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**Reduction of Reticuloendotheliosis in Foundation
Breeding Flocks of Chickens: A Combined
Immunological and Molecular Biological Approach**

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Reduction of reticuloendotheliosis in foundation breeding flocks of chickens:

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ABSTRACT

Reticuloendotheliosis virus (REV) is an avian retrovirus that can cause immunosuppression, growth retardation and tumors. An attempt to define the extent of the economic damage to the poultry industry that it causes is discussed in this report. In addition to losses experienced by commercial laying flocks, reduced rates of hatchability and embryo developmental disorders were demonstrable due to vertical transmission of the virus.

I. Eradication of REV

In this project a comprehensive national program was applied for the eradication of REV from Israeli breeding stocks by the elimination of antibody-positive birds from the breeding program. The prevention of REV-infected breeders entering Israel was also implemented by serological examination of imported day-old chickens and turkeys for maternal antibody. At the same time commercial breeding flocks in Israel were surveyed routinely to measure the extent of environmental exposure to REV throughout Israel.

II. Economic factors associated with vertical transmission on breeders and progeny

It was observed that on some poultry farms exposure of breeding flocks to viral infection, if it occurs when the birds are immunocompetent, leads only to a seroconversion event. In these flocks no differences were demonstrated between the performances of seronegative and seropositive birds. When the F1 generation was selected according to seronegativity of the parents, all the progeny were seronegative, indicating that tolerantly infected birds did not form a significant proportion of the parent flock. In sharp contrast, breeding flocks that became exposed to the virus about the point of lay or during the laying period, shed virus vertically for a brief period of time through the egg. Our epizootiological observations lead us to conclude that the progeny (laying pullets) becomes tolerantly infected and are immunosuppressed as they increase in age. Increased mortality and susceptibility to intercurrent diseases were recorded.

III. Comparison of Israeli and US isolates

We have compared the immunogenic proteins of Israeli isolates with those of prototype REV, notably chick syncytial virus (CSV). We have also demonstrated that some REV isolates have an increased pathogenicity for chick embryos, which is unconnected with their replicative ability in the embryo.

IV. Differential diagnosis

To develop sensitive and specific methods for differential diagnosis for virus-induced tumors in chickens and turkey the PCR and histochemistry using monoclonal antibodies were applied in Israel and the USA, respectively. In Israel, a large scale survey of chicken and turkey flocks in Israel was carried out, and in the USA controlled experimentally-induced tumors and some field cases were examined histologically. The need to intensify efforts in differential diagnosis by evaluating novel methods is justified because of the overlapping clinical findings and pathology in the four main tumor diseases of the chicken and turkey: Marek's disease, lymphoid leukemia, lymphoproliferative disease and RE. Moreover, diagnosis by virus isolation can be time-consuming, especially from the tumors themselves. Overall, the PCR was shown to be a very reliable method to differentiate between these viruses, and was especially valuable in cases of mixed infections, while the histochemical approach seemed very promising in single or in mixed infections.

GENERAL INTRODUCTION AND OBJECTIVES

At the onset of the program, it had been documented that serological evidence of REV infection of domestic poultry seemed to be widely disseminated in countries with poultry industries, although chronic neoplastic diseases were infrequently recognized in chickens and turkeys. On the basis of reported clinical disease, the economic importance of RE in turkeys was considered to be minor and in chickens, negligible (Witter, 1991). However, under experimental conditions, field isolates were described to be highly immunodepressive and oncogenic (Witter and Johnson, 1985). In 1990 we initiated a study of the prevalence of REV infection in commercial flocks and found many examples of infection, as well as various forms of the disease, ranging from immunosuppression through neoplastic disorders. Based on these findings the objectives of the original proposal were:

- I. Establish an effective program for the eradication of REV from Israeli breeder flocks based on the identification and elimination of infected breeders and the protection of progeny from horizontal infection.
- II. Evaluate the economic impact of vertically-transmitted REV infection on production traits through development of pilot infected and uninfected breeder flocks and evaluation of both breeders and progeny.
- III. Compare biological properties of US and recent Israeli isolates by infectivity experiments on embryos and on day old birds.
- IV. Evaluate the effectiveness of different tests for the differential diagnosis of lymphoid tumors of viral etiology in commercial chicken flocks.

The final report is divided into sections which parallel the objectives of the project. In section I we describe the General Methods used throughout the project, and in each section, the specific Methods including experimental designs that were used. In each section we include an introduction and discussion.

Final Research Report

Section I: Eradication of REV from Israeli breeder flocks

Introduction

To adopt an eradication program, an initial survey has to include a method of identifying infected breeding flocks, from which positive birds can be removed from the breeding population. Because of the large numbers of flocks involved, very simple tests are needed, and the ELISA has served to detect REV antibody positive birds. Infection with retroviruses normally leads to production of antiviral antibodies after an "window" time interval, when viremia is present but antibody levels are not yet detectable. Four combinations are possible: bird can be positive both virologically and serologically, positive for either the virus alone (tolerance), specific antibodies or negative for both. In order to fulfill the present objective several serological examinations were performed on the same birds, over an extended period of time, if they were negative at the first examination. This will eliminate the possibility of tolerant infection. In view of the findings that some imported breeding flocks were massively infected with REV the Israeli Veterinary Services introduced a regulation requiring that all importations be tested serologically. Only REV-antibody negative flocks, denoting that the parent birds were not exposed to REV in the country of origin, are allowed to enter Israel for breeding purposes.

In parallel, routine examination of chicken and turkey breeding flocks is being done at KVI in order to monitor the possibility of horizontal infection in certain geographical localities.

General Methods

Isolation of REV in tissue cultures

Birds were bled aseptically from the wing vein into heparin as anticoagulant. When PCR was also performed, the anticoagulant was 2% EDTA. The blood sample was lightly centrifuged and the plasma was separated into two fractions: one, as enriched buffy coat and, two, as a

leukocyte fraction. The first fraction was processed to isolate the virus by mixing it with freshly trypsinized chick embryo fibroblasts (CEF) from specific pathogen free (SPF) embryonated eggs in 60 mm plastic dishes and cocultivated for 7 days with medium changes as necessary. The medium used was Leibovitz and McCoy (1:1) supplemented with 4% fetal calf serum (FCS) until monolayers were formed, and 1% FCS, afterwards. The monolayer was then frozen and thawed three times and the resulting lysate was mixed with fresh CEF and seeded into new dishes containing glass cover slips. After a further 7-day incubation period the cover slip was removed and fixed in cold acetone. The growth of REV was determined by indirect immunofluorescence (IF) using a monoclonal antibody to REV.

REV antibody detection by ELISA

The ELISA was performed as described previously (Davidson et al, 1985). Briefly, polystyrene plates (Nunc, Denmark) were coated (18 hr at 4°C) with 100 µl of REV-infected CEF lysate and with uninfected CEF lysate, diluted 1:400 in 0.05M carbonate-bicarbonate buffer, pH 9.6. After 3 washes in phosphate-buffered saline (PBS) containing 0.05% Tween 20, sera were diluted 1:400 in PBS containing 0.1% bovine serum albumin and were then incubated for 1 hr at 37°C. After 3 additional washes, alkaline phosphatase-labelled rabbit anti chicken IgG conjugate was incubated for 1–2 hrs at 37°C. Finally, 1 mg/ml of substrate (p-nitrophenyl phosphate; Sigma) in 10% diethanolamine buffer (pH 9.8) was added and absorbencies were read in an ELISA reader. A reaction was considered positive when the optical density difference (O. D. D.) of the readings of a serum reacted with the positive and negative antigens was >0.1. In each plate positive and negative control sera were included and the reaction was read when the positive serum reached an a O.D.D. of approximately 0.6.

REV antibody detection by IF

Determination of REV-Ab in the plasma of day-old chicks was performed at a 1:20 dilution of plasma. Uninfected and REV-infected CEF were trypsinized, washed in PBS and dotted on

microscope slides. After fixation in acetone, diluted plasma were reacted for 30 min. on both cell types. After 3 washes in PBS, FITC-labelled rabbit anti chicken IgG diluted 1:32 was reacted as before. Following a further wash, the slide was counterstained with 1% Evans blue, washed in distilled water and a drop of 90% glycerol (pH 8.0) applied before covering. The slides were examined in a Nikon epifluorescence microscope.

Polymerase Chain Reaction (PCR)

DNA from whole blood was extracted according to Sambrook et al (1989). The primers used for the detection of REV were previously described (Aly et al, 1993; Davidson et al, 1995) and were based on sequences of the proviral SNV-LTR (Shimonoto et al, 1980):

Upper primer: CAT ACT GGA GCC AAT GGT T

Lower primer: AAT GTT GTA CCG AAG TAC T

The expected amplified DNA band size is 291 bp spanning the entire length of the unique 5' (U5), repeat (R) region and includes 118 bp of the unique 3' (U3) of the LTR.

Total DNA (1ul) was mixed in a 25 ul PCR reaction with 10 mM Tris-HCL (pH 8.5), 50 mM KCl, 1.5 mM MgCl₂, dATP, dCTP, dGTP and dTTP each at 200 uM, primers (1.0 uM each) and 0.5 units AmpliTaq DNA polymerase (Perkin Elmer). PCR cycling parameters were 31 cycles of 1 min at 94°C, 55°C for 1 min and 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. REV mixtures lacking templates were routinely included as negative controls. Positive control was included with SNV-DNA as the template.

Results and Discussion

A. Eradication of REV based on the identification of infected grandparents (broiler breeds) and grandparent (light breeds) and their elimination from the breeding program

This objective required the establishment of a comprehensive screening program covered the foundation flocks of the primary breeding organization – the Poultry Breeders Union (PBU). In the first year of the program all the g.g.p. female lines housed on their separate farms were

examined. In addition, the four foundation g.p. lines of light breeds that were located on one farm were also bled. During the second year of the program the F1 generations of the following flocks were thereafter tested using ELISA alone or in conjunction with virus isolation in cell culture (viremia) and the PCR on whole blood DNA.

1. Light breeders

The four primary breeding lines belonging to PBU were housed in individual cages (hens) or caged in groups of two to four (roosters). The flock is located on one farm (M. H.) in a shed housing over 7000 birds. In October and November 1992, 80 hens and 20 roosters from each line were bled and none of the 400 birds was viremic. The cell-free fraction from each bird was analysed by ELISA for REV antibodies. Six of the 400 plasmas were weak positives. These were retested at 1:20 by IF and none was positive. In August 1993, the same farm was reexamined, this time after being repopulated with a fresh foundation flock. Both sexes of all four lines were bled and examined by ELISA. None of the 80 birds tested had antibodies to REV.

In November 1993, the same breeding farm that had housed the parent lines, now housed the F1 generation. Whole blood was taken from a total of 60 hens and examined as above. Four birds were REV antibodies positive and two were PCR positive. No viremic birds were detected. Although the flock was examined several times over a period of three months and individual birds were retested, no evidence of lateral spread of infection was established. No evidence of vertical transmission of REV through the egg was demonstrable using the antigen trapping ELISA on egg albumen.

2. Heavy (broiler) breeders

The PBU maintains the female line dams in individual laying cages and their sires are housed similarly in small groups. The male line foundation flock is kept on litter. Throughout 1993, broiler breeders located on five separate farms were tested virologically and serologically.

Four flocks tested negative throughout. The fifth flock that comprised two sub-lines was bled in August 1993 and found to have a high proportion of seropositive birds in each subline (45%). The numbers bled were 179 and 459, respectively. When the index of performance (body weight, age at 1st egg, % egg production) was calculated for each serological status, no significance differences were found for each subline. 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. The F1 progeny of these matings were examined throughout the 2nd year using ELISA. Day-old chicks and the F1 breeding flock, newly housed and now in-lay, were tested extensively for REV antibodies. In June 1994, 80 birds of the two sub-lines were shown to be ELISA negative. It should be noted that this new flock was located on the same farm as its parents, but was now housed on litter. Therefore, there was no evidence for lateral or other external sources of REV infection on these premises.

Two additional farms with female broiler breeder lines belonging to the PBU were surveyed. On one farm, over 100 birds each of 3 sub-lines were bled. None was REV antibody 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, the PBU decided to eliminate this flock.

3. Broiler breeder-sire lines

Day-old chicks of two male sub-lines - "Cornish", were examined serologically. The birds originated from two separate flocks. No evidence of passively acquired antibodies was found.

Conclusion

On the basis of the extensive survey of the PBU's 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.

B. Examination of imported day-old birds

In 1993, the Israeli Veterinary Services introduced a regulation requiring that all importations be tested serologically for REV antibodies. Table 1 summarizes the results of testing imported day old chicks and turkeys in the period 1993–1995.

Table 1: ELISA examination of imported day-old flocks

Year	1993		1994		1995	
Flocks	No. +ve flocks/Total	Total tested	No. +ve flocks/Total	Total tested	No. +ve flocks/Total	Total tested
Chickens	1/15	150	2/15	295	2/24	520
Turkeys	NT		NT		0/7	127

NT – Not tested

Table 1 shows that the number of imported day-old flocks increased annually and in 1995 the imported turkey flocks were included. Occasional positive flocks were detected each year, and these were destroyed by statutory order. Some had been imported from W. Europe and others from USA.

C. Examination of local breeding flocks

From November 1993 periodical examinations of chicken and turkey breeding flocks are performed at KVI to monitor other possible sources of infection through breeding.

Table 2 shows the monthly examinations done in November 1993 through April 1994 on chicken flocks expressed as the number of positive birds over the total number of birds examined from each flock. The table shows that in 7 flocks some birds were seropositive. The results demonstrated that a more comprehensive study was needed to monitor the situation in Israel, therefore the number of flocks increased markedly in the following year.

Table 2: ELISA examination of breeding flocks in 1994

Flock	November 1993	January 1994	February 1994	April 1994
1				0/5
2				0/5
3				0/5
4			2/10	1/5
5				1/4
6			3/10	0/5
7				2/4
8				4/4
9				0/4
10				0/4
11			0/10	
12			7/10	
13			1/5	
14		0/9		
15		1/12		
16		0/10		
17		0/16		
18	0/8			
19	0/8			
20	0/8			
21	0/8			
No. of +ve flocks/ No. examined	0/4	0/4	1/5	1/10

Table 3: ELISA examination of chicken breeding flocks in 1995

Flock	March 1995	May 1995	June 1995	July 1995	August 1995	September 1995	November 1995
1A			0/6	0/9	0/9	0/9	0/10
1B		0/16		4/8			
2			0/6		8/8	7/7	
3			0/6		1/9	0/9	0/10
4						0/9	0/10
5A	0/9		0/6	0/8			0/10
5B						0/9	
6A		0/13	0/12	0/9	0/9	0/9	
6B				0/9			
6B	0/13			0/9			
7	0/20		0/6	0/9	7/7	9/9	10/10
8	0/20		0/8		0/8	1/18	2/11
9				0/9	1/9	5/9	4/10
10					0/8		0/11
11			0/6			0/9	0/10
12A	0/20	0/16	0/12	0/9		0/9	0/11
12B			0/6		0/9	0/9	0/10
12C	0/13				0/9	0/9	0/10
13A				0/9	1/9		
13B			0/6				8/11
14						0/9	
15A	0/20	0/16	0/6		0/8	0/9	0/11
15B	0/19						0/11
16	0/19			0/9	0/8	1/18	1/11
17							0/11

18	14/47			9/27	0/15	0/25	0/11
19				0/8	8/8	7/9	
20					0/7	0/9	0/10
21						0/9	
22	0/22						0/24
23					0/8		
24					0/8		0/10
No. of +ve flocks/ No. examined	1/11	0/4	0/9	2/11	3/18	4/20	2/21

Table 3: continued

Table 3 show the results of the survey done in 1995. As many as 10 out of the 24 flocks were REV positive. However, only in 6 was a high rate of seropositivity observed (Flocks no. 2, 7, 9, 13B, 18 and 19). A seasonality effect of late summer was notable.

