks of these two broiler breeders.

TABLE 3. Neutralization profile of ADOL-Hc1 and HPRS-103 ALV with antibodies to various subgroups of ALV.

Antibody	ALV/Neutralization*	
	HPRS-103	ADOL-Hc1
A	-	-
B	-	-
C	-	-
D	-	-
E	-	-
HPRS-103 (J)	+	-
ADOL-Hc1	+	+
Control negative	-	-

*- = no neutralization; + = virus is neutralized. HPRS-103 = Prototype of subgroup J ALV; ADOL-Hc1 = Prototype of US ALV-J.

TABLE 4. Frequency of subgroup J-like ALV and antibody in flocks from two different broiler breeders.

Breeder #	Flock #	ALV (virus isolation) No.+/no. tested (%)	Antibodies to*	
			ALV-J	ALV-A
			No.+/no. tested (%)	
A	1	13/40 (33)	31/40 (78)	0/40 (0)
	2	105/217 (48)	NT	NT
	3	4/25 (16)	18/25 (72)	0/25 (0)
B	1	14/31 (45)	27/31 (87)	0/31 (0)

*Incidence of antibody to ALV-A and ALV-J was determined by virus neutralization tests using strain Rous associated virus-1 (RAV-1) of subgroup A ALV and strain ADOL-Hc1 of ALV-J, respectively.

CONCLUSIONS

The data presented in this paper indicate the isolation of a nonsubgroup A ALV from flocks from several broiler breeders that experienced an increase in PCR myeloid leukosis mortality. Serological and molecular characterization of the new isolate suggest that it is related to strain HPRS-103, the prototype of subgroup J ALV, an ALV previously not reported in flocks raised in the USA.

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CURRENT STATUS OF DIAGNOSIS, EPIDEMIOLOGY AND CONTROL OF ALV-J

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INTRODUCTION

In 1989, an avian leukosis virus (ALV) of a new envelope subgroup, designated J, was isolated by workers at the Institute for Animal Health from commercial meat-type chickens in the UK (7). The prototypic strain is designated HPRS-103 ALV. The virus has been completely sequenced (2,3). It has *gag* and *pol* genes which show high homology to these genes in ALVs of other subgroups, but an *env* gene whose gp85 (SU) domain shows only about 40% homology with other subgroups. The *env* gene shows higher (75%) homology to *env*-like sequences of the E51 clone of the recently discovered EAV family of endogenous avian retroviruses (4,5) and EAV family endogenous sequences (designated EAV-HP) with very high homology (96%) to HPRS-103 *env* have been found in all chickens studied. These findings suggest that HPRS-103 is a recombinant between another ALV and endogenous EAV sequences.

The virus has a wide host range, all of 11 lines of chickens studied being susceptible to infection. Amongst 12 species of poultry and game birds, only chickens, jungle fowl, and turkeys were susceptible (8). In the field, presence of the infection is associated with the occurrence of myelocytomatosis, and that tumor, renal tumors and other rarer tumors are induced by experimental infection of chickens (9). Susceptibility to tumors varies markedly between different lines of chickens, but meat-type lines are particularly prone to tumors.

From some field or experimental cases of myelocytomatosis ALVs can be isolated that acutely transform cultured chicken bone marrow cells or monocytes (10). HPRS-103 itself does not transform these cells *in vitro*. HPRS-103 lacks a viral oncogene and presumably induces myelocytomatosis by insertional mutagenesis (3). Preliminary work suggests that the acutely transforming isolates have transduced the cellular *myc* oncogene.

Studies on the cell tropism of HPRS-103 indicate that, in contrast to subgroup A ALV, it has a tropism for cells of the myelomonocytic series, but low tropism for bursal cells, consistent with its induction of myeloid, and not lymphoid, leukosis (1).

DIAGNOSIS

Diagnosis of ALV-J infection and related diseases involves pathological identification of tumors and virological identification of ALV-J infection.

Pathologically, myelocytomas comprised usually of well-differentiated myelocytes are present on the inner sternum, vertebrae and ribs, and myelocytomatous infiltration of the liver, spleen and kidneys usually occurs, causing their enlargement. Occasionally blast-cell tumors and histiocytic sarcomas occur in the field.

ALV-J virus can be isolated from materials such as tumors, serum, and cloacal or vaginal swabs by culture in C/E chick embryo fibroblasts in a microculture system followed by gs-antigen ELISA testing of cell extracts for evidence of virus growth (9). Isolates are identified as subgroup J ALV by neutralization with J subgroup chicken antiserum. The ALV group-specific antigen (gsa) ELISA is also used to demonstrate increased gsa levels in egg albumen and cloacal or vaginal swabs from certain classes of infected birds (6).

ALV-J antibodies in serum are identified by neutralization of ALV-J in the microculture system (9), or in antibody ELISA tests using plates coated with lysate of HPRS-103 infected cells or with gp85 envelope glycoprotein of HPRS-103 produced in insect cell cultures infected with a recombinant baculovirus (13). Use of ELISAs based on lysate or recombinant antigen gave similar results but within an infected flock the correlation between virus neutralization and ELISA results was low, probably because of detection of different epitopes.

As an adjunct to conventional tests for ALV-J, we have developed subgroup J ALV-specific PCR tests based on the sequence of HPRS-103. These tests have been used to detect ALV-J proviral DNA in tumor samples and in CEFs infected with ALV-J. By incorporation of a reverse transcriptase step the PCR can be used to detect viral RNA.

Of 12 recent isolates of ALV-J from commercial flocks, only four were amplifiable by the PCR test initially developed for HPRS-103, but all 12 were identified with a second PCR test in which a modified primer was used. Sequence analysis of the *env* gene of these isolates revealed significant nucleotide substitutions encompassing up to 3% of the *env* gene. The nucleotide changes tended to be concentrated in the hr1, hr2, and possibly other regions of the *env* gene. Only two of these variant viruses were neutralized by HPRS-103 antisera. Thus, genetic and antigenic variation occurs amongst ALV-J isolates.

The relative sensitivities of conventional methods of identifying ALV infected birds (by virus isolation, serum antibody and cloacal swab tests for gsa) and detection of proviral DNA and viral RNA in different sample materials by PCR were studied in experimental embryonal and post-hatching HPRS-103 infections of meat-type and Brown Leghorn chickens. Test materials for PCR assays were whole blood, serum and feather pulp. Following embryonal infection of both lines of chickens, conventional tests and PCR tests for proviral DNA in blood and feathers seemed equally sensitive. Following contact infections of meat-type birds, the proviral PCR tests on blood and feathers appeared to be marginally more sensitive. In contact-infected Brown Leghorns, none of the test materials were PCR positive, even in birds which had developed virus-neutralizing antibodies.

EPIDEMIOLOGY

Study of an experimental HPRS-103 infection indicated that the virus behaved like ALV of other subgroups. In this and other experiments we routinely classify infected birds by presence (+) or absence (-) of viremia (V), serum antibodies (A), and gsa in cloacal or vaginal swabs (S). Thus, embryonal infections lead to tolerant viremic shedders (V+A-S+). Post-hatch infection often leads to immune non-shedders (V-A+S-), although in meat-type birds early post-hatch infection often leads to viremic shedders (V+A-S+) and sometimes to immune shedders (V-A+S+). In a preliminary study we have detected low numbers of antibody-forming cells in spleens in V+A-S+ meat-type birds infected post-hatching, whereas such cells were not present in V+A-S+ birds infected as embryos (11). Thus immune tolerance in the former type of birds may not be complete.

As with other ALV subgroups, it appears that ALV shedders (to albumen or vaginal swabs) are likely to be congenital transmitters of ALV to their progeny. Of 25 hens in a naturally infected flock which produced eggs with gsa positive albumen, 80% produced chicks with gsa positive meconium; of 74 hens which produced eggs with gsa negative albumen, only 3% produced chicks with gsa positive meconium (9).

Experimentally myelocytomas and other tumors occur mainly in birds which are infected embryonally and develop a tolerant viremia. In the field, tumor mortality can vary greatly according to the location of infected stock, suggesting that as yet unknown predisposing factors influence tumor formation.

ERADICATION

The finding that HPRS-103 behaved like a typical exogenous ALV suggested that well-established ALV eradication procedures based on the work of Spencer (12) and others should be effective in eradicating ALV-J from infected flocks. Accordingly an eradication scheme was developed and applied commercially. The steps applied to pedigree birds in this scheme are as follows:

1. 20 weeks: Gsa test on cloacal swabs (both sexes). Reject positive birds.
2. 22 weeks: Antibody and viremia test (both sexes). Reject viremia positive birds.
3. 23 weeks: Gsa test on first two egg albumens/hen. Reject positive hens.
4. 26 weeks: Gsa test on meconiums from first chicks/hen. Reject positive dam progeny group and the hen.
5. 40 weeks+: Gsa test on two egg albumens/hen. Reject positive hens.

For testing multiplication flocks, gsa tests on cloacal swabs are carried out at 20 and 23 weeks, rejecting positive birds. At 26 weeks plus, gsa tests are carried out on samples of egg albumens and meconiums to establish transmission levels.

In two typical infected flocks, application of this scheme over 6 years of a continuous pedigree program has reduced the prevalence of hens with gs-antigen in cloacal swabs plus egg albumen from over 50% to less than 5%, and the prevalence of gs-antigen in meconium after swab and albumen testing from 5% to 0%. Falling prevalences in flocks of positive swabs, albumen and meconium and low or negative antibodies provide evidence of progress in ALV-J eradication.

We have speculated that vertical transmission of ALV-J could occasionally occur from hens which are negative in the swab and albumen tests. Such an occurrence would hinder eradication by providing residual infected birds. It was to detect such transmitters that the PCR tests described above were developed and currently these tests are being evaluated commercially.

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RETICULOENDOTHELIOSIS VIRUS: AN OVERVIEW OF CURRENT ISSUES

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INTRODUCTION

Reticuloendotheliosis (RE) is the name given to the various pathological manifestations consequent to infection with reticuloendotheliosis virus (REV). The name derives from an early description of the acute reticulum cell neoplasia induced by the replication-defective, oncogene-containing strain T (9). However, this name has also been applied to the runting disease syndrome and lymphoid neoplasia, both induced by replication-competent strains.