Table 4: ELISA of turkey breeding flocks performed in 1995.

Flock	October 1995	November 1995
1	0/11 females	0/10
2	0/11 males	1/11
3	0/10	0/9
4		0/10
5	0/4 females, 0/9 males	

Table 4 shows that all the turkey breeding flocks examined were seronegative.

Conclusion

Of the flocks bled at fairly regulated intervals in the second half of 1995, three out of 18 appear to have undergone seroconversion in this period.

D. Protection of progeny from horizontal infection

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 5 month old laying pullets in the first part of 1994. In February and May 1994, two episodes of neoplastic disease were diagnosed in birds that had been hatched as two batches. The birds belonged to the same commercial group, and were from the same breeding farm although not from the same breeding flock. The two parent flocks had been imported as day-old chicks which were found to be free of passive antibodies on that day. Because of the unusual epizootiology features of the two episodes, an effort was made to investigate them as fully as possible, viz, by serology, virology, PCR and histopathology.

In the first episode, five poultry farms were involved (Table 5). In all instances the birds were raised from day-old (the same hatch date). During the growing period, one of the flocks suffered excessive mortality due to fowlpox. At the point of lay, a tumor problem was diagnosed in all 5 flocks with mortality rates of between 0.5% and 2% per week. This attrition persisted for about 6 weeks into lay, although the laying rate was not unduly affected. Table 5 shows that all 5 flocks were seropositive, although virus was not isolated from the peripheral blood of any of the birds. In contrast, 6 of the 24 blood DNAs from the sick birds (with tumors) were PCR positive for REV. An additional 3 blood samples had MDV templates while 5 other DNAs had templates of both viruses. No MDV-DNA was found in the 13 healthy control bled as in-contact controls, but 4 were positive for REV (the same proportion as for the sick group). When tumor DNA was examined by PCR, 6/34 were MDV positive, 2/34 were REV positive and one tumor DNA had both templates.

In the second episode over 20,000 replacement pullets were raised from day-old on one farm and distributed among 19 poultry farms, several weeks before they were due to lay. These farms were located within a 150 km radius of the grower farm, and most were 40 km

away. Of the 19 laying flocks, 6 were affected enough clinically for the poultrymen to seek veterinary assistance. The complaint took the form of excessive mortality in the 1st month of lay (2% on average). Table 6 shows that 5 of these flocks were REV antibody positive and in two of them, PCR examination of blood DNA was positive for both REV and MDV.

Histopathological examination of tumors from both episodes revealed infiltrations typical of MD, RE and in some instances LL (In some birds bursal tumors were found).

Discussion

Examination of the records of the two parent flocks showed that the first flock was in peak production at the time when the eggs of the first REV-affected hatch were set. However, the second flock was at an early stage of its laying cycle. Both flocks had been bled at monthly intervals and tested for REV antibodies. In the first instance, all the samples were strongly positive, while in the second, the flock was experiencing a seroconversion event. The epizootiological significance of these findings is accentuated by further examination of other flocks that were raised in previous years in the same poultry houses; all were found to be REV antibody positive and the seroconversions occurred during the late summer-fall months.

Conclusions

These observations have led us to conjecture that RE virus may persist in favorable ecosystems, in as yet, undetermined animal hosts. Recently introduced flocks are being followed with the expectation that the event will repeat itself and the horizontal phase of infection of these parent flocks can be detected.

Further studies are needed in order to understand the extent of horizontal transmission of REV and the biological vector/s that are involved. It is also important to focus on the role of mechanical transmission of REV by arthropods, especially in the light of the study of Motha et al (1984) who described virus transmission by mosquitoes in Australia, and the fact that the specific farm under discussion is located in the vicinity of orange groves, irrigated areas and

also the seasonal seroconversions have occurred there each year.

Episode I

Table 5: Diagnosis of tumor-bearing chicken flocks, progeny of one REV antibody positive parent flock

Flock	Health status	REV-Abs ELISA	PCR-Blood DNA			PCR-Tumor DNA			Histopathology
			MDV	REV	MDV+REV	MDV	REV	MDV+REV	
1. C (6 mo.)	Sick	3/3 (*)	1/3	0/3	0/3	0/5	0/5	0/5	RE
	Healthy	5/5	0/5	1/5	0/5	NA	NA	NA	
C (7 mo.)	Sick	3/3	0/3	2/3	0/3	0/4	2/4	0/4	RE/LL; RE
2. Q (6 mo.)	Sick	6/6	3/6	0/6	0/6	2/5	0/5	0/5	MD, RE, RE/LL
Q (7 mo.)	Sick	3/3	1/3	0/3	0/3	NT	NT	NT	RE
	Healthy	4/4	0/4	1/4	0/4	NA	NA	NA	
3. WB (6 mo.)	Sick	6/6	1/6	1/6	4/6	3/8	0/8	0/8	RE/LL
	Healthy	4/4	0/4	2/4	2/4	NA	NA	NA	
4. UD (6 mo.)	Sick	3/3	0/3	0/3	1/3	1/12	0/12	1/12	RE
Total									
	Sick	24/24	6/24	3/24	5/24	6/34	2/34	1/34	
	Healthy	13/13	0/13	4/13	2/13	NA	NA	NA	

(*) – REV was not isolated in tissue culture.

NT – not tested; NA – not applicable

Episode II

Table 6: Diagnosis of tumor-bearing chicken flocks, progeny of one REV antibody positive parent flock

Flock	Health status	REV-Abs ELISA	PCR-Blood DNA			Histopathology
			MDV	REV	MDV+REV	
1. YT (8 mo.)	Sick	4/4 (*)	0/4	1/4	0/4	RE (x2), LL, mixed erythromyeloblastoma
2. BY (8 mo.)	Sick	4/4	0/4	0/4	0/4	NT
3. GJ (8 mo.)	Sick	4/4	0/4	0/4	0/4	NT
4. KZ (8 mo.)	Sick	4/4	0/4	1/4	0/4	RE
5. KJ (8 mo.)	Sick	3/3	0/3	1/3	0/3	RE, MD
Total						
	Sick	19/19	0/19	3/19	0/19	

(*) – REV isolation in tissue culture was not performed

NT – not tested

Section II: **The economic impact of vertically transmitted REV infection**

A. Experimental infection of adult laying birds

Introduction

Both horizontal and vertical modes of virus transmission are recognized for REV. In this experiment we investigated viral transmission in experimentally infected adult hens. In particular, we focused on transmission to the progeny of non-tolerantly infected breeding chickens, which may occur to the progeny of commercial poultry exposed to horizontal transmission by insects.

Vertical transmission has been reported as being uncommon (Witter, 1991). The exception being in a commercial turkey breeder flock, where only a transmission rate of 1.8% was detected among 46% viremic birds (Witter and Salter, 1989). In an earlier study, Witter et al (1981) had found that eggs and progeny chicks from non-tolerantly infected hens were always negative for virus and group specific (gs) antigens.

Vertical REV transmission from tolerantly-infected birds has been reported to occur at very low rates. McDougall et al (1980) isolated REV from 2 of 25 embryos from tolerantly infected turkey hens. Very low rates of viral shedding and transmission have been demonstrated also in tolerantly infected chickens, even in spite of using SPF birds (Bagust and Grimes, 1979); Bagust et al., 1981). However, Motha and Egerton (1987) described efficient REV transmission from tolerantly infected laying type hens when the eggs were incubated within 24 hours of lay, which is not a very common practice in the field.

We used PCR for the detection of REV-LTR (Aly et al., 1993) as this method is more sensitive than virus isolation for detecting REV infection (Davidson et al., 1995). In parallel, virus isolation was attempted.

The REV-antibody (REV-Ab) levels in the adult hens and the transmission of maternal REV-

Abs to the progeny were followed at weekly intervals. Gs antigens in egg albumen and feather follicle extracts (FFE) were analysed to evaluate their sensitivity as diagnostic materials. No infectious virus was reisolated from the adult hens, but REV-LTR was detected by PCR. REV was transiently detected in the progeny over two weeks. REV-Abs were produced in the hens and were transmitted to the chicks over two weeks. Very little gs antigen was found in the egg albumen and none in the FFE.

Materials and Methods

Chickens and Viruses

Six months old Lohman strain of layers were used. They were REV-Ab negative before injection with the REV-M isolate that was isolated from 3 months old replacement laying pullets (Davidson et al., 1995).

Experimental design

Twelve hens and one male were injected with 0.5 ml medium of a cell culture infected with REV-M whose titer was 10^{-4} . The birds were bled in 2%EDTA on the day of injection and at weekly intervals through five weeks. The plasma served for REV-Abs determination by ELISA and IF, whole blood for extraction of DNA for PCR and the buffy coat for inoculation of CEF for REV reisolation. A few feathers were plucked from each bird weekly, the tips were cut in TE buffer (1mM EDTA, 10 mM Tris HCL, pH 8.0) and the gs antigens were extracted by sonication. The eggs were collected daily. A sample of 0.5 ml albumen was taken, then the eggs were sealed, stored at 18°C for 1–6 days and incubated at weekly intervals. all the hatched chicks were bled and ELISA, IF and PCR were performed.

Virus isolation in tissue cultures, Polymerase chain reaction, REV antibody detection

The methods are described in Section I

REV gs antigen detection in egg albumen and in FFE

Polystyrene plates were coated with a mixture of monoclonal antibodies (mAbs) 11A25 and

11C37 (received from Dr. LF Lee, ADOL, MI, USA). The mAbs were diluted at 1:1000 in carbonate-bicarbonate buffer. Buffers and wash procedures were as described for REV–Ab ELISA. Egg albumen samples (0.1 ml) were placed in the wells and reacted for 18 hr at 4°C. Following 3 washes the IgG fraction of rabbit anti REV serum (received from Dr. R. L. Witter, ADOL, East Lansing, MI, USA) at a concentration of 20 ug/ml was reacted for 1 hr at 37°C. The next steps were the reaction with goat anti-rabbit IgG (diluted 1:1000) and the substrate, azino di-3-ethyl-benzthiazoline sulphonate (ABTS) (Sigma) at 1 mg/ml in 0.1M citrate-0.2M phosphate buffer pH 4.0, supplemented with 0.003% H₂O₂.

For the determination of gs antigens in FFE, the same ELISA system was used except to place 0.1 ml of FFE extract in the wells (Davidson et al, 1986).

Results

Detection of REV and REV-antibodies in hens

To analyse the infection of the hens with REV the birds were examined for virus and REV–Abs in their plasma. REV was not reisolated from any bird throughout. In contrast, the REV–PCR was positive for birds #91 and #92 at 12 and 14 days p.i., respectively (Table 7). The plasmas of 12 hens and one rooster were analysed weekly over 35 days p.i. by ELISA for the presence of REV–Abs (Table 7) and few hens were bled at 70 days p.i. for REV–Abs. As seen in Table 7, the production of REV–Abs began at 14 days p.i. in almost all the hens and their levels rose throughout 35 days p.i. At 70 days p.i. even higher levels of REV–Abs were detected in the 4 birds sampled. Bird #91 died at 12 days p.i. No seroconversion was observed in the rooster serum.

The hatchability and fertility of eggs

Infection of hens did not influence the number of eggs laid in each of the 5 weeks p.i., and between 57–61 eggs were laid each week (Table 8). In contrast, the fertility of the embryonated eggs was affected, as indicated by the number of infertile eggs (Table 8). The

initial and final infertility percentages in weeks 1 and 5 were approx. 20%. This level rose to 46% during by week 3 p.i. while the number of dead embryos was about the same throughout. The percentage of chicks that hatched declined from 63% in week 1 to 37% in week 3, but recovered to 73% in week 5 p.i.

Table 7: REV detection and REV-antibodies in adult hens

Day p.i.	0	7	14	21	28	35	70
O.D.D. – positive control	0.68	0.68	0.68	0.60	0.65	0.68	0.55
Bird #83F	0	0	0.08	0.19	0.25	0.44	0.72
#84F	0	0.03	0.10	0.26	0.34	0.39	NT
#85F	0	0	0.08	0.24	0.19	0.25	NT
#86F	0	0	0.06	0.08	0.15	0.26	NT
#87F	0	0	0.05	0.08	0.14	0.36	0.55
#89F	0	0	0.07	0.17	0.21	0.35	NT
#90F	0	0	0.12	0.42	0.40	0.69	0.73
#91F	0	0*	Died				
#92F	0	0	0.05*	0.31	0.19	0.31	NT
#93F	0	0.01	0.05	0.09	0.11	0.32	NT
#94F	0	0	0.06	0.14	0.19	0.41	0.72
#95F	0	0.02	0.07	0.21	0.15	0.12	NT
#96M	0	0.01	0.02	0.05	0.05	0.07	NT

NT – not tested * – REV-PCR positive; F – female; M – male

Table 8: Fertility of eggs and hatchability of embryos

	Weeks post infection				
	1	2	3	4	5
No. of eggs laid	57	58	59	61	59
No. of infertile eggs (%)	14 (24%)	22 (38%)	27 (46%)	21 (34%)	11 (19%)
No. of dead embryos	7	4	10	9	5
No of hatched chicks (%)	36 (63%)	32 (55%)	22 (37%)	31 (51%)	43 (73%)

Detection of REV and gs antigen in day old chicks

Vertical transmission was determined by conducting REV-PCR on whole blood DNA (Table 9). PCR positive chicks were analysed for 4 weeks p.i., and were detected commencing at day 12 p.i. The ELISA performed on egg albumen samples was occasionally positives on days 6, 15, 16, 23 and 26 p.i. Examination of FFE for REV gs antigens was negative throughout.

Table 9: REV infection of day old chicks

Weeks post infection	1	2	3	4	5
No. of REV-PCR positive chicks (%)	NT	1/23 4.3%	8/21 38%	8/31 26%	NT
No. of REV-Ab (ELISA) positive	0/25 0%	0/23 0%	9/21 43%	9/31 29%	2/42 5%
No. of REV-Ab (IF) positive	0/24 0%	2/14 14%	20/21 95%	16/24 67%	10/42 24%
REV antigenemia in egg albumen	1/43 2.3%	0/35 0%	2/53 4%	2/26 7.7%	0/16 0%

NT – not tested

Detection of REV-Abs in the blood of the day old chicks

The day old chicks were bled as they hatched and maternal REV-Ab level were determined by ELISA and IF. Commencing at day 15 p.i. REV-Abs appeared in the blood but their levels declined gradually until they were undetectable on day 32 p.i. Since the titer of REV-Abs in the chicks' blood was much lower than in the hens' blood, we also determined the presence of REV-Abs by IF. Positive samples commencing 12 days p.i. through 32th day p.i. were detected.