REV has a broader host range than other avian tumor viruses. The virus was first isolated from turkeys, but probably is now more prevalent in chickens. A variety of other avian species are susceptible, including most species of domestic poultry and game birds.

In RE, infection only rarely results in disease and, thus, these two parameters need to be considered separately.

As other speakers will be focusing on specific issues with RE, the purpose of this paper will be to introduce some of the relevant issues and evaluate its current and future importance.

INFECTION FREQUENCY

An early 1980s survey (11) identified a higher incidence of seropositive flocks in southern states and in a subsequent study of broiler breeder flocks in Mississippi, 6 of 6 seroconverted during the observation period (10). No comprehensive survey has been published recently, but anecdotal information suggests that the relative frequency of REV antibodies has increased in recent years, especially in some of the northern states. REV infection appears endemic in several foreign countries.

DISEASE FREQUENCY

Outbreaks of RE in turkeys, once seen at least occasionally, seems now to be extremely rare. However, one outbreak in a breeder complex in Pennsylvania was particularly severe (12) and proved so intractable to control that the operation is no longer in business. Natural outbreaks of disease in chickens are extremely rare, except when associated with the use of contaminated biologics. One such case was recently reported by Fadly et al. (4) where lymphomas were induced in two chicken flocks inoculated with a contaminated fowl pox vaccine. However, in

certain foreign countries, the current potential for disease consequent to natural infection of chickens or turkeys appears to be significant. The possibility that similar problems might develop in the United States warrants consideration.

ISSUES

The relatively high visibility of RE among poultry diseases may be surprising given the infrequency of clinical manifestations. The following is a partial list of issues that may explain the high level of interest by researchers and industry personnel.

• **Differential diagnosis.** The close similarity of lymphomas induced by REV to those of both Marek's disease and lymphoid leukosis are a real problem to those concerned with definitive diagnoses of avian tumors. PCR assays for REV have been proposed as a diagnostic tool, but can give confusing results unless other etiologic agents can be excluded.

• **Export of breeder progeny.** Commercial breeders are now required to evaluate parent flocks for REV antibodies prior to the export of progeny to certain foreign countries. Most breeders have found at least a few seropositive parent flocks or have had shipments of chicks rejected upon entry into the country of destination. In the process of conducting the required testing, usually with a commercial ELISA test kit, occasional equivocal or false positive results have been encountered. Confirmatory testing procedures are needed. Moreover, procedures sufficient to guarantee freedom of commercial flocks from REV exposure are lacking and the reservoir of infection remains an enigma. Mosquito transmission has been described (8), but its role in the United States has not been established.

• **Vaccine quality control.** Although the propensity of REV to show up as a contaminant of poultry biologics is well documented, most of these cases, until recently, had occurred in other countries and there was no standard requirement for certification of licensed biologics in the United States for freedom from REV. However, following the identification of a contaminated fowl pox vaccine in 1994, industry concerns prompted APHIS to issue guidance to biologics companies on testing procedures. At present, all poultry biologics companies are testing master seed stocks for REV.

• **Vertical transmission.** The frequency of vertical transmission of REV from seropositive flocks is problematic. Although vertical transmission has been reported in both chickens and turkeys, the frequency of this transmission following contact infection in the field is probably very low.

• **Endangered species.** A flock of Attwater Prairie Chickens, an endangered species, was found infected with REV and efforts towards eradication were attempted (3).

• **Eradication.** Although some aspects of the epidemiology suggest that procedures used successfully for avian leukosis virus eradication would also work for REV, there has been no

field testing of such a procedure. More importantly, there is no commercial source of ELISA kits to detect REV antigen, a test of potential value in any such eradication procedure (1,12).

• **Basic science.** REV has intrigued many retrovirologists who have used this virus as a model for basic studies. Recently, REV proviral sequences were found to have spontaneously integrated into the genome of the Marek's disease herpesvirus during cocultivation in cell culture (5), thus providing a novel method of insertional mutagenesis. REVs also have been used as potential vectors for human gene therapy (2).

• **Public health.** The wide host range of REV prompts speculation on the potential of this virus to cause disease in humans. Johnson et al. (6,7) reported reactivity of human sera with REV antigens and speculated on a role of REV in human disease, but the data remain inconclusive.

CONCLUSIONS

REV has the potential to be a serious disease of poultry. Some aspects of this potential threat have been realized in other countries. It will be prudent to maintain surveillance and sufficient expertise to insure that REV-related problems do not adversely affect the economic viability of the United States poultry industry.

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RETICULOENDOTHELIOSIS VIRUS INFECTION IN TURKEYS: INDUSTRY PERSPECTIVE

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Initially REV was isolated in 1958 from turkeys with visceral lymphomas in the USA. Similar viruses, spleen necrosis (SN) and duck infectious anemia (DIA) were later isolated from ducks. It was not until 1975 that outbreaks of leukosis in turkeys were associated with REV in the UK and the USA.

Condemnation for leukosis and tumors in young turkeys has been very low. Observations in 1980 indicated that the incidence of condemnations due to lymphoid tumors was one bird per 10,000 in the U.S. processing plants. In the 1990s, annual survey on turkey health status, no report on the presence of REV tumor disease in turkeys were given to the United States Animal Health Association (USAHA) Sub-committee on Turkey Health Survey. This indicates that the disease is of very low prevalence in the U.S. turkeys. It is fair to say that REV has received uncommon attention by the researches especially in the 1980s and 1990s considering that it has not been an economically significant disease of turkeys. Also, it has no public health significance.

A group of syndromes are induced by the retroviruses of the REV group. The disease syndromes include acute reticular cell neoplasia, a runting disease syndrome and a chronic neoplasia of lymphoid and other tissues. Strain "T" REV was found to be acutely oncogenic for young chickens, turkeys, and Japanese quail. This strain of REV also contains a helper REV that replicates in chicken fibroblast tissue cultures possessing no oncogenic properties. The helper virus has been designated as REV-A or as non-defective strain "T".

The chicken cyncytial (CS) virus and strain "T" REV are found to be antigenically related to DIA and SN viruses. These three viruses are related and have been isolated from turkeys, chickens, ducks, pheasants, and geese indicating the possible existence of a wide range of reservoirs which could cause sporadic outbreaks of REV in turkeys (4,16).

However, the chronic neoplastic disease sporadically appears to cause significant mortality and condemnation losses in commercial turkey flocks. REV is also a potential contaminant of poultry vaccines such as HVT (5), MD (6) and Pox (2). Testing and purifying commercial egg propagated fowlpox vaccines has reduced the presence of REV in flocks.

In studies on vertical transmission by McDougall, et al. (7,8) turkey hens were inseminated with infected semen from REV tolerant male turkeys or with semen from uninfected control males. REV was detected in 27.5% of the embryos and in 8.7% of 1-day-old poults from hens

inseminated by REV infected semen. When the very same hens were inseminated with uninfected semen, REV was detected in only 0.7% of the embryos examined. REV spread readily to uninfected poults maintained in contact with progeny of hens inseminated with REV-infected semen. It appeared that the incidence of egg transmission is increased by REV infected semen.

A non-defective REV was isolated by Witter and Salter (14) from a commercial turkey breeder flock. The virus was isolated from 59 hens (46%). Only 4 out of 45 hens transmitted REV to their progeny during a 6-week observation period. The overall incidence of REV transmission was 1.8%.

It appear that tumors do not play a major role in vertical (14, 15) transmission. Non-defective REV has sporadically been isolated from turkeys suffering from lymphoid neoplasia.

Immunofluorescence (IF) (1), complement fixation (CF) (11), serum neutralization (SN) (3) and agar gel precipitation (AGP) (9) assays in the chick embryo fibroblast cell tissue cultures have commonly been used in the diagnosis of REV. Direct ELISA (10, 12) on albumen is probably considered to be the best method for detection of non-defective REV.

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EPIDEMIOLOGY AND CONTROL OF RETICULOENDOTHELIOSIS VIRUS IN CHICKENS AND TURKEYS IN ISRAEL

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INTRODUCTION

Reticuloendotheliosis virus (REV) is an exogenous C-type retrovirus that infects chickens, turkeys and other avian species and is transmissible both vertically and horizontally (6). The vertical mode of transmission from one generation to the next occurs either via the germ cells or by oviduct infection. Horizontal infection is generally a result of environmental contamination from either various body excretions of infected birds or other external vectors. Biting insects such as mosquitoes have been implicated as possible vectors (3), but other fomites cannot be excluded. Contaminated poultry vaccines are notorious sources of REV (4, 5). For these reasons, efforts to control REV infection of commercial flocks have to be coordinated by taking these modes of transmission into consideration. On one hand, day-old chicks can be monitored for possible exposure of the parent flock to REV, and on the other, affected farms can be examined for evidence of environmental infection.

EPIDEMIOLOGY

Serology and virology

In 1990, RE was diagnosed in a number of pullet and broiler breeder flocks in Israel. The outbreak was traced to importation of 1-day-old chickens from Europe in 1989, but because of its sporadic incidence, REV was only isolated in the following year. In the interim between importation and diagnosis, some local flocks became infected resulting in economic losses from neonatal and adult mortality and reduced egg production in layers. With the possible exception of Australia, REV had not been commonly recognized in breeding chickens or turkeys in any country, and no recognized procedures for eradication of the infection have been established other than testing poultry vaccines for REV contamination.

Epidemiology in the first years of the study (1990-91) was based on demonstration of ELISA antibody in tumor-affected, stunted or immunodepressed flocks, and by attempts to isolate the virus in cell cultures. Later on, commencing in 1993 we introduced the PCR for surveillance of REV involvement in tumor formation and at the same time we monitored for REV antibodies. The PCR also served for differential diagnosis of avian tumors. These included three oncogenic viruses of chickens (MDV, REV and LLV) and three of turkeys (MDV, REV and LPDV) (1, 2). *A flock was regarded as REV positive on the basis of either PCR or ELISA, or both tests.*

Table 1 shows the total number of flocks that were diagnosed as REV positive by ELISA alone or in conjunction with PCR. During 1990, antibodies were found in 18/92 (20%) tumor-affected chicken and in 1/19 (5%) turkey flocks, while in 1991, 48/99 (48%) chicken and 2/18 (11%) turkey flocks carried REV antibodies. In 1989, we isolated REV three times and during 1990 and 1991 there were 6 and 5 isolations from chickens, respectively. In 1992, one isolate was made from a turkey flock. These data show that REV infection was widespread in 1990 and even more so during 1991.