Discussion

In the present experiment we show that REV can be transmitted vertically from non-tolerantly infected adult hens. To our knowledge very few studies have been performed on such chickens, an exception being of Witter (1981), who found no evidence that it occurs. The importance of the vertical transmission to the progeny rose lately because of the demonstration of REV infection in commercial birds (Davidson et al, 1995). In contrast to previous studies where REV infection was demonstrated by virus isolation, in this experiment we used PCR for detecting the virus. We cannot exclude the possibility that only REV-LTR sequences were present in the chick blood because we did not attempt to reisolate virus, however our attempts to isolate REV from the adult hens were also unsuccessful. In this instance, the REV-PCR was positive, indicating that the PCR is more sensitive than virus isolation. The production of REV-Abs in the hens and the transfer of maternal REV-Abs to the chicks demonstrated that infection has taken place. We can assume, that infectious virus was also transmitted to the chicks because gs antigen was detected in a low percentage of egg albumens.

Based on the findings of Motha and Egerton (1987) who demonstrated the presence of REV in day old blood, whole blood DNA was checked for REV sequences by PCR. The vertical mode of REV transmission described previously was based on detecting infective virus in cloacal swabs (Witter et al, 1991). Here, we have demonstrated REV-LTR sequences in the whole blood DNA. In parallel with the transient expression of REV-LTR for a 3 days period (days 12–14 p.i.) in the hens, REV-LTR in the hatched chicks were detected commencing at day 12, during the third week p.i., and declined afterwards. This may indicate a higher rate of REV transmission than originally thought in commercial breeder flocks.

REV gs antigens in the egg albumen were detected sporadically in parallel with REV-LTR detection in the chicks, albeit at very low levels. It therefore seems unfeasible to apply an

REV eradication program to breeding flocks on the basis of an egg albumen assay for gs antigen. This contrasts with LLV gs antigens which are abundant in the egg albumen of LLV-infected laying hens (Spencer et al, 1976).

The infertility data and the viability of the day old chicks were affected in this experiment and these results should be included as sequelae to REV flock infections.

B. Economic impact of vertically transmitted REV in breeding flocks

Observations on the impact of vertically-transmitted REV on economic traits were made throughout the 2nd year of the project on affected flocks. These included two female sublines of broiler breeder g.g.p.s of which 45% were seropositive. In these flocks no differences were demonstrable between seropositive and seronegative subgroups regarding body weight, egg production (% laying) and the age at which the 1st egg was layed. These parameters were measured while the birds were being selected for breeding.

Because of economic problems, the PBU was unwilling to set aside an infected flock for experimental purposes. In the meanwhile, the performances of other broiler breeding flocks that are located on "REV high-risk" farms were observed.

Vertical transmission of REV was suspected in two episodes involving laying pullets that were described above. In these flocks the overall losses were confined to the period of onset of lay, when mortality due to neoplasia occurred. In one flock an outbreak of fowlpox was diagnosed during the growing period, while in other flocks facial abscesses affecting some birds and unlinked to neoplasia were seen. On average, the mortality in these flocks was about 2% per month. Later into the laying season, an occasional bird with neoplastic lesions could be found. In most affected flocks no complaints were made concerning their laying performance.

C: The pathogenicity of Israeli isolate M-91

The objectives of this experiment were to estimate the pathogenicity of one Israeli isolate (M-91), to determine its immunosuppressive activity in chickens immunized and challenged with Marek's disease virus (MDV) and its immunosuppressive effect on the serological response of birds vaccinated with four inactivated viral vaccines.

Methods

Experimental design

Six-days old embryonated specific pathogen free eggs were inoculated with M-91 into the yolk sacs. On the day of hatching, part of each group was immunized with MDV vaccine strain Rispens. Ten days later MDV challenge virus (strain Y/91) was injected into four subgroups: those inoculated with REV as embryos; REV-inoculated + MDV-vaccinated; uninoculated + MDV-vaccinated chickens and uninoculated controls. In addition, a fifth subgroup (REV + MDV + Rispens) was left unchallenged at day 10; a sixth subgroup (REV+) that was neither vaccinated nor challenged, and a seventh subgroup (REV-) that was neither vaccinated nor challenged. When the birds were 23 and 67 days old, all were vaccinated revaccinated, respectively with a quadrivalent, inactivated, oil-adjuvanted vaccine containing the following viruses; Newcastle disease (NDV), infectious bursal disease (IBDV), reovirus (viral arthritis-VA) and infectious bronchitis (IBV). The experiment was terminated when the birds were 1-year old and during this time they were bled and weighed at frequent intervals over the first 160 days of the experiment. The following parameters were measured: body weight, isolation of REV from the peripheral blood, measurement of the immune response to REV, IBDV, VA and IBV by ELISA and IF (REV only), measurement of the immune response to NDV by hemagglutination-inhibition (HI). In addition, all sick and dead birds were necropsied and their organs examined histologically.

Results

Body weights: Overall, the chickens that received the two field viruses (REV and MDV) weighed less than all the other treatment groups. However, those receiving REV alone weighed less than those receiving MDV alone.

Morbidity and mortality: All REV+ groups eventually died at a slow uneven rate whether they were immunized or not with MDV-Rispens and challenged or not with MDV-Y/91. In contrast, morbidity in the MDV challenge subgroup was virtually complete by the 4th month. In effect only one bird died (out of 14) in the subgroup immunized and challenged with MDV, whereas all 10 REV+ birds succumbed. The clinical findings in birds dying from mixed infection ranged from neoplasia to emaciation; some were also paralysed.

Viremia: In the subgroups receiving REV alone or in combination with MDV, a viremia was registered throughout the experimental period, no REV antibody was detectable in these birds.

Serology: Compared to the control subgroup that received only the inactivated vaccine viruses, the birds that were REV+ and REV+Rispens had unaffected primary responses to NDV and VA. However, the secondary response to VA, as well as both the primary and secondary responses to IBDV and IBV were depressed. In contrast, the secondary response to NDV remained intact. Overall, the groups vaccinated and challenged with MDV demonstrated the least immunosuppression and especially those challenged by contact. Unvaccinated, MDV-challenged birds were extremely immunodepressed, although only the secondary response to NDV of the contact-challenge group was undetectable. Also, the subgroup receiving both virulent viruses retained a normal primary response to NDV.

Discussion

The effects of this non-defective REV isolate on the bird were quite profound. First, all the birds pretreated as embryos eventually died after challenge with the moderately virulent field strain of MDV. These birds were REV-tolerant throughout and had a markedly reduced

antibody response to two of the four viral components of the vaccine. This is in sharp contrast with the excellent protection displayed by the conventionally vaccinated+challenged group in which only one bird died. The moderate pathogenicity of the REV isolate also manifested itself in the prolonged death time and in the observation that several REV-tolerant pullets layed several eggs before eventually dying. The preferential destruction of T and B subsets by REV including memory cells of both types would be a possible explanation for the differential response to the four viral antigens. The induction of prolonged immunological tolerance to REV indicates that this phenomenon is not accompanied by absolute immunosuppression. The other pathological feature of REV infection, viz., reduced growth rate, paralysis and eventually emaciation point to a more generalized infection of the host in the absence of tumor formation.

Section III: **Comparison of the biological properties of US and Israeli isolates**

A. Pathogenicity and viral replication in chick embryos

Reticuloendotheliosis viruses (REV) are avian C-type retroviruses that can transform pre-B and pre-T lymphocytes and cause B- or T-cell lymphomas, growth retardation and immunosuppression in susceptible species of birds. The REV group of viruses are immunologically, morphologically and structurally distinct from the leukosis/sarcoma group of avian retroviruses (reviewed by Witter, 1991). The REV group includes the replication-defective prototype strain T virus (REV-T) (Robinson and Twiehaus, 1974), a replication-competent member isolated from transforming strain-T stocks (REV-A) (Hoelzer et al, 1979), chick syncytial virus (CSV) (Cook, 1969), duck infectious anemia virus (DIAV) (Ludford et al, 1974), duck spleen necrosis virus (SNV) (Trager, 1959), and other strains isolated from turkeys (Paul et al, 1977), pheasants (Dren et al, 1983) and ducks (Li et al, 1983). All members of the group are antigenically related and indistinguishable by immunofluorescence (IF) using convalescent chicken serum (Purchase et al, 1973).

Few reports have been published regarding the pathogenicity of these REV prototypes, and to our knowledge no comparative studies have been performed among REV prototypes and isolates.

Purchase et al (1973) studied the pathogenicity of SNV, DIAV and CSV for chickens and ducks and found that the first is the most pathogenic of the three, whereas the last is the less pathogenic of the three. Theilen et al (1966) that described the pathogenicity of REV-T for chick embryos found an increased death of chick embryos proportional with the virus amount and also described lesions in infected embryos that were similar to those found in REV infected chickens. Motha (1987) described the pathogenicity of the Q 1/77 non-defective REV strain inoculated into 6 day-old embryonated eggs. The main effect was the reduced

hatchability, a reduced body weight of REV-infected chickens, feathering defects, viremia and antigenemia.

Our aim in this section was to characterize the comparative pathogenicity of three Israeli REV isolates and three REV prototypes (CSV, SNV and REV-T) for chick embryos and to relate it to the replicative properties of the viruses in the various organs of the embryos. The viral replication was surveyed by the recently described PCR method for the amplification of the REV provirus (Davidson et al, 1995) and by the organ print method followed by immunofluorescence (IF) detection using monoclonal antibodies to REV (Davidson et al, 1994). We could demonstrate that two of the three local isolates possess the property of extensive replication in vivo, where only one of them have an increased virulence for embryos of two sources. All three REV prototypes replicate to a moderate level in the embryos, but REV-T retains its well known virulent capacity in embryos of both sources.

Materials and Methods

Embryonated eggs

Two types of embryonated eggs were used; eggs from a SPF breeding flock (SPAFAS, Norwich, CT) and eggs from a clinically normal commercial Leghorn flock. The eggs were kept for 19 days in a heated, humidified, rotating incubator and transferred to a hatching incubator for the three last days of incubation.

Eggs were injected with sterile media of REV-infected cultures:

REV isolate S originated from a 5 month-old broiler breeder chicken flock; Isolate M originated from a 3 month-old replacement laying pullet flock; Isolate D originated from a 3.5 month-old meat type turkey flock. REV prototypes SNV, CSV and REV-T were grown similarly and were received from Dr. R. L. Witter, ADOL, MI, U.S.A.. The six viruses were titrated in CEF grown on cover slips using mAb 11A25 (received from Dr. L. Lee, ADOL, MI, U.S.A.). Titration was made by infecting a series of cultures with ten-fold dilutions of the

infected culture media, and IF analysis after 6 days of cultivation.

Experimental design

Groups of four embryonated eggs at the 6th day of embryonic development were injected with 0.5 ml of a 1:5 dilution of infected culture media of each infecting virus. The embryonated eggs were injected into the animal side with a 3 ml syringe and a 23 G needle directly into the yolk sac. After injection, the eggs were inspected daily under illumination and pathological changes in the embryo development were observed.

At the 19th day of embryonic development the SPF eggs of each group were gathered in a separated aerated box and kept in the hatching incubator. At the 22th day of incubation all hatched chicks and unhatched eggs were bled in the presence of EDTA, as an anticoagulant and dissected. The organs of each embryo, in separate, were separated with sterile scissors and disposable blades. Half of each organ was frozen in a sterile ependorf tube for further extraction of DNA, and the other half served for the preparation of the organ impressions. Three slides were prepared from each organ of each embryo. At the time of dissection the pathological changes of each embryo were recorded.

The commercial type injected embryos were treated similarly at the first passage in vivo. For the second and third passage, the embryos were dissected at the 19th day of embryonic development and the liver and spleen were homogenized to serve as the inoculum for the third in vivo passage. All injections were made at the 6th day of embryonic development.

Indirect immunofluorescence (IF)

Acetone-fixed organ impressions were incubated at 37°C for 30 min with mAb 11A25 (1:400). After three washes in PBS, the slides were incubated at the same conditions with fluorescein isothiocyanate (FITC) labeled rabbit antibody to mouse IgG (BioMakor, Rehovot, Israel) diluted 1:32. Following a further wash, the slides were counterstained with 1% Evans Blue, washed in distilled water and a drop of 90% glycerol (pH 8.0) applied before covering. The slides were

examined at x400 and x1000 magnification in a Nikon epifluorescence microscope and ASA 400 film was used for photography throughout. The approximate size of the impression was 0.5 cm² and the whole area was scanned for the enumeration of IF-positive cells. Only areas where the impression was of one monolayer thickness were examined and photographed.

DNA preparation and the Polymerase Chain Reaction

PCR was performed as described in Section I.

Preparation of RNA and Reverse Transcriptase – Polymerase Chain Reaction

RNA was extracted with guanidinium thiocyanate protocol (Chomcynski and Sacchi, 1987) using Tri ReagentTMLS (Molecular Research Center, Inc, Cincinnati, OH, USA) according to manufacturer's instructions. Briefly, 0.25 ml culture medium was mixed with Tri Reagent and kept 5 min at room temp. Next, 0.2 ml of chloroform was added, mixed and stored 2–3 min at room temp. The mixture was centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase containing the RNA was separated and the RNA was precipitated with isopropanol. After washing in 75% ethanol and drying, the RNA was dissolved in 20 µl of water. First, the RNA was denatured at 90°C for 5 min. and then reversed transcribed in a 20 µl reaction using 3.3 µg 6 mer random primer, 200 µM of dATP, dCTP, dGTP and dTTP, 33U RNase inhibitor and 25U avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wisconsin, USA). The reaction was allowed to proceed for 10 min at 23°C, followed by incubation at 42°C for 30 min. The temp. was then raised to 98°C for 7 min, after which the sample was rapidly cooled to 4°C. For PCR 3 µl of the RT reaction was used and the reaction was performed as described above.

Results

Quantification of the virus inocula

Culture media from uninfected CEF and from CEF infected with each of the six REV_s were harvested and served as the inocula for the in vivo experiments in embryos. The quantification

of the REVs was made in two ways:

- a) Infection of tissue cultures with ten-fold dilutions of the culture media and IF examination using mAbs to REV.
- b) Estimation of the virus quantity in the culture media by RT-PCR.

The IF examination of REV-infected CEF monolayers grown on coverslips revealed that the media of cultures infected with all the six viruses produced a heavy infection of the new CEF cultures up to a 10^{-3} dilution, showing that the six inocula contained considerable amounts of viruses.