TABLE 1. Diagnosis of REV infection of tumor-bearing flocks

Year	Chickens (No. of flocks)		Turkeys (No. of flocks)	
	PCR (%)	ELISA (%)	PCR (%)	ELISA (%)
1990	Not done	18/92 (20)	Not done	1/19 (5)
1991	Not done	48/99 (48)	Not done	2/18 (11)
1993	2/33 (6)	2/33 (6)	0/5 (0)	0/5 (0)
1994	24/37 (65)	22/37 (59)	5/8 (62)	5/8 (62)
1995	12/33 (36)	17/33 (51)	1/7 (14)	1/7 (14)
1996	6/22 (27)	7/22 (32)	0/5 (0)	0/5 (0)

In 1993, there were only two positive chicken flocks, while in 1994 the number of infected flocks increased dramatically. In 1995-1996 this trend was reversed, especially in 1996. Affected turkey flocks were frequently encountered in 1994.

From November 1993, screening by ELISA was extended to include healthy breeding flocks to assess the degree of environmental contamination, and a flock was considered REV-positive if 50% or more sera in the sample were ELISA positive. Sporadic positive flocks were identified in February, March, and April 1994. From July 1995 through November 1995 the number of positive flocks was fairly constant: 2/11 in July, 3/18 in August, 4/20 in September and in November - 2/21. From January 1996 the rate decreased, with only one flock in January 1996, and no more positive until October 1996. The data suggest that a periodic increase in the rate of environmental exposure may occur during the summer and late fall months.

Environmental contamination and evidence of vertical transmission

The possibility that some poultry farms are located in geographical areas where natural reservoirs of the virus exist was examined in some detail in the light of two episodes of tumors affecting two batches of 5 month-old laying pullets in February and May 1994. These were the progeny of two imported parent flocks that were REV-antibody free as one day-old chicks. In the first episode, in which five poultry farms were involved, all the flocks suffered from tumors, an increased mortality (0.5-2% per month), were seropositive and had a positive REV-PCR.

In the second episode over 20,000 replacement pullets raised from day-old chicks on one farm were distributed among 19 farms. In six flocks the birds were affected clinically with tumors and excessive mortality (about 2% per month), five of them seroconverted and in two flocks REV was detected by PCR.

The epizootiological significance of these findings is emphasized by the examination of four parent flocks that were raised by the same breeding farm; all were found to have REV antibodies, with the seroconversion event having occurred during the late summer-fall months (Table 2).

Insect studies

Being aware of Motha's reports on the role of biting insects in REV transmission during the warm season (3) we have initiated a controlled study employing the PCR for the demonstration of REV in insects. The first step was to establish sensitive methods of REV detection in mosquitoes and then to monitor REV in insects trapped on infected farms. Experimental feeding of mosquitoes was made over 96 hr, by giving REV-infected culture media mixed with chicken blood. Samples were taken from the satiated mosquitoes and the feeding mixture remaining in the cage, and analyzed by; a) RT-PCR with REV-LTR and

TABLE 2. A serological study on horizontal transmission of REV on one breeding farm

Year	1992		1993								1994			
Month	8	10	1	3	5	7	8	9	10	11	6	8	9	10
Flock A	-	+	+	+	+	+			+					
Flock B							-	-	+	+				
Flock C											-	-	+	+
Flock D												-	-	+

REV-gag primers; b) REV-antigenemia by ELISA, and c) virus isolation in cell culture. Virus was detected in the insects up to 5 hr post feeding, in contrast to 96 hr in the feeding mixture. It seems that mosquitoes can transmit REV for short periods of time after feeding, and contamination in the field can be expected if a blood meal is shared between infected and uninfected birds. A study on insects trapped on locations with active REV infections is currently being performed and the data will be presented.

CONTROL

Importation of 1-day-old chicks

In 1993, the Israeli Veterinary Services introduced a regulation requiring that all importations of 1-day old chickens be tested serologically for REV antibodies. The regulation was based on our previous survey showing that 18.5% of the flocks imported between 1990 and 1993 carried REV antibodies (Table 3). Since 1993, the number of imported positive flocks has decreased annually and an occasional positive one was detected over the following 3 years, and in 1996 none were positive. Importation of turkeys has been monitored since 1995, and as yet, no positive flocks have been found. The REV positive flocks, which were destroyed by statutory order, had been imported from West Europe or from the USA. With the implementation of these regulations, the awareness of breeding flock producers has increased, leading to the import of only clean birds. The effective control of the REV infection in Israel during the last year is surely a reflection of the coordinated activity of state laboratory and field services.