By RT-PCR (Fig. 1), amplification of REV-LTR sequences were obtained with all REVs, except of REV-T and the media of uninfected CEF. However, the negative result with the REV-T-infected culture media indicated that a sequence change might have been contained in the proviral REV-T LTR, rendering unreactiveness by PCR, rather than the absence of virus from the inocula, as it produced extensive infection of the CEF cultures.

Pathogenicity of the REV isolates and prototypes

Table 10 describes the virulence of the three REV isolates and the three REV prototypes used in the present study that was analysed in SPF embryonated eggs. The hatchability of the chick embryos until the 22nd day of incubation serves as an important parameter as it predicts the hatching potential of naturally infected eggs and that is an economical factor. One of the six uninfected embryos and one of the four embryos infected with the S, M, SNV and CSV viruses did not hatch probably as either a non-specific, background feature, or a normal delay in hatching. In contrast, none of the REV-T infected embryos hatched and that uniformity reflects on the specific virulence of REV-T.

We examined the number of development defective or dead embryos, both among those that hatched and both in the unhatched ones (Table 10). All the embryos of the uninfected

Figure 1

RT-PCR quantification of REV virus content in the REV-infected culture media that served for embryo inoculation. As a control, medium of uninfected CEF was used for RNA extraction.

The infective viruses are marked on top of each lane.

group, including the one that delayed in hatching had a normal appearance and organ development. In contrast one of the 4 embryos infected with the S isolate, that did not hatch, had a defective development of the yolk sac and the embryo's eyes. Another S-injected embryo died at 13 days post infection (the 19th day of incubation).

Among the M-infected embryos one did not hatch and in addition, it had a defective yolk sac membrane. Like the control embryos, all the four D-infected embryos had a normal development.

One of the four embryos infected with the SNV prototype did not hatch and the embryo was defective, as it had a foamy digestive tract and a solified yolk sac.

Among the four embryos infected with CSV, one embryo that did not hatch had a hydrocephalus syndrome.

The most drastic pathological effect showed up with the REV-T infected embryos; None of the embryos hatched, and their dissection showed one embryo with undeveloped digestive tract and other with an unswollen yolk sac.

Table 11 describe the pathogenicity of the REVs for embryos derived from commercial embryonated eggs. Three consecutive passages were performed, where the first included media from infected cultures as inoculum, and the other two passages included homogenized embryos from the previous in vivo passage. The embryos injected with uninfected control media and those infected with the M isolate were unaffected throughout the three in vivo passages. However, the embryos infected with all other REVs showed pathognomic lesions, and one REV-T-infected embryo died. These effects were observed only in the first in vivo passage, with the exception of SNV-infected embryos that showed defective development signs and lesions on the second in vivo passage. All these four embryos had enlarged livers, two of them with a green color change, and in the other two the stomach wall did not close. Most of the lesions involved the liver; 3 out of the 4 of both S-infected and D-infected

Table 10: Pathogenicity of REV in SPF chicks: Hatchability, defective development of embryos and mortality

Infective virus	No. of chicks hatched/Total	No. of defective embryos (alive at 21d)/ Total	Embryo mortality
Uninfected	5/6	0/6	0/6 (d)
S	3/4	1/4 (a)	1/4 (c)
M	3/4	1/4 (b)	0/4
D	4/4	0/4	0/4
SNV	3/4	1/4	0/4
CSV	3/4	1/4	0/4
REV-T	0/4	2/4	0/4

(a) – all organs were with lesions; this is the same embryo that did not hatch.

(b) – The same embryo that did not hatch

(c) – That is a different embryo than that that did not hatch

(d) – Embryo mortality before the 21th day of incubation.

Table 11: Pathogenicity of REV in commercial embryonated eggs: defective development of embryos and mortality

Passage No./ Infective virus	Ist passage		IInd passage		IIIrd passage	
	Lesions	Mortality	Lesions	Mortality	Lesions	Mortality
Control	0/4	0/4	0/4	0/4	0/4	0/4
S	3/4	0/4	0/4	0/4	0/4	0/4
M	0/4	0/4	0/4	0/4	0/4	0/4
D	3/4	0/4	0/4	0/4	0/4	0/4
SNV	1/4	0/4	4/4	0/4	0/4	0/4
CSV	1/4	0/4	0/4	0/4	0/4	0/4
REV-T	0/4	1/4	0/4	0/4	0/4	0/4

embryos had enlarged livers with a brownish discoloration. Two D-infected embryos were smaller than the others. One SNV-infected embryo had a retarded growth, and one CSV-infected embryo had a liver color change.

Detection of embryo infection with REV using the PCR and the IF techniques

The six uninfected embryos and the 6 groups of 4 embryos each, injected with various REVs were analysed by PCR and by organ printing and IF examination. Table 12 illustrate the results obtained with each embryo individually, and Tables 13 and 14 reflect the cumulative results of all the four embryos injected with one virus. The results in Tables 12–14 were expressed as numerical scores that range between 1 and 3, for different extends of the specific positive reactions, or no signal (scored as zero). As the six uninfected control embryos were unreactive by either PCR or IF, Tables 12–14 incorporated only four of the six embryos, to unify that group to the other 6 groups that contained only 4 embryos each. The PCR and IF scores of all the uninfected control embryos were zero, that denotes that no virus replication occurred in these embryos. A PCR score of 3 denotes a strong band of PCR product; a score of 2 denotes a PCR product banded at a moderate intensity and a score of 1 denotes a weak PCR band.

The evaluation of the IF examination in scores considered the number of times that the organ print preparate was positive out of the three triplicates prepared from each organ of each embryo, therefore it is between 1 and 3. In the IF examination we have also to consider the number of cells positive in the each examination field.

Individual examination of embryos

The four uninfected control embryos were negative by PCR and by IF (Table 12). In contrast, all four embryos infected with the S isolate were abundant in REV provirus, as most of the organs analysed achieved a high PCR score (Table 12). Embryo S1 had a PCR score of 3 in the blood, liver, spleen, bursa and thymus but the yolk sac membrane was negative. Embryo

S2 died at the 19 day of incubation, therefore blood was not available for PCR. That embryo was less reactive in PCR than S1, as its liver was negative, the spleen and membrane had a score of 2, the thymus – 3, and the bursa was scored as 1. Embryos S3 and S4 had high scores of 3 in most organs, except of the blood and membrane of S3 that had a score of 2 and the membrane of S4, scored as 1. By IF the spleen of S1 was scored as 3, and the liver, spleen and bursa of S3, as well as the spleen and bursa of S4 were scored as 1.

The IF examination of the organ prints of the S-infected embryos was very interesting; The REV-IF positive cells in all the spleen prints examined were numerous in each observation field, and their number could be estimated as approx. 100 (Fig. 2). All other IF-positive organs in that study, either of the S-infected embryos, or embryos infected with other viruses, contained less positive cells, and these could be estimated as about 10 or even less in each examination field.

The PCR scores of the M-infected embryos were much lower than the former, and by IF no positive cells could be detected. Embryo M1 was PCR positive only in the spleen and thymus.

Table 12: Detection of embryo infection with six REV prototypes and isolates by the PCR and the IF techniques

Embryo No.	Organ	Control		S		M		D		SNV		CSV		REV-T	
		PCR	IF	PCR	IF	PCR	IF	PCR	IF	PCR	IF	PCR	IF	PCR	IF
#1	Blood	0	**	3	**	0	**	0	**	0	**	0	**	1	**
	Liver	0	0	3	0	0	0	0	1	2	0	0	1	1	1
	Spleen	0	0	3	3	2	0	0	2	1	2	1	1	1	1
	Bursa	0	0	3	0	0	0	0	0	1	0	0	0	0	0
	Thymus	0	0	3	0	1	0	0	0	1	0	0	0	0	0
	Memb.*	0	0	0	1	0	0	0	0	1	1	0	0	1	0
#2	Blood	0	**	NT	NT	0	**	2	**	0	**	2	**	0	**
	Liver	0	0	0	NT	0	0	0	0	0	0	1	0	0	0
	Spleen	0	0	2	NT	0	0	3	0	2	1	1	0	1	0
	Bursa	0	0	1	NT	0	0	3	0	1	0	0	0	0	1
	Thymus	0	0	3	NT	0	0	3	0	2	1	0	0	0	1
	Memb.*	0	0	2	NT	1	0	1	0	0	0	1	0	0	0
#3	Blood	0	**	2	**	0	**	0	**	0	**	0	**	0	**
	Liver	0	0	3	1	1	0	0	0	0	0	0	0	0	0
	Spleen	0	0	3	1	1	0	1	0	0	0	2	0	1	1
	Bursa	0	0	3	1	0	0	0	1	0	1	0	0	0	0

#4	Thymus	0	0	3	0	0	1	0	0	0	0	0	0	1	0
	Memb.	0	0	2	0	0	0	0	0	1	0	0	0	0	0
	Blood	0	**	3	**	2	1	**	0	**	0	**	0	0	**
	Liver	0	0	3	0	0	0	0	3	0	0	0	0	0	1
	Spleen	0	0	3	1	0	0	0	3	1	0	0	0	1	1
	Bursa	0	0	3	1	0	0	0	3	1	0	0	0	0	1
	Thymus	0	0	3	0	0	0	0	3	0	0	0	0	0	0
	Memb.	0	0	1	0	0	1	0	0	1	0	0	0	0	0

* Yolk sac membrane

** Organ print is not feasible for blood

Evaluation of results

PCR: 3 – strong band

2 – moderate band

1 – weak band

IF: 3 – positive in 3 replicates

2 – positive in 2 replicates

1 – positive in 1 replicate

0 – negative

Figure 2

IF figures of REV-infected cells in the organ impression of REV-infected embryos.

A- Spleen impression of a S-infected chick embryo x40.

B- Spleen impression of a S-infected chick embryo x100.

C- Spleen impression of a CSV-infected embryo x100.

D- Bursa impression of a D-infected embryo x100.

(scores of 2 and 1, respectively); only the membrane of embryo M2 was weakly PCR positive; a score of only 1 was found in the liver, spleen and thymus of M3, and in the blood and membrane of M4.

Two of the four embryos infected with isolate D were much reactive by PCR than those infected with isolate M. In embryo D1 no PCR positive organs were detected. Embryo D2 had a PCR score of 2 in the blood, 3 in the spleen, bursa and thymus and 1 in the membrane. Embryo D3 was slightly PCR positive only in the spleen, but D4 was similar to D2, except of the liver reaction with a score of 3, and the unreactiveness of the membrane. In spite of the negative results of D1 by PCR, by IF we could detect positive cells in the liver and spleen. Embryo D2 that was positive by PCR was negative by IF. In embryo D3 IF-positive cells could be seen only in the bursa, but in D4 they were detectable in all organs but the thymus.

Only two of the embryos infected with the SNV prototype were reactive by PCR and had relatively low scores; whereas the blood of embryo SNV1 was negative, its liver was scored with 2 and all other organs with 1; the spleen and thymus of embryo SNV2 were moderately PCR positive and its bursa had a weak reaction. By IF we could detect positive cells in the spleen and membrane of SNV1, the spleen and thymus of SNV2, the bursa and membrane of SNV3 and the membrane of SNV4.

The CSV-infected embryos were moderately PCR positive and only in the spleen of embryo CSV1 positive cells could be detected by PCR. In embryo CSV2 the blood, liver, spleen and membrane were PCR positive. In embryo CSV3 only the spleen was moderately positive and no positive PCRs were recorded with the organs of embryo CSV4. By IF only the liver and spleen of embryo CSV1 were scored as 1.

The analysis of REV-T prototype resulted in basically the same weak reactivity as with the SNV and CSV prototypes; PCR scores of 1 were obtained in all organs of embryo REV-T1, except of the bursa and thymus; in embryos REV-T2 and REV-T4 only the spleen reacted,

but in embryo REV-T3 both the spleen and the thymus were weakly positive. By IF, the REV-T1 embryo was positive in the liver and spleen, embryo REV-T2 – in the bursa and thymus, REV-T3 in the spleen and REV-T4 – in the liver, spleen and bursa.

The PCR reaction including all the organs of one embryo of each isolate is illustrated in Fig. 3. These are the following embryos: S3, M4, D4, SNV1, CSV2 and REV-T1. The intensive reactions with the organ of S- and D-infected embryos is prominent compared to the moderate or weak reactions of the embryos infected with the M-isolate and with the three REV prototype viruses, and compared to the negativity of the uninfected embryo.

Cumulative analysis of all embryos infected in each group

The individual analysis of each embryo is important to reflect the degree of variability within the group, but to reflect the general trend of the group Tables 13 and 14 present the summing of the individual scores of PCR and IF, respectively, of all 4 embryos within each group. Table 13 shows that none of the uninfected embryos' organs were positive by PCR. In contrast, the embryos infected with the S isolate have the highest scores in all organs tested, that reflect the highest replication ability of that isolate in chick embryos. Isolate D have the second highest PCR scores after isolate S, but no viral sequences could be detected in the membrane. The prevalence of isolate M sequences, and those of the three prototypes is similar in the blood, liver, spleen and membrane. Among these viruses only the SNV sequences could be detected both in the bursa and in the thymus, where the sequences of isolates M and REV-T could be detected only in the thymus, and the CSV sequences could not be detected in neither of these organs. It seems, therefore, that according to the PCR examination isolates S, D and SNV replicate both in the thymus and in the bursa, whereas isolate M and REV-T have affinity for the thymus only. Among the six organs analysed by PCR, for all the groups, it seems that the spleen is the preferential organ in the embryo for the virus replication. The other organs surveyed and the blood are similar in their efficacy for use

in PCR.

Figure 3

REV-PCR of representative embryo from each group, as indicated on the left of each illustration. For each embryo the PCRs of the blood, liver, spleen, bursa, thymus and yolk sac membrane is shown. In each block both negative and positive control DNA were included.

Table 13: Cumulative scores of PCR in REV-infected SPF embryos

Infecting virus/ Organ analysed	Blood	Liver	Spleen	Bursa	Thymus	Membrane
Uninfected	0 (a)	0	0	0	0	0
S	8	9	11	10	12	5
M	1	1	3	0	2	2
D	4	3	7	6	6	0
SNV	0	2	3	2	3	1
CSV	2	1	4	0	0	1
REV-T	1	1	4	0	1	1

(a) – PCR scores as detailed in Table 1.

Table 14: Cumulative scores of IF in REV-infected SPF embryos

Infecting virus/ Organ analysed	Liver	Spleen	Bursa	Thymus	Membrane
Uninfected	0 (a)	0	0	0	0
S	1	5	2	0	1
M	0	0	0	0	0
D	4	3	2	0	2
SNV	0	3	1	1	3
CSV	1	1	0	0	0
REV-T	2	3	2	1	0

(a) – IF scores as detailed in Table 1.

Table 14 shows the cumulative scores of the IF examination for each group. By IF the spleen of the embryos infected with the S isolates have the highest cumulative score, reflecting the fact the its organ prints were the richest in IF positive cells. Isolate S could be detected also in other organs, except the thymus. The same findings appeared in the D-infected embryos. SNV- and REV-T- infected cells were detected both in the bursa and the thymus, but CSV replicated only in the liver and spleen and a minimal score was obtained. The infection with isolate M could not be traced by the organ print method.

The cumulative scores of the IF examination are lower than those of the PCR, and fewer organs are positive, due to the lower sensitivity of that method compared to PCR. However, by that method the morphology of the REV-infected cells could be vizualized.

Discussion

Reticuloendotheliosis viruses are known to produce T and B-cell lymphomas in chickens and turkeys, often after an immunosuppressive state and a growth retardation in affected flocks (Witter, 1991). Since REVs were described to be disseminated both horizontally and vertically (Witter, 1991), we sought now to define whether REVs are potential damaging for the early stages of embryonic development, the hatchability potential and the death of embryonated eggs. The parameter of morbidity and mortality might play an important economical factor in the hatcheries' production. To gain an overall notion of that factor, we included in that analysis three REV Israeli isolates and three REV prototypes. The Israeli REVs were previously characterized as being antigenically identical and similar to CSV, whereas SNV and REV-T differed antigenically (Davidson et al, 1996). To broaden the pathogenicity analysis we examined their effect by infecting either SPF or embryonated eggs from a commercial source. Isolate S was the most harmful to SPF eggs, as one embryo had specific lesions and other died. Isolate M caused a minor defective development and isolate D did not affect the health of the injected eggs. The REV-T prototype was the most harmful virus, whereas CSV and

SNV had a minor effect. In the commercial eggs REV-T retained its pathogenicity, as it caused the death of one embryo. CSV and SNV affected the embryos at the first in vivo passage to the same degree as the SPF embryos, but SNV caused a defective development of all embryos at the second passage in vivo. We anticipated that an enhanced replication of the virus occurred on the first passage, therefore at the second passage, the effect rate was increased. In contrast to SNV, all other REVs seemed to be attenuated by the first passage in vivo. An alternative explanation might be also some technical reasons. However, with the three local isolates, an increased rate of lesions was observed with isolates S and D. Both sources of eggs differed in their susceptibility to isolates M and D, as the former was not pathogenic at all for embryos from the commercial source, while that was the situation of isolate D for SPF embryos. We assume that this effect is related to the different genetic background of the embryos.

These results indicate that in spite of the small numbers of eggs used, the infection of chicken embryos with REV is not innocent, and might have economical implication. Our results are concordant to those of Motha (1987). However, to our knowledge that is the first study that focus on field REV isolates in relation to REV prototypes in terms of pathogenicity. As such, we also sought to analyse the differences in the replication ability of the six REVs in SPF embryonated eggs and to define the embryonic target organs. Our analysis employed two innovative techniques, i.e., PCR for amplifying proviral LTR (Davidson et al, 1995) and the organ print method and visualization of the antigen producing cells after IF with mAbs to REVs (Davidson et al, 1994). The PCR proved itself as the sensitive one, but by organ printing and IF, the biology of the REV infection could be illustrated.

By both methods isolate S especially, but also isolate D, had an intensive replication ability in the chick embryos. Isolate M and the 3 prototypes also replicated in the embryos, but to a lesser extent. The main target organ for all REV is the spleen, and the extensive replication

ability of isolate S is demonstrated not only by the highest scores in PCR and IF, but also through the fact that the S-infected spleens possess the highest number of REV-infected cells.

The analysis of the bursa and the thymus can provide an indication on the T- or B-cell affinity of the viruses and thus it can serve as an additional biological means of characterization. The virus affinities are based on the results of the PCR, as it was most sensitive than IF in that study. Compared to isolates S and D, as well as SNV, that replicate in both organs, isolate M and REV-T could be detected only in the thymus, while CSV was not found in either of these organs.

In conclusion, the present study demonstrate the difference in pathogenicity, in vivo replication ability and difference in organ distribution of various REVs.

B. The immunodominant proteins of REV

Introduction

All members of the REV group are related antigenically and indistinguishable by immunofluorescence (IF) using convalescent chicken serum (Purchase et al, 1973). Previous studies on REV proteins described two env gene-encoded glycoproteins, gp90 and gp20 (Tsai et al, 1986; Tsai and Oroszlan, 1988) and five gag gene-encoded core proteins, p12, pp18, pp20, p30 and p10 (Tsai et al, 1985). The two glycoproteins are the surface and transmembrane proteins, respectively, while the C' epitope of gp90 is exposed on the outer surface of the infected cell. A rabbit antibody to the gp90 peptide located on the C' segment was able to cause lysis of the cell in the presence of rabbit complement (Tsai and Oroszlan, 1988). In addition to the predicted role of gp90 in eliciting the production of antibody-mediated cytotoxic effects, Federspiel et al (1989) showed that env gene expression confers at least a 1000-fold increase in resistance to REV superinfection. They constructed two REV env-gene expression plasmids, containing the REV-A or the SNV env gene, and followed the levels of gp90 and gp20 in transfected cell lines. The expressed gene-product protected single cells from further infection with REV-A or SNV. Earlier studies gave slightly different mol. wts. for the env gene-encoded proteins, 71/73 and 22 kD (Mosser et al, 1975) or 73 and 19 kD (Maldonado and Bose, 1975, 1976), and some workers reported that the 29 kD gag gene-encoded protein is probably the major structural core protein responsible for the group-specific antigenicity (Maldonado and Bose, 1976; Tsai et al, 1985, Mosser et al, 1975).

Antigenic differentiation between various REV isolates was described by Cui et al (1986) who analysed them using a panel of 11 monoclonal antibodies (mAbs) to REV-T strain. They described three distinct epitopes (A, B and C), one type-specific (A) from REV-T but not from CSV-infected cells and two type-common (B, C) from both REV-T and CSV-infected cells. The 62 kD glycoprotein presents both B and C epitopes, the 21 kD glycoprotein presents only one

type-common epitope while the 54–72 kD glycoprotein presents the A epitope. In their study, rabbit anti-REV antiserum immunoprecipitated 6 virus-specific proteins (21–62 kD) of strains REV-T and CSV. Chen et al (1987) studied a total of 26 isolates and showed that isolates of subtype 1 (including REV-T) possessed epitopes A, B and C, subtype 2 (including SNV) contained only epitope B and subtype 3 (including CSV) contained epitopes A and B. Nevertheless, viruses of subtypes 1 and 2 could not be differentiated by receptor interference based on the env-gene products, gp90 and gp20 (Federspiel et al, 1989).

None of these studies analysed native immunogenic REV proteins since the env gene-encoded proteins were characterized with anti-peptide, mAbs and rabbit antiserum but not with chicken serum. For this reason the present study was designed to characterize native viral proteins that are exposed to the avian immune system during infection. Convalescent chicken serum was therefore used to immunoblot cell lysates of three prototype REV strains, REV-T, CSV and SNV representing the three subtypes described by Chen et al (1987) and eight Israeli isolates (Davidson et al, 1995). Using this approach we have also compared the antigenic profiles of these viruses using three mAbs, 11A25, 11C237 and 11C100 (Cui et al., 1986), a rabbit antiserum raised to REV-T whole virus (Cui et al., 1986) and a rabbit antibody to the gag gene-encoded p30 of REV-A (Tsai et al, 1985).

Materials and Methods

Viruses

Prototype REV strains REV-T, CSV and SNV were provided by Dr. R. L. Witter.

The Israeli REV isolates B, BI, EG, L, M, S and RA were obtained from commercial chickens, and isolate D from a commercial turkey flock (Davidson et al, 1995).

Antisera

Chicken convalescent serum was obtained from a 18.5-month-old bird infected experimentally with isolate REV-M 10 weeks earlier.

The rabbit anti-REV serum and the mAbs 11A25, 11C237 and 11C100 (Cui et al., 1986) were provided by Dr. L. F. Lee, ADOL, East Lansing, MI, U. S. A..

The rabbit antiserum to REV-A p30 was a gift of Dr. S. Oroszlan (Tsai et al, 1985).

Propagation of viruses in tissue culture

Chick embryo fibroblasts (CEF) were grown in Leibovitz-McCoy medium (1:1 v/v) (Bet Haemek, Israel) supplemented with antibiotics, antifungals and 4% fetal calf serum (FCS) until confluent monolayers were formed and with medium containing 1% FCS afterwards. Secondary CEF monolayers were infected with REV-infected medium at a ratio of 1:10 and grown for 6 days with changes of medium every other day. The inoculum was titrated by seeding it in ten fold dilutions (up to 10^{-4}) on monolayers grown on coverslips in small plates (60mm²) for 6 days. The slips were fixed in acetone and examined by IF using mAb 11A25 as described (Davidson et al, 1995). All inocula were used at a 10^{-4} titer as determined by IF.

The CEF were harvested by scraping and kept at -70°C until examined. The cell pellets were solubilized by mixing in RIPA buffer (0.5% (w/v) sodium deoxycholate, 0.5% (v/v) Nonidet 40 (NP-40), 1mM phenylmethylsulphonyl fluoride (PMSF), 2ug/ml Aprotinin and 2 mM Na₂EDTA). They were then kept on ice for 30 min, centrifuged (13,000g at 4°C), and the supernatant stored at -70°C .

Gel electrophoresis and immunoblotting

SDS-PAGE was performed using the buffer system of Laemmli (1970); 10% gels were used throughout and loaded with 150 ug CEF lysate protein per lane (Bradford protein determination kit, Bio Rad). Prestained SDS-PAGE low molecular weight standards (Bio Rad) containing Phosphorylase B (142.9 kD), Bovine serum albumin (97.2 kD), Ovalbumin (50.0 kD), Carbonic anhydrase (35.1 kD), Soybean trypsin inhibitor (29.7 kD) and Lysozyme (21.9 kD) were included in each run.

Electrotransfer to 0.45um nitrocellulose (NC) sheets (Towbin et al ,1979) was carried out at room

temperature for 3 hr using a Hoeffer TE 70 Semiphor apparatus. The blots were then incubated with PBS+2.5% calf serum+2.5% casein (blocking buffer) at 4°C for 18 hr. All further steps of immunoblotting were performed at room temperature on a rocking platform. The first antibody was diluted in blocking buffer and incubated for 1–2 hr; the chicken convalescent serum was used at a 1:300 dilution, the rabbit anti-REV at 1:150, the mAbs 11A25, 11C237 and 11C100 and the rabbit anti p30 serum at 1:400 dilution. The blot was washed twice in blocking buffer diluted 1:1 with PBS, followed by two washes with blocking buffer diluted 1:4 with PBS. Peroxidase-linked secondary antibodies (rabbit-anti-chicken IgG, rabbit anti-mouse IgG, goat anti-rabbit IgG, Bio Makor, Israel) were diluted 1:1000 in blocking buffer:PBS (1:4) and incubated for 1–2 hr. The blots were then washed three times with PBS and reacted with the substrate, 0.05% 3,3'-diaminobenzidine (Sigma), 0.03% cobalt chloride and 100 μ l of 30% H₂O₂ in 100 ml PBS. The reaction was terminated by immersing the blot in distilled water.

Determination of REV antigen concentration in cell lysates by ELISA

Polystyrene plates were coated (18 hr at 4°C) with 0.1 ml of a mixture of the mAbs 11A25 and 11C237 (diluted each at 1:1000 in 0.05M carbonate-bicarbonate buffer). After 3 washes in phosphate-buffered saline (PBS) containing 0.05% Tween 20, 0.1 ml of the CEF lysates diluted in PBS + 0.1% bovine serum albumin were incubated for 1 hr at 37°C. The CEF lysates were diluted from an initial protein concentration of 2 μ g/ml in threefold dilution series. After 3 washes as before, the IgG fraction of rabbit anti-REV (at a concentration of 20 μ g/ml) was reacted for 1 hr at 37°C. The next steps were three PBS-Tween washes, reaction with goat anti-rabbit IgG (diluted 1:1000), three additional washes, and incubation with the substrate, azino di-3-ethyl-benzthiazoline sulphonate (ABTS) (Sigma) at 1 mg/ml in 0.1 M citrate-0.2 M phosphate buffer pH 4.0, supplemented with 0.003% H₂O₂. The optical absorbances were measured in a Dynatech ELISA reader at 405/490 nm.

Results

Total REV antigenic activity of the infected lysates

In order to detect total antigenic activity the ELISA was designed to incorporate antisera reactive with all three neutralizing epitopes, and therefore included the two mAbs and the rabbit anti-REV (whole virus) serum.

Cell lysates of uninfected CEF and of CEF separately infected with the REV prototype strains CSV, SNV and REV-T and the eight local isolates were quantified by ELISA (Table 15).

As shown in Table 15, in comparison with the absorbance of the uninfected lysate the infected cell lysates had endpoint antigenic activities of approximately 0.07 ug/ml total protein.

REV proteins immunoblotted by chicken convalescent serum

The major REV proteins in infected cell extracts blotted with the whole convalescent serum are shown in Fig. 4 and summarized in Table 16. Under both reducing and non-reducing conditions, i.e. in the presence and absence of DTT of SDS-PAGE, a diffuse major band of 75–100 kD was visible in all the infected cell lysates. The uninfected lysate did not react with the chicken serum.

REV proteins immunoblotted by mAb 11A25

Blots of all cell lysates separated under reducing conditions were unreactive with mAb 11A25. In contrast, under non-reducing conditions of electrophoresis all REV-infected cell lysates were reactive, with the notable exception of SNV and the uninfected control. Fig. 5 shows that a diffuse band of 75–100 kD was predominant.

REV proteins immunoblotted by mAb 11C237

Under reducing conditions only REV-T was reactive with mAb 11C237, which gave a well-defined band of 100 kD (Fig. 6 and Table 16). All the infected lysates, except SNV, were reactive with mAb 11C237. Under these conditions the immunoblot reactivity was more intensive than with mAb 11A25; a diffuse 75–100 kD band was apparent, and in addition, three high mol. wt. antigens of approximately 200, 180 and 140 kD were present. The 140 kD antigen was less sharp

than the other two bands.

REV proteins immunoblotted by mAb 11C100

Blots of all cell lysates were reacted with mAb 11C100 in the presence and in the absence of DTT. Only REV-T lysate reacted with it under non-reducing conditions to reveal an antigen of 75–100 kD (Blot not shown, results summarized in Table 16).

REV proteins immunoblotted by the rabbit anti-REV (whole virus) serum

A more complex pattern of REV antigens was revealed with the rabbit anti-REV serum (Fig. 7 and Table 16). Under reducing conditions, the specific REV antigens included a broad band of 55–65 kD, a doublet polypeptide of 35 kD and a narrow band of 25 kD. In the REV-T cell lysate an additional faint 100 kD antigen was detected. Under non-reducing conditions a major diffuse antigen of 75–100 kD was present in the infected cell lysates. In addition a faint, but sharp, band of 65 kD, a strong 58 kD band, a faint doublet antigen of 36 kD and a well-demarcated 30 kD antigen were seen in all the cell lysates. The 58 and 65 kD antigens resembled the reduced 55–65 kD antigens, which were more diffuse under these conditions of electrophoresis. The 30 kD antigen resemble the 25 and 28 kD antigens that were revealed under the reducing conditions of electrophoresis.