Selective breeding

In 1992, we initiated an REV eradication program, based on identification and removal of infected grandparents (broiler and light breeds) from the breeding program. In the first year of the program, all the g.g.p. female lines and the four foundation g. p. lines of light breeds were tested by ELISA. In the second year, the F1 generation was screened by ELISA, viremia and PCR.

TABLE 3. ELISA examination of imported day old chicks

Year	Chickens			Turkeys		
	No. +ve flocks/No. tested	% positive flocks	Total sera examined	No. +ve flocks/No. tested	% positive flocks	Total sera examined
1991-1992	5/27	18.5	384	NT		
1993	1/15	6.7	150	NT		
1994	2/15	13.3	295	NT		
1995	2/24	8.3	520	0/7	0	127
1996	0/27	0	618	0/9	0	192

Light breeds: 80 hens and 20 roosters (housed in individual cages) from each of the four inbred lines were analyzed by ELISA and found negative for viremia. Six plasmas were weakly positive by ELISA, but negative for IF antibodies. The same farm was repopulated with a fresh foundation flock, reexamined and found negative, as was the F1 generation.

Heavy breeds: The female line dams were maintained in individual laying cages, their sires were housed in small groups and the male line foundation flock is kept on litter. Throughout 1993, broiler breeds located on five separate farms were tested virologically and serologically. Four flocks tested negative throughout, while the fifth flock, that comprised two sublines had a high proportion of seropositive birds in each subline (45%). Roosters of each subline were tested by ELISA; one group was almost 100% positive, and the other almost all seronegative. On the basis of the ELISA data, only the seronegative hens were retained for breeding and only seronegative roosters were used for insemination. One-day-old chicks and the F1 breeding flock of these matings were examined extensively during the second year using ELISA. Among 80 birds of the two sublines no antibody positive birds were identified. As the new flocks were located on the same farm as their parents, and housed on litter, there was no evidence for lateral or other external sources of REV infection on this farm.

Two additional farms with female broiler breeders lines were surveyed. On one farm, over 100 birds from each of the three sublines were bled and none were REV positive. In contrast, on the second farm all 70 birds examined were positive. Because it was not possible to initiate a reduction program on these premises, that flock was eliminated.

In summary, on the basis of an extensive survey of pedigree lines, it would seem that it is possible to reduce and even eradicate REV from the breeding cycle if only serologically negative birds are used for breeding. The protection of their progeny from horizontal infection appears to depend to a great extent on the geographical location of the poultry farm.

CONCLUSIONS

Epidemiological studies and attempts to control REV infection were conducted in Israel during the past 7 years (1989-1996). The results suggest that effective monitoring of disease using the ELISA and PCR in conjunction with strict surveillance of importations of 1-day-old chicks has resulted in a marked reduction in the incidence of REV and has increased the profitability of flocks. Nonetheless, the analysis of environmental vectors of REV contamination continues with the hope that further studies will add to our knowledge of local ecosystems and their impact on poultry health.

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METHODS FOR DETECTION OF RETICULOENDOTHELIOSIS VIRUS CONTAMINATION IN POULTRY VACCINES

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SUMMARY

The recent discovery of reticuloendotheliosis virus (REV) in a licensed poultry live virus vaccine has made apparent the need for improved product surveillance. This is the first known occurrence of REV contamination of a USDA licensed vaccine. Eggs used in the production of such vaccines must come from specific-pathogen-free (SPF) flocks monitored serologically for extraneous REV. The greatest risk of contaminating live virus vaccines with REV occurs primarily during the isolation of the initial master seed vaccine virus (MSV) from an REV-infected source. Currently, no Standard Requirement (SR) exists for testing licensed avian vaccines for REV contamination. However, the USDA has recently established testing guidelines for the detection of REV in MSVs. The three recommended testing methods are: *in vivo* virus amplification combined with *in vitro* virus identification; serologic testing for REV-specific antibodies in inoculated birds; and direct detection of REV-specific DNA using polymerase chain reaction (PCR). An efficient procedure for the detection of REV-specific DNA using a PCR assay is described. This assay is currently used at the Center for Veterinary Biologics Laboratory (CVB-L) to evaluate MSVs and prelicense vaccine serials for the presence of extraneous REV.

INTRODUCTION

REV is a retrovirus of poultry that can cause neoplasia and immunosuppression in experimentally infected birds. While many commercial chicken flocks have serologic titers to REV, genuine clinical outbreaks of reticuloendotheliosis (RE) are infrequent and self-limiting.

Extraneous REV has been discovered in some poultry vaccines, although infrequently (3,5). Until recently, REV was not detected in USDA licensed vaccines. However, accidental exposure of young susceptible flocks to REV through contaminated live virus vaccines could cause a high morbidity rate of clinical RE. The primary danger of contaminating live virus vaccines with REV occurs during the initial vaccine virus isolation. That is, the most likely sources of extraneous REV are MSVs that come from REV-infected material. Embryonated eggs or tissue culture substrates used for manufacturing poultry vaccines come from specific-

pathogen-free (SPF) flocks monitored serologically for extraneous REV. Thus, contamination with REV is not likely to occur during vaccine production. Therefore, APHIS recommends analyzing vaccine MSVs for extraneous REV.