REV proteins immunoblotted by the rabbit anti-p30 polypeptide

Similar patterns of reactivity were obtained with the rabbit anti-p30 antibody under both reducing (not shown) and non-reducing conditions of SDS-PAGE (Fig. 8 and Table 15). The major difference was the failure of this antibody to detect the unreduced 75–100 kD antigen. As seen in Fig. 8, the following antigens were demarcated well in the infected lysates: doublets of 50 and 60 kD, 36 and 39 kD and a 28 kD antigen. The latter band was strongly blotted, indicating its identity as p30, initially used for antiserum production.

Table 15: Determination of the REV antigenic activity of CEF lysates by ELISA

(Optical density values at 405/490 nm)

Cell lysate protein (ug/ ml)	CEF*	CSV	SNV	R-T	B	BI	EG	L	M	S	RA	D
0.0**	.20	.18	.19	.20	.19	.21	.18	.19	.22	.18	.21	.22
2.0	.16	.65	.92	.73	.57	.70	.66	.49	.60	.61	.44	.67
.20	.18	.35	.50	.38	.31	.38	.38	.32	.33	.34	.27	.41
.07	.21	.25	.33	.26	.22	.26	.27	.24	.22	.24	.23	.32
.02	.16	.18	.26	.24	.22	.22	.21	.21	.22	.21	.23	.27

R-T - REV-T

* - uninfected culture; ** - Cell blank

Table 16: Detection of viral proteins of eleven REV isolates under nonreducing and reducing SDS-PAGE by chicken serum, three mAbs and two rabbit antisera.

MW	Chicken		mAb 11A25	mAb 11C100		mAb 11C237	Rabbit anti-REV-T (whole)		Rabbit anti-p30 (gag)	
DTT	+	-	+	-	+	-	+	-	+	-
75-100 (env)	all	all	none	all but SNV	none	REV-T only	REV-T	all but SNV	none	none
22(env)	none	SNV only	none	none	none	none	none	none	none	none
55-65 35, 25 (gag)*	none	none	none	none	none	none	all	all	all	all

all/none - refers to REV's: CSV, SNV, REV-T, REV-B, REV-BI, REV-EG, REV-L,

REV-M, REV-S, REV-RA, REV-D.

* - proteins of MW 65, 58, 36, 30, and 60, 50, 39, 36, 28 detected by the rabbit anti-REV serum in the absence of DTT and by the rabbit anti-p30 antiserum under both conditions of SDS-PAGE.

Figure 4

Immunoblot analysis of cell lysates of uninfected CEF, of CEF infected with the REV prototype strains CSV, SNV, REV-T and Israeli REV isolates: B, BI, EG, L, M, S, RA and D using chicken convalescent serum. The cell lysate type is identified on the top of the blot. The proteins were separated by 10% SDS-PAGE in the presence and absence of 0.1M DTT (as marked on the top of each blot). The molecular weight standards are marked.

Figure 5

Immunoblot analysis of cell lysates as in Fig. 1, electrophoresed in the presence and absence of DTT, as in Fig.1, and blotted with mAb 11A25.

Figure 6

Immunoblot analysis of cell lysates as in Fig. 1, electrophoresed in the presence and absence of DTT, as in Fig.1, and blotted with mAb 11C237.

Figure 7

Immunoblot analysis of cell lysates as in Fig. 1, electrophoresed in the presence and absence of DTT, as in Fig.1, and blotted with rabbit anti-REV (whole virion) serum.

Figure 8

Immunoblot analysis of cell lysates as in Fig 1, electrophoresed in the absence of DTT and blotted with rabbit anti p30 serum.

Discussion

In the present report we have characterized the immunodominant proteins of REV using a serum from an experimentally infected chicken. The REVs included three prototype strains CSV, SNV and REV-T, in addition to eight Israeli isolates. The convalescent chicken serum detected a very diffusely reactive antigenic band of 75–100 kD under both reducing and non-reducing conditions of electrophoresis, while the two rabbit antisera and the three mAbs used now yielded continuous and discontinuous epitopes on the reduced and unreduced 75–100 kD antigen. The present report also analyses the S–S bond content of REV antigens recognized by the chicken and their conformational epitopes. Heat-labile epitopes were also investigated and no evidence was found for their presence (data not shown).

The rabbit antisera and 3 mAbs detected only the unreduced form of the 75–100 kD antigen and these may be discontinuous epitopes. Chen et al (1987) had showed that only mAb 11A25 reacted with SNV by IF and this finding was confirmed now. In contrast to IF we showed now that in immunoblots neither mAb was reactive with SNV. Only mAb 11C237 was active under reducing conditions in recognizing continuous epitopes of the 100 kD antigen of REV-T. This result differentiated the two mAbs, in contrast to Chen et al (1987) who showed that 11A25 and 11C237 reacted with REV-T, which had been used to immunize the mice (Cui et al., 1986). Although mAb 11A25 was produced in the same way (Cui et al, 1986), its unique specificity in this study was demonstrated by its lack of reactivity for the reduced form of REV-T and the unreduced three high mol. wt. antigens (140, 180 and 200 kD) of all REVs, except REV-T.

In support of our findings, Cui et al (1986) showed by the competitive ELISA immunoassay that the two type-common mAbs 11A25 and 11C237 reacted with two different epitopes (B and C) on the 62 kD glycoprotein. Our study with mAb 11C100, described by Cui et al (1986) as being REV-T type specific, further defines the 54–72 kD antigen that they described by showing that this mAb reacted only with unreduced form of REV-T i.e., with discontinuous epitopes.

The comparison of the reactivities of the three mAbs 11A25, 11C237 and 11C100 led us to identify the immunoblotted 75–100 kD antigen as being the same as the immunoprecipitated type-specific 54–72 kD (of REV-T) and the type-common 62 kD antigen of the rest, described by Cui et al (1986). Difference in MWs can be attributed to the methods used to separate the proteins. REV-T infected cell lysates yielded a 100 kD antigen when immunoblotted with the 11C237 mAb, but a diffuse antigen of 75–100 kD with the chicken convalescent serum, both separations being performed under reducing conditions. The differences in molecular weight reflect different epitopes being recognized by the two sera, but they are basically present on the same immunodominant complex.

Cui et al (1986) concluded that the major immunogenic protein of REV is the 62 kD antigen, and not the 29 kD structural core protein (Tsai et al, 1986), because the former reacted with their panel of mAbs raised to intact virions. The fact that the chicken antibodies found in the convalescent serum reacted with the 75–100 kD antigen, together with the behaviour of the mAbs and the rabbit anti-REV-T whole virus in this study, leads us to conclude that it is the immunodominant REV antigen.

In spite of minor differences in its molecular weight, the 75–100 kD antigen is probably the same as the env gene-encoded antigen gp90 (Tsai et al, 1986; Tsai and Oroszlan, 1988; Federspiel et al, 1989), the 71/73 kD antigen of Mosser et al (1975), the 73 kD antigen of Maldonado and Bose (1975, 1976) and the 62 or 54–72 kD antigens of Cui et al (1986) and Chen et al (1987). Tsai et al (1986), however, suggested that the molecular weight of gp90 is higher than expected, probably due to modification by unusually large size N- and O-linked carbohydrate moieties. In contrast to our findings, in the studies of Tsai and Oroszlan (1988) and Federspiel et al (1989), purified gp90 was detected under reducing conditions of electrophoresis using a rabbit antiserum to whole virus. By using crude whole cell lysates as antigens, their conformational analysis was affected by reducing agents in this study.

An additional 22 kD antigen was detected only in the unreduced SNV lysate by the chicken convalescent serum. This could be the second env gene-encoded antigen, gp20, (Tsai et al, 1986; Tsai and Oroszlan, 1988), the 22 kD antigen of Mosser et al (1986), the 19 kD antigen of Maldonado and Bose (1985, 1986) and the 21 kD antigen of Cui et al (1986) and Chen et al (1987). Whereas only the SNV 22 kD antigen was detected in our study, in other studies this antigen was shared by the REV-A, REV-T and CSV.

The gag antigens of 30–65 kD detected by the rabbit antiserum were not affected by DTT, when blotted with anti-REV (Cui et al, 1986) and the anti-p30 antisera (Tsai et al, 1985). The resemblance of the gag gene-encoded proteins, reported by Tsai et al (1985), is attributable to disruption of the REV virions and exposure of structural proteins in the process of immunizing the rabbits.

In this study we have demonstrated differences between CSV, SNV and REV-T, while all 8 Israeli isolates analysed were identical antigenically with CSV, thus resembling subtype 3. This similarity is important for further work aimed at developing vaccines against REV, and in epidemiological studies.

Section IV: **Evaluation of the effectiveness of different tests for the differential diagnosis of lymphoid tumors**

A. PCR: Detection of REV-LTR

Introduction

Four oncogenic viruses are recognized in the etiology of avian tumors of: Marek's disease (MDV) (Calnek and Witter, 1991), reticuloendotheliosis (REV) (Witter, 1991), lymphoid leukosis (LLV) (Payne and Purchase, 1991) and lymphoproliferative disease (LPD) (Biggs, 1991).

MDV, REV and LLV affect chickens, while turkeys are affected by MDV, REV and LPD. MDV is a herpesvirus that causes malignant transformation of T cells in the chicken (Witter, 1991), whereas in the turkey, either the B cell (Elmubarak et al, 1981; Nazerian et al, 1982) or the T cell (Powell et al, 1984) may be the targets for transformation; REV, LLV and LPD are C-type retroviruses that transform pre-B or pre-T cells (REV and LPD) or B cells (LLV). In addition to their oncogenicity, the viruses are also immunosuppressive, can cause growth retardation and have contaminated poultry vaccines.

Viral-induced tumors are the most important neoplasms of chickens and turkeys because: a) The lifespan of domestic fowl is not long enough for natural tumors to develop, and: b) Domestic fowl develop tumors at a much lower rate than mammals, except of tumors for the reproductive tract (Fredrickson and Helmboldt, 1991).

The differential clinical diagnosis of the four diseases is problematic since the location, gross and the histological appearances of the lesions found at necropsy overlap. In chickens both MDV and REV cause visceral lesions that consist of a lymphoproliferation of pleomorphic cells, while tumors caused by LLV, and occasionally by REV in chickens, consist of monomorphic cells. In turkeys, visceral lesions composed of pleomorphic cells are caused by MDV and LPD, while the monomorphic type is found in REV infection.

The classical methods of differential diagnosis are based on virus isolation and

demonstration of specific antibodies. However, virus isolation is time-consuming and is also hindered by the presence of humoral antibodies. Moreover, LPD cannot be propagated in cell culture. For these reasons we have applied the PCR to demonstrate directly the presence of MDV and of proviral REV, LLV and LPD sequences in the blood and tumors of naturally infected chickens and turkeys. The development of the four systems of PCR was intended to provide a fast and more accurate means of diagnosis of avian neoplasms. The utility of applying the PCR to distinguish MDV and REV, mainly in chicken neoplasms, has been reported earlier (Davidson et al, 1995). In the present report we present the results of a two-year survey on both chicken and turkey commercial flocks, and we also included the PCR for proviral LPD and LLV.

The primers of REV, LLV and LPD were designed to amplify the proviral LTR of these viruses. Our rationale in using primers that amplify the proviral LTRs of the three retroviruses was based on their ability of rapid integration into the host DNA, while large parts of the provirus, except the LTR, are excluded over time. The LTRs, therefore, are the most stable of the integrated sequences (Robinson and Gagnon, 1986).

The present report will illustrate the prevalence of each of the four oncogenic viruses in the formation of tumors in the domestic fowl.

Materials and Methods

Chicken and turkey flocks

The flocks presented in this study were sampled due to clinical disease over a two years interval (1993–1995), and includes a variety of flocks and birds of various ages. The birds were bled (using EDTA as anticoagulant), necropsied and the tumors were examined histopathologically. The plasma was examined for REV antibodies by ELISA and the packed blood cells and tumor tissues served for DNA preparation for PCR (Davidson et al, 1995).

Oligonucleotide primers

The PCR primers for MDV were selected to detect only the oncogenic serotype 1 viruses, and were designed to amplify the 132 bp tandem repeats of the BamH1–H fragment of the MDV genome (Davidson et al, 1995; Becker et al, 1992).

The direct primer was: 5' TACTTCCTATATAGATTGAGACCGT and the reverse primer was: 5' GAGATCCTCGTAAGGTGTAATATA. The direct primer is located 65 bp 5' to the tandem 132 bp repeat and the reverse primer is 105 bp downstream. The expected amplified DNA band size with a double 132 bp repeat is 434 bp and 566 bp for a triple repeat.

The primers used in the REV–PCR system were described previously (Aly et al, 1993) and are based on sequences of the proviral SNV–LTR (Shimonoto et al, 1980).

The direct primer was: 5' CATACTGGAGCCAATGGTT and the reverse primer: 5' AATGTTGTACCGAAGTACT. The expected amplified DNA band size is 291 bp spanning the entire length of the unique 5' (U5), repeat (R) region and includes 118 bp of the unique 3' (U3) of the LTR.

The primer sequences for the LLV–LTR (Chen and Barker, 1984; Habel et al, 1993) were designed by Dr. E. J. Smith, ADOL, MI, U. S. A., and were:

Upper primer #26 5' – AAGTAAGGTGGTACGATCGTGCCTTA

Lower primer #30 5' – CTGCTTCATT CAGGTGTT CGCAAT

The expected amplified DNA band size is 182 bp.

The primer sequences for the LPD–LTR were designed by us based on the sequence of LPDV (Sarid et al, 1994; Gak et al, 1991). The direct primer was: 5'

CTTGCTGTTTTAAGCCACAT and the reverse primer: 5' AGCTATAGGCTCCGCGTCAA. The expected amplified DNA band size is 356 bp spanning most of the U3, the R and the U5 regions of the LTR.

The polymerase chain reaction

DNA was mixed in a 25 µl PCR reaction with 10 mM Tris–HCl (pH 8.5), 50 mM KCl, 1.5 mM

MgCl₂, dATP, dCTP, dGTP and dTTP each at 200 mM, and primers (1.0 mM each) and 0.5 units of Taq polymerase. For all PCR systems we used AmpliTaq DNA polymerase (Perkin-Elmer, NJ, U. S. A.), except of MDV and LPD in turkeys, where Thermostable DNA polymerase (Advanced Biotechnologies LTD, Surrey, U. K.) was used. PCR cycling parameters were 31 cycles of 94C for 1 min, 55C for 1 min and 72C for 1 min, followed by a final elongation at 72C for 10 min. PCR samples were mixed with gel loading buffer (0.25% bromophenol blue tracking dye in 40% sucrose). Electrophoresis was carried out in 1.5% agarose gels with ethidium bromide, 0.5ug/ml, in 0.5xTris-Borate-EDTA buffer (TBE) at 100 Volts/cm (Sambrook et al, 1989). The molecular weight markers for DNA gel analysis were a 1 kilobase DNA ladder (BRL, Bethesda, USA).

Results

Chickens

The advantages of applying the PCR for MDV and REV, on chicken neoplasms, have been reported earlier (Davidson et al, 1995) and were based on a one year sample. In the present report we have included the results of a two-year survey on chicken and turkey commercial flocks. In addition a PCR for proviral LLV was included. Table 17 shows that 82 chicken flocks out of a total of 113 flocks sampled because of clinical disease, were positive by PCR. In 39 out of the 82 PCR positive flocks (48%) and in 9 out of the 31 PCR negative flocks (29%), REV antibodies were detected. PCRs for MDV, REV and LLV were performed on a total of 551 birds using DNA purified from whole blood and on 116 DNAs prepared from tumors. Regarding the PCR, about half of both sets of blood and tumor samples were positive (63% of the tumor DNAs and 46% of the blood DNAs derived from flocks that some of the birds were PCR positive), 59% positive tumors out of a total 123, and the 189 (34%) positive of the 551 blood samples tested.

Some birds with two species of oncogenic virus, were detected and two flocks containing all

three species were identified.

Of the flocks with MDV only, the virus was detected preferentially in the tumor DNA (77% positive) over blood DNA (43% positive). The MDV tumors had a pleomorphic type of cellular infiltration in 10 out of the 14 flocks examined. For REV, the PCR is best performed on blood DNA, as in 43% of those cases it was positive, compared to 20% of the cases when tumor DNAs were used. In these cases the tumors had a monomorphic cellular infiltration. The PCR for LLV was positive in only 4 flocks, but tumors were not examined histologically. Table 17 presents 18 flocks in which a mixed infection with two viruses was detected; 11 flocks had MDV and REV in both tumors and blood in equal (41% and 51%). In two flocks both REV and LLV were detected, in blood only, and in 5 flocks both MDV and LLV were detected, in blood and tumor, 62% and 81%, respectively. In mixed infections both types of lesions were found in different organs.

In two flocks all three viruses were detected, and about half the blood and tumors samples were PCR positive.

We conclude that PCR is more useful if based on tumor DNA for MDV, but blood DNA is of greater value for LLV and REV.

Turkeys

Table 18 shows the 40 turkey flocks analysed using PCR for MDV, REV and LPD. Among the 22 PCR positive flocks, in 5 REV antibodies were detected by ELISA. A total of 174 birds were analysed for blood DNA, and in addition, 40 tumors were examined. 21% of the 174 blood DNA samples were positive, compared to 55% of the 40 tumor DNA samples. MDV was detected by PCR in 21 out of the 33 tumors examined (64%) and in 10 out of the 41 blood DNAs sampled (24%). Only 2 out of the 12 flocks in which MDV was detected were positive for REV antibodies. Like the chicken data, MDV could be detected preferentially in tumor DNA. The tumors that were examined histopathologically showed a pleomorphic cellular

infiltration. These results are detailed in Table 19, and are of unusual interest, since only a few reports exist on turkey lymphomas caused by MDV (Witter et al, 1970; 1974; Powell et al, 1984; Paul et al, 1977).

Table 20 depicts the turkey flocks in which REV was detected by PCR. In 17 out of 38 birds the REV-PCR was positive, but only in the blood DNA. Although the number of tumor DNAs examined in this category was low, we are impressed that as in the chicken, REV can be detected preferentially in the blood DNA and one third of these flocks had REV antibodies. We do not have histopathological data for the type of cellular infiltration in the tumors.

Unexpectedly, some of the turkey flocks showed three types of mixed infection (Table 21). We detected a turkey flock that was positive for both MDV and LPD, a second one that was positive for MDV and REV and a third flock that was positive for all three viruses (MDV, LPD and REV). Among these flocks same birds were found with sequences of two viruses (one bird with MDV+LPD, another with REV and LPD and two birds with the three viruses, MDV+REV+LPD). Histopathologically the tumors of the flock with MDV+LPD had pleomorphic cellular infiltration, which can be caused by either virus.

Only in one of the four flocks with a mixed infection we could detect REV antibodies.

Comparison of blood and tumor DNA for their relative value in diagnosing avian tumors by PCR

Among the 82 PCR positive chicken flocks analysed, in 31 of them both blood and tumor DNAs were analysed by PCR (Table 17). In 13 of the 31 flocks, the blood gave better results than the tumor DNA (42%), in 10 flocks the DNA from both sources gave identical results (32%), and in 8 flocks the analysis of the tumor DNA gave better results (26%). When analysing the results shown in Table 17 for each virus, in MDV-positive flocks 77% of the tumor DNA samples were positive, as compared to 43% of the blood DNA samples. In contrast, in REV-positive flocks 43% of the blood DNA samples were positive, compared to

only 20% of the tumor DNA samples.

Among the 22 PCR positive turkey flocks, in 8 flocks using both sources of DNA were examined. In 4 flocks the blood DNA was a better source for the PCR, in 2 flocks both sources were the same and in 2 flocks the tumor DNA was better. According to the PCR results (Table 18), we observed that MDV was diagnosed by PCR in 64% of the tumor DNAs, compared to 25% of the blood DNAs. In contrast, REV was found only in the blood DNA, but only a small number of tumors were found in these flocks.

The above analysis led us to conclude that MDV sequences can be found preferentially in tumor DNA, whereas REV – in the blood DNA. Nevertheless the best diagnosis could be made by the analysis of both blood and tumor DNA. However, in cases when the birds remain alive, blood sampling can provide an adequate diagnosis even for MDV, which, according to our findings in chicken and turkeys, 24–43% of the cases would be positive. Based on the numbers of positive samples, of 3–5 birds examined from a flock would provide a reliable result.

Distribution of PCR positive flocks according to the class of farm of origin

In Israel two main types of agricultural settlements exist, Kibbutz and Moshav. In the first, the farm is managed as a collective, and in the second as individual farms (small holdings).

During the project veterinarian assistance was sought concerning clinical problems regarding both neoplastic and immunosuppressive conditions on both types of farm. We have classified the incidence of those diseases in terms of the class of farm of origin. Table 22 shows this distribution by PCR on each type of farms including the flocks shown in Tables 17 and 18. To analyse the differences in prevalence of PCR positive flocks in Kibbutz and Moshav, we performed a statistical analysis (chi square), for chickens and turkeys separately. The difference was not statistically significant in either case. However, most of the positive flocks were raised in Moshavs, an indication of poor sanitation and isolation of the poultry houses.

Table 17: Commercial chicken flocks examined by PCR and ELISA

November 1993–November 1995

Flock status by PCR	REV-ELISA positive flocks		PCR positive birds detected in:				Histopathology– type of cellular infiltration		
			Tumor DNA		Blood DNA		No. flocks tested	Pleo– morphic (+)	Mono– morphic (++)
	No. positive/ No. tested	%	No. positive/ No. tested	%	No. positive/ No. tested	%			
MDV	8/31	26	27/35	77	65/150	43	14	10	4
REV	19/27	70	3/15	20	52/122	43	5	0	5
LLV	1/4	25	NT		6/15	40	NT		
MDV+REV	8/11	73	9/22	41	35/68	51	10	8	1
LLV+REV	2/2	100	NT		6/12	50	2	1	1
MDV+LLV	0/5	0	30/37	81	18/29	62	3	1	2
MDV+REV+LLV	1/2	50	4/7	57	7/14	50	2	1	1
Total PCR positive	39/82	48	73/116	63	189/410	46	36	19	16
Negative	19/31	29	0/7	0	0/141	0	9	4	3

+ Chickens – MDV/REV

++ Chickens – LLV/REV

Table 18: Commercial turkey flocks examined by PCR and ELISA

November 1993–November 1995

Flock status by PCR	REV-ELISA positive flocks		PCR positive birds detected in:				Histopathology– type of cellular infiltration		
			<u>Tumor DNA</u>		<u>Blood DNA</u>		No. flocks tested	Pleo– morphic (+)	Mono– morphic (++)
	No. positive / No. tested	%	No. positive/ No. tested	%	No. positive/ No. tested	%			
MDV	2/12	17	21/33	64	10/41	24	9	9	0
REV	2/6	33	0/5	0	17/38	45	NT		
MDV+REV	1/2	50	1/1	100	4/13	31	NT		
MDV+LPD	0/1	0	NT		4/6	67	1	1	0
MDV+REV+LPD	0/1	0	NT		4/6	67	NT		
Total PCR positive	5/22	23	22/39	56	37/104	38	13	12	0
Negative	4/18	22	0/1	0	0/70	0	3	2	0

+ Turkey – MDV/LPD

++ Turkey – REV

Table 19: Differential diagnosis of commercial turkey flocks

PCR analysis – MDV

Flock No.	REV-ELISA	PCR-Blood DNA	PCR-Tumor DNA	Histopathology
1	0/4	1/4	4/4	Pleomorphic
2	0/6	4/6	2/6	Pleomorphic
3	0/6	2/6	1/3	NT
4	0/6	1/6	3/3	Pleomorphic
5	0/7	0/7	2/2	Pleomorphic
6	12/13	0/5	3/3	Inconclusive
7	0/4	1/4	2/3	Pleomorphic
8	1/3	1/3	NT	NT
9	0/5	NT	4/4	NT
10	0/5	NT	1/1	Pleomorphic
11	0/5	NT	1/1	Pleomorphic
12	0/13	NT	2/2	Pleomorphic
13	NT	NT	1/1	Pleomorphic
14	NT	NT	1/1	NT
15	NT	NT	1/1	NT

Table 20: Differential diagnosis of commercial turkey flocks

PCR analysis – REV

Flock No.	REV-ELISA	PCR- Blood DNA	PCR-Tumor DNA	Histopathology
16	0/8	2/8	0/8	NT
17	0/10	5/10	0/1	NT
18	2/5	1/5	NT	NT
19	0/5	4/5	NT	NT
20	0/6	4/6	NT	NT
21	3/4	1/4	NT	NT

Table 21: Differential diagnosis of commercial turkey flocks having a mixed infection

PCR analysis -- MDV+REV

Flock No.	REV- ELISA	PCR - Blood DNA			PCR - Tumor DNA			Histopathology
		MDV	REV	MDV+REV	MDV	REV	MDV+REV	
22	2/5	1/5	1/5	0/5	NT	NT	NT	NT
23	0/8	NT	2/8	NT	1/1	0/1	0/1	NT

PCR analysis - MDV+LPD

Flock No.	REV- ELISA	PCR - Blood DNA						Histopathology
		MDV	REV	LPD	MDV+REV	MDV+LPD	REV+LPD	
24	0/6	3/6	0/6	0/6	0/6	1/6	0/6	Pleomorphic

PCR analysis - MDV+REV+LPD

Flock No.	REV- ELISA	PCR - Blood DNA						Histopathology
		MDV	REV	LPD	MDV+REV	MDV+LPD	REV+LPD	
25	0/6	1/6	0/6	0/6	0/6	0/6	1/6	2/6 NT

Table 22: Epidezootiology of avian oncogenic viruses, according to positive flocks in Kibutz and Moshav in Israel

PCR status	Kibbutz	Moshav	Total flocks
Chickens			
MDV	2	29	31
REV	3	24	27
LLV	1	3	4
MDV+REV	0	11	11
MDV+LLV	0	5	5
REV+LLV	1	1	2
MDV+REV+LLV	0	2	2
Total positive	7 (64%)	75 (74%)	82 (70%)
PCR negative	4	27	31
Total examined	11	102	113
Turkeys			
MDV	1	11	12
REV	1	5	6
MDV+REV	2	0	2
MDV+LPD	0	1	1
MDV+REV+LPD	0	1	1
Total positive	4 (50%)	18 (56%)	22
PCR negative	4	14	18
Total examined	8	32	40

Discussion

This section presents the results of the two-year survey of commercial flocks affected by oncogenic viruses. In most of the cases both PCR and ELISA for REV antibodies were carried out. Histopathological examination of the tumors was done wherever possible and in about half the cases virus isolation was attempted.

In the light of these results it appears that the PCR is an efficient method for the differential diagnosis of avian oncogenic viruses, and is of a special value in the detection of concurrent infection with more than one virus, which is more likely to occur in commercial flocks. By using the classical methods of virus isolation it would be very difficult and time consuming to attain such results, and considering the relative short time required to run the reaction, diagnosis by PCR is recommended for screening clinically affected birds and flocks.

B. PCR: Detection of the REV-env gene

In that section we describe an additional PCR system aimed to detect nondefective REVs based on the sequence of the REV-env gene (Wilhelmsen et al, 1984). That PCR system would be useful as an alternative to the LTR amplification system, and it can also serve to determine the occurrence of the REV-env gene integration phenomena into the cellular genome. That is important in the light of studies with avian retroviruses that showed a rapid integration into the host DNA, while large parts of the provirus, except the LTR, are excluded over time (Robinson and Gagnon, 1986). We also report the application of a non-radioactive, psoralen-biotin label for the probes, for Southern blotting and a sensitive colorimetric method of detection. That method was applied as a means to increase the sensitivity of detection and to confirm specificity of the PCR products, by avoiding the disadvantages imposed by using radioactively labeled probes, such as a limited half life and disposal problems. Both PCRs are now compared for their sensitivity in detecting REV's genome in infected chicken embryo fibroblasts (CEF).

Materials and Methods

Viruses

A total of 11 REV isolates were studied; these included three prototype strains, the replication-defective turkey isolate, REV-T (Robinson and Twiehaus, 1974), the nondefective chick syncytial virus (CSV) (Cook, 1969) and duck spleen necrosis virus (SNV) (Trager, 1959) provided by Dr. R. L. Witter (ADOL, East Lansing, MI, U.S.A.); and seven Israeli REV isolates, B, BI, EG, L, M, S and RA from commercial chickens, and isolate D from a commercial turkey flock (Davidson et al, 1995).

Control samples

DNA samples prepared from whole blood of birds infected with other avian oncogenic viruses and were used as controls for the specificity of the env primers for REV.

MDV-Y – a Marek's disease virus isolate from a 6-month-old egg type breeder flock.

ALV– a lymphoid leukosis virus isolate from a 6-month-old egg type breeder flock, and characterized as a LLV subtype A or C virus.

LPDV– a lymphoproliferative disease virus of turkeys obtained from Prof. A Yaniv, Tel Aviv University, Israel.

DNA preparation and the polymerase chain reaction

The PCR for REV-env employed primers designed according to the sequence of REV-A (Wilhelmsen et al, 1984):

The forward primer: TGA CCA GGC GGG CAA AAC C

The reverse primer: CGA AAG GGA GGC TAA GAC T

The predicted product size was 807 bp and spanned the region of 5999–6806 bp of the env gene. The primers used to amplify the REV-LTR and the PCR method were described previously.

Preparation of the hybridization probes

The internal probe for the env gene was prepared by PCR using the reverse primer and an additional primer at the 6528 bp of the env gene: TTC GGA AGA ATC TGT GCG GAA GGA C, using template DNA prepared from CEF infected with isolate REV-B. The predicted size of the product, named e3, was 278 bp. The nested PCR product was purified by phenol/chloroform extraction and its concentration was estimated after electrophoresis in 1.5% agarose gel by U.V. illumination in comparison with the 1kb DNA ladder run in parallel. The probe for REV-LTR comprised of the PCR product itself that was purified using the

Wizard PCR preps DNA purification system kit (Promega, USA).