The three methods recommended by APHIS for detecting REV in vaccines are: *in vivo* virus amplification combined with *in vitro* virus identification; serologic detection of REV-specific antibodies in inoculated birds; and direct detection of REV-specific DNA using PCR (7). All three methods are designed to amplify low levels of REV that may be present in the test sample.

IN VIVO VIRUS AMPLIFICATION

Vaccine viruses are usually cytopathic to tissue cultures, making it difficult to amplify extraneous REV. Filtering cannot be used to remove large vaccine viruses (e.g., fowl pox virus) before inoculating susceptible cultures, since filtering can reduce the amount of extraneous REV to an undetectable level (3,4). Thus, REV-susceptible chicks are used to more effectively amplify low levels of REV (8). One-day-old REV-susceptible chicks are inoculated with unfiltered MSVs. This is followed by *in vitro* REV identification 2 weeks post-inoculation (PI) by inoculating blood samples inoculated onto susceptible tissue cultures. These cultures are evaluated for the presence of REV using an enzyme immunoassay (ELISA), fluorescent antibody assay (FA), or PCR (4,8).

SEROLOGIC DETECTION

Exposure to REV can also be detected in inoculated birds by testing for the production of REV-specific antibodies. SPF chicks are inoculated twice (at 2 and 4 weeks of age) with untreated MSVs, and then serum samples are evaluated for REV-specific antibody 3 to 4 weeks PI using ELISA or FA (7,8).

POLYMERASE CHAIN REACTION

MSVs can be analyzed simply and rapidly by PCR for extraneous REV-DNA. The REV-PCR employs primers specific to the target proviral long terminal repeat common for most strains of REV (1,2,3,5,6). To extract REV-DNA from specimens, the samples are incubated in a lysis buffer. The extracted DNA is then amplified by Taq DNA polymerase with the two REV-specific primers.

Of the three methods recommended by APHIS, the PCR evaluation of MSV samples is the test of choice at the Center for Veterinary Biologics Laboratory. Here we describe the steps of this assay, and the results of its use in our laboratory.

REV DNA extraction from test samples

A 1 μl sample of test material is added to 9 μl lysis buffer [50 mM KCL, 10 mM Tris-HCL (pH=8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, 0.25 mM

$CaCl_2$, and 100 μg/ml Proteinase K]. The preparation is incubated for 1 hour at 37°C and then 10 minutes at 99°C to heat inactivate the proteinase K.

Primers

The PCR primers were synthesized at the Iowa State Nucleic Acid Facility, and were purified by high performance liquid chromatography. The primers were designed previously (1). The sequence of the direct PCR primer (*rev-1*) is 5'-CATACTGGAGCCAATGGTT-3', and the sequence of the reverse primer (*rev-2*) is 5'-AATGTTGTACCGAAGTACT-3'.

DNA amplification

To the 10 μl extraction preparations (REV DNA templates) the following are added: 9 μl 10X PCR buffer [500 mM KCL and 100 mM Tris-HCL, pH 8.3], 8 μl dNTPs [2 μl (0.2 μM) each of 4 nucleotides], primer *rev-1* [5 μl of a 50 μg/500 μl H_2O (10 μM)], primer *rev-2* [5 μl of a 50 μg/500 μl H_2O (10 μM)], 4 μl $MgCl_2$ (25 mM) and Taq Polymerase (2.5 units in 0.5 μl).

PCR cycling parameters

The first denaturation is at 95°C for 5 min. This is followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 47°C for 45 sec, and elongation at 72°C for 1 min (initially set for 1 min for the first cycle and then extended by 1 sec increments per cycle). A final elongation is set at 72°C for 10 min.

Analysis of amplified REV DNA by agarose gel electrophoresis

After amplification 18 μl samples of the PCR mixtures are each added to 2 μl of gel loading buffer (0.25% bromophenol blue tracking dye in 10 ml of 1M Na_2EDTA, 20% Ficol 400, 1% sodium dodecyl sulfate, and 0.25% xylene cyanol). Electrophoresis is carried out in 3.0% agarose gels (1% LER + 2% Nu Sieve) in Tris-Borate-EDTA (TBE) buffer with 20 μl ethidium bromide added to the melted agarose. The gel is run at 70 volts for 2 hours.

Analysis of Amplified REV DNA

The REV-PCR assay detected REV-DNA in the MSV, production seed, and vaccine serial of one USDA licensed fowl pox vaccine. The PCR products were the expected size (291 bp) for REV, based on agarose gel electrophoresis. We have tested 30 MSVs and 8 prelicense serials submitted to our laboratory since establishing this assay in 1995. The results of this testing showed that all samples were negative for REV-DNA by this PCR assay.

CONCLUSIONS

To improve product surveillance, we report the use of PCR for the detection of extraneous REV in live virus vaccines. Currently, all poultry vaccine MSVs submitted to CVB-L are examined

for extraneous REV prior to licensure. MSVs can be analyzed for extraneous REV-DNA by the PCR method described here. Biologics firms are encouraged to test new MSVs and MSVs from their currently licensed products for extraneous REV.

The use of REV-free SPF substrates is important in preventing REV contamination during vaccine production. However, serologic methods used to monitor SPF flocks may miss viremic birds that may be free of detectable antibodies. To improve biologic security, SPF firms could use REV-PCR to monitor their flocks for REV infection. PCR would detect an REV outbreak before serological responses could be detected, by that preventing the use of REV contaminated substrates for vaccine production.

Our laboratory has not conducted studies to compare the relative sensitivities of the different methods recommended by APHIS for detecting REV. This is an area that needs further investigation.

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DERMAL SQUAMOUS CELL CARCINOMA AND MULTICENTRIC HISTIOCYTOSIS IN CHICKENS

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INTRODUCTION

Dermal squamous cell carcinoma (DSCC) and multicentric histiocytosis (MH) are diseases of broiler chickens. Tables 1 and 2 show the important features and facts concerning these two diseases. Both DSCC and MH have been widely regarded as neoplasms; therefore, chickens are condemned at processing.

For DSCC, recent research reinforces findings from experiments done in the 1950s; namely, that DSCC is a regressing lesion of the feather follicle epithelium that appears to be grossly and microscopically similar to the benign tumor known as keratoacanthoma in human beings. Keratoacanthoma is a regressing benign tumor that grossly and microscopically resembles a shallowly-invasive squamous cell carcinoma. It should be noticed with interest that the keratoacanthoma was previously known as multiple self-healing squamous epithelioma (MSHSE) (9). The cause and cure for DSCC are not known.

For the disease recognized as MH, it is still not known if the proliferating cells are either neoplastic or hyperplastic. Recent work indicates that MH can be transmitted from naturally ill chicks to specific-pathogen-free Leghorn chicks and commercial broiler chicks. However, the cause and cure for MH are not known.

DERMAL SQUAMOUS CELL CARCINOMA

TABLE 1. Features and facts concerning dermal squamous cell carcinoma in chickens.

Feature	Facts
History	Since 1800s (1,3,4,7,8,19,22,28)[A] Various names: squamous cell carcinoma (SCC) (5,6,29,30) dermal SCC (5,6,20,29,30) carcinomatoid tumor (26) avian keratoacanthoma (13,14,23) keratoacanthomas are regressing tumors that arise from follicle epithelium and that grossly and microscopically resemble SCC (2,21)
Incidence/ distribution	Varies $< 0.01 > 0.10$ (14,20,29,30) ↓ in summer (14,30) ↑ in dusty environs (11) ↑ young birds (14) ↑ new houses (11) ↑ certain companies (11)
Etiology	Naturally-occurring lesion not known Experimentally: · carcinogen → regressing lesions SCC (25) · carcinogen → regressing lesions carcinomatoid tumor (26) · carcinogen → regressing lesions keratoacanthoma (24) · carcinogen + fowl pox + testosterone → SCC → regressed, metastasized (10)
Gross pathology	Lesions in live birds & carcasses appear similar (13,14): keratin-filled nodules and craterform ulcers occur in feather tracts
Micro pathology	Nodules = outgrowths of feather follicle epithelium (FFE) (13,14) = cystic FFE = hyperplastic FFE Cavitated ulcers are lined by squamous epithelium, filled with keratin Transmission electron microscopy = no virus seen (5,13)

TABLE 1 -- continued

Feature	Facts
Pathogenesis	Complete pathogenesis may not be known: FFE growth expands → forms nodule or cyst → infiltrates adjacent dermis → fibroplasia, inflammation → ulceration → central core is lost → regression to dermal scar → reepithelialization
Diagnosis and differential	Gross and microscopic pathology Ulcerative dermatitis
Prevention and control	None

[A]Numbers in parentheses are referenced literature citations.

MULTICENTRIC HISTIOCYTOSIS IN CHICKENS

TABLE 2. Features and facts concerning multicentric histiocytosis in chickens.

Feature	Facts
History	Since 1991 (12,16,17,18)[A] Various names because of gross appearance (12): "Big-spleen Marek's disease" (12,16,17,18) "Reticuloendotheliosis-like syndrome" (18) "Multicentric histiocytosis (MH)" (12,16,17)
Incidence/ distribution	Recognition/spread (12,17): 1991: Four states in the southeast U.S. 1992: Seven states from PA to ARK 1993: Eleven states from PA to TEX, MINN 1991-94: MH increased, surpassed diagnoses of Marek's disease Current incidence and distribution can not be known with certainty.

TABLE 2 -- continued

Feature	Facts
Etiology	Not known Experimentally: transmissible (15) Virus isolation/PCR results have been variable
Gross pathology	Natural and experimental lesions appear identical (12,15,16,17) small birds may be pale splenomegaly, hepatomegaly; miliary 0.5-to-5.0 mm nodules
Micro pathology	Natural and experimental lesions appear identical (12,15,17,18,27) anemia-low PCV (15,27) nodules in most organs consist of spindle cells with ample eosinophilic cytoplasm, oval to bizarre nuclei; use of markers to identify cell lineage underway (15) Transmission electron microscopy: no virus seen; proliferating cells appear to be histiocytes
Pathogenesis	Not known
Diagnosis and differential	Gross and microscopic pathology None
Prevention and control	None

[A]Numbers in parentheses are referenced literature citations.

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