Nonradioactive labelling of the probes using psoralen-biotin

The PCR probes were psoralen-biotin labelled using the Rad-Free system for probe labelling and detection (Schleicher & Schuell, Germany). The e3 probe concentration was 60 ng (in 27 μ l). It was denatured to single strands in TE buffer (10 mM Tris, 1mM EDTA) by boiling for 10 min and immediately cooled on ice and spinned down. Biotin-psoralen was added at a volume ratio of 1.1 μ l to 10 μ l DNA, and the mixture was placed in a microtiter plate well. The reaction was UV-crosslinked using a long-wave (365 nm) UV lamp with the plate on ice. The mixture was transferred to a clean microfuge tube and 2 volumes of H₂O-saturated n-butanol was added. The tube was vortexed and the top n-butanol phase was discarded. The extraction was repeated twice to remove unincorporated biotin-psoralen. The probe was then stored at 4°C or frozen until use.

Hybridization of blots and the colorimetric detection

The blots were crosslinked in a microwave oven for 2 min and prehybridized with 5 ml 5% Rad-Free blocking powder, 1% sodium dodecyl sulphate (SDS) and 5xSSC for 3–4 hr at 55°C. For the next step, the biotin-psoralen labelled probes, at an approximate concentration of 65 ng/ml were removed, diluted in 100 μ l TE buffer, boiled to denature and filtered through a 0.45 μ m filter directly into a new hybridization bag containing the filter and new prehybridization solution. The bags were incubated for 18 hr at 55°C with gentle agitation. After hybridization the filter was washed twice for 10 min with 2xSSC, 0.1%SDS at RT and two additional washes of 10 min. each with 0.1xSSC, 0.1% SDS at 55°C. The hybridization reaction on the NC filter was detected with the BCIP/NBT colorimetric substrate as described by Sun and Rigas (1994). First, the filter was incubated at RT with 0.2 mM NaCl, 0.1M Tris-HCl, pH 7.5, 0.05% Triton x-100 and 3% bovine serum albumin. This was followed by 25 min incubation in buffer (0.2 mM NaCl, 0.1 M Tris-HCl, pH 7.5, 0.05% Triton x-100 and 1:1650 streptavidin-alkaline phosphatase conjugate (included in the Rad-Free kit). The filters were washed 3 times for 10 min with 50 ml buffer I (0.2 mM NaCl, 0.1 M Tris HCl, pH 7.5, 0.05% Triton x-100), for 2 min with buffer II (0.1M NaCl, 0.1 M Tris-HCl, pH 9.5, 50 mM MgCl₂) and for 30 min in buffer III (0.1M NaCl, 0.1M Tris-HCl, pH 9.5, 50 mM MgCl₂, 0.32 mg/ml NBT, 0.16 mg/ml BCIP) under reduced illumination. The progress of the color reaction was monitored by visual inspection and was terminated by washing in 1mM EDTA.

Results

Detection of REV in infected CEF by the REV-LTR and REV-env primers

Fig. 9 shows the PCR reactions for the REV-LTR, the REV-env gene and the Southern blot of the product with the biotin-psoralen-labelled e3 probe, using DNA from uninfected CEF and from CEFs infected with the 11 REV strains. The REV-env PCR yielded the expected product of 807 bp that was intensely stained with the biotin-psoralen-labelled e3 internal probe. DNA from all virus-infected CEF produced a similar PCR product. The reaction with REV-T can be attributed to the sequences of REV-A, the coinfecting-nondefective virus

Figure 9

Amplification of REV-LTR and of REV-env gene by PCR in DNA prepared from uninfected CEF and from CEF infected with three REV prototype strains and with eight Israeli REV isolates, as indicated on top of each lane. The REV-specific PCR product is marked with an arrow on the right side of the figure. Top: REV-LTR PCR; Middle: REV-env PCR; Bottom: Southern blot of the REV-env PCR using the psoralen-biotin-labelled e3 probe.

of REV-T (Hoelzer et al, 1979).

The REV-LTR PCR yielded the expected product of 291 bp in all infected CEF preparations, while REV-T yielded a fainter product. It is notable that in the study of Aly et al (1993) the REV-T strain also yielded a PCR product of a lower intensity compared to other REVs. As that result was consistent, and as by the immunofluorescence analysis of REV-T-infected CEF, an intense signal was obtained with monoclonal antibodies to REV, we might assume that a sequence change exists in REV-T-LTR compared to the LTRs of other REV strains. Hybridization with the LTR probe resulted in specific staining pattern, identical to that obtained in the determination of the PCR specificity previously performed (Aly et al, 1993).

To determine the specificity of the env primers for REV we analysed DNAs of whole blood from birds infected with other avian oncogenic viruses; Marek's disease virus, isolate Y, lymphoid leukemia virus and lymphoproliferative disease virus. Only the DNA of REV infected chickens were reactive (data not shown).

The sensitivity of the two PCR systems for REV

To determine the sensitivity of REV detection by PCR using the proviral LTR primers and the REV env gene primers template DNA purified from CEF infected with the REV-B isolate was used. Seven tenfold dilutions of template DNA from 23.5 ng to 23.5 fg were used in both systems. Fig. 10 and Fig. 11 show the EtBr-stained PCR (panels A) and the hybridization reaction for the LTR and the env fragments (panels B), respectively. In both systems the same sensitivity of detection was achieved; By ethidium bromide staining of the PCR products it was 23.5 pg total DNA, and after the hybridization about 2.35 pg total DNA was detected. DNA from uninfected CEF was negative in both systems.

In Fig. 10, panel B, lane 1, the 291 bp PCR band is missing, but a 1230 bp product, found as minor in lanes 2 and 3, is evident, and is not specific. The reason for the absence of the 291 bp band is artifactual, as seen from lane 2.

Figure 10

Sensitivity of the REV-LTR PCR using DNA from uninfected CEF and from REV-B-infected CEF at the following DNA concentrations: lane 1–23.5 ng; lane 2–2.35 ng; lane 3–235 pg; lane 4–23.5 pg; lane 5–2.35 pg; lane 6–235 fg; lane 7–23.5 fg. Panel A – EtBr staining of the gel; Panel B – Southern blot using the LTR probe.

Figure 11

Sensitivity of the REV-env gene PCR using the same samples as in Fig. 10 and the e3 probe.

Application of the REV-env PCR system in comparison to the REV-LTR on blood samples

To assess the comparative efficiency of the two PCR systems for detecting REV infection, we analysed several chicken and turkey flocks and various organs of six REV-infected embryos (blood, liver, spleen, thymus, bursa of Fabricius and yolk sac membrane). As seen in Table 23, the blood samples of five flocks were negative for both the REV-LTR and REV-env genes; In six flocks the blood samples were positive for REV-LTR at a higher rate than for REV-env, denoting of a possible exclusion of the REV-env gene from the genomic DNA. That trend was found also in three of the six embryo analysed, where in the other three of the six embryos analysed, an opposite result was obtained, not yet understood.

Table 23: Analysis of whole blood and organs from REV-infected chicken and turkey flocks and from REV-infected chick embryos.

Flock. No.	Type	LTR-PCR	env-PCR
1	Chickens	0/3 (a)	0/3
2		0/3	0/3
3		0/4	0/4
4		4/6	0/6
5		3/4	1/4
6		1/4	0/4
7		4/5	2/5
8		4/5	0/5
9	Turkeys	0/3	0/3
10		0/3	0/3
11		4/5	1/5
S1	Chick embryos	5/6	2/6
D1		0/6	1/6
D3		1/6	5/6
D4		5/6	5/6
SNV3		0/6	1/6
REV-T4		1/6	0/6

(a) No. positive/ Total examined from that group

Discussion

We aimed to develop an additional PCR for the detection of infection with REV based on the amplification of the REV-env gene sequence. In this study a specific internal probe was labelled non-radioactively using psoralen-biotin in conjunction with a colorimetric method of detection. We showed that the primers aimed to amplify the 807 bp env gene sequence were effective in detecting REV infected-CEF with 3 REV prototype strains and with 8 Israeli REV

isolates. That PCR system was developed to provide an additional parameter to assess the infection with REV, and to determine which genomic part of the REV provirus has been integrated into the cellular genome. That system would be useful also in future studies that may employ a DNA vaccine that would contain only the REV-env gene. We show that the REV-env PCR is of similar sensitivity of detection in REV-infected CEF, compared to that previously reported system, in which proviral REV-LTR sequences were amplified (Aly et al, 1993; Davidson et al, 1995). In both systems we could amplify a specific PCR product from as little as 23.5 pg total DNA, and after hybridization with the relevant probes, a positive signal was obtained with ten times less DNA. The lower limit of sensitivity determined by Aly et al (1993) was less than 100 infectious SNV particles per ml of serum from 10 week old chickens. Using plasmid DNA that contains the proviral SNV the visual endpoint for EtBr stained bands corresponded to 15 fg or 600 copies of SNV and Southern blots detected products with 1.5 fg of target SNV. The difference in the results between the present and the study of Aly et al (1993) reflects the difference in titre of the infectious virus and the nature of the DNA used. Where Aly et al (1993) used plasmid DNA for the sensitivity determination, we used genomic DNA of CEF, that had the major contribution to the quantity.

The Rad Free non-radioactive probe labelling system used in this study utilizes a non-enzymatic probe labelling method employing the photoreactive reagent, psoralen-biotin, which intercalates specifically into DNA. When irradiated with long-wave UV light (365 nm) psoralen-biotin forms a covalent bond. Due to the non-enzymatic labelling strategy the variabilities and inconsistencies associated with nick translation and random primed labelling are eliminated. The method is simple, fast and highly efficient. Detection was performed by the addition of streptavidin-alkaline phosphatase conjugate and visualization using the colorimetric method described by Sun and Rigas (1994). Use of the colorimetric method of detection instead of chemiluminescence provided a simple alternative to the use of x-ray films and development process. With this method we have shown a high sensitivity of about 2 pg total DNA (compared with 0.1pg for plasmid DNA using chemiluminescence as reported by the manufacturer). As seen in Figs 10 and 11 use of the labelled probe enhanced the sensitivity of the PCR reaction by tenfold.

That study employed a non-radioactive method of probe-labelling which is advantageous compared to the commonly used radioactive labelling reagents. The advantages are the long-time stability of labelled probes, that ensure uniformity throughout the study, saves working

time, and also eliminates disposal problems of radioactive waste and workers exposure.

C. Immunohistochemical methods using monoclonal antibodies (USA)

Leaders: RL Witter, AM Fadly, RF Silva, EJ Smith and LF Lee (ADOL); plus W Reed (MSU)

Title: Improved methods for diagnosis of avian lymphomas

Objective: To develop improved methods for the differential diagnosis of Marek's disease, lymphoid leukosis and reticuloendotheliosis (2 types) in chickens (nonbursa lymphomas – RE–NBL) and bursal lymphomas (RE–BL).

Background and rationale

The realization that REV is widespread in poultry and can cause neoplastic lesions that resemble very closely both MD and LL has made the differential diagnosis of avian tumors extremely difficult, if not impossible, by conventional methodology.

A comprehensive collaborative project has been initiated to provide better diagnostic tests that will discriminate between tumors of different etiologic types. Ideally, these tests will be simple and can be applied in diagnostic laboratories. However, even more complicated tests will be welcome if they have the requisite specificity.

The approach is to develop a number of test systems, each based on unique monoclonals or DNA probes/PCR, and evaluate each for sensitivity and specificity against a battery of tumor and control materials. This approach emphasizes a uniform system for evaluating each candidate procedure, and the development of appropriate types of tissue controls.

Acquisition of tumor materials

Chickens were experimentally treated to create five classes of tumor and control materials for analysis. At appropriate times, chickens were killed and materials taken for virology, histopathology (paraffin sections) histochemistry (frozen sections), cytology (smears) and molecular analysis (tissues for DNA extraction). In the third year of the project, birds from three field cases have been added to group E4. However, more cases are needed. The classes are given in Table 24.

Selection of most useful antibodies

A battery of 25 antibodies was assembled and characterized for reactivity against bursa and spleen cells at various dilutions. Of these, 21 antibodies were selected for use representing T–cell markers, B–cell markers and other (IgM, macrophage, panleukocyte and MHC class II) and were obtained from Hyun Lillehoj, Chen–lo Chen and Don Ewert. Antibodies against viral

antigens were obtained from Dr. Lucy Lee; these included MATSA, pp38, ALV and REV. Table 25 presents the battery of seven antibodies that provide a good basis for differential diagnosis of lymphomas.

Development of histochemistry assay

An immunoperoxidase assay using the ABC complex procedure (Vector Labs) was developed, based on the imbedded in O.C.T. and snap frozen. Sections were cut at 4U with the cryostat and fixed in cold absolute alcohol for 5 min. The tissues were then dried at -10°C for 1 hour. Storage if needed was limited to 1-399 days at -70°C . The staining procedure involved sequential treatments with blocking sera, primary antibody, biotinylated secondary antibody, ABC complex working solution, and DAB substrate – each step separated by washes in PBS. The slides were then counterstained with hematoxylin.

Table 24: Categories of lymphomas and other tissues

<u>Code</u>	<u>Description</u>
A1	MD lymphoma
A2	LL lymphoma
A3	RE (bursal lymphoma)
A4	RE (nonbursal lymphoma)
A5	spontaneous lymphoma (ev21, alv6, line 0)
B1	non-tumorous, MDV-infected spleen (S1)
B2	non-tumorous, MDV-infected spleen (S2)
B3	non-tumorous, MDV-infected spleen (S3)
B4	non-tumorous, ALV-infected spleen
B5	non-tumorous, REV-infected spleen
C1	non-tumorous, non-infected spleen
C2	non-tumorous, non-infected spleen (with FCA+SRBC)
D1	MD lymphoma+ALV+REV (MD lymphoma expected)
D2	LL lymphoma+MDV (S1, S2) (LL lymphoma expected)
D3	RE NBL+MDV (S1)+ALV (RE NBL expected)
D4	RE BL+MDV (S1) (RE BL expected)
E1	MD/RE-NBL
E2	LL/RE-BL
E3	MD/LL
E4	field cases (>20 wks)

Table 25: List of the monoclonal antibodies panel

	Monoclonal antibody	Lymphoma type			
		MD	LL	RE-NBL	RE-BL
Lymphoma	CT3	+++	-	+++	+
	CB1	-	+++	-	+++
	CTLA3	+	-	++	++
	H19	(+)	-	-	-
	11B118	-	-	++	++
	16AL42	-	+++	-	-
Nerve	CTLA3	+		++	
	K-1	+		++	
	11B118	-		++	

Confirmatory studies on T versus B lymphomas.

Three T-cell monoclonals and three B-cell monoclonals were tested against a battery of defined tumors (see below).

Tumor	Bird	CT3	CT4	TRC-2	CB1	CB10	M4
MD	004	+++	+++	++	-	-	-
	006	+++	+++	+++	-	-	-
RE (NBL)	279	+++	++	++	-	-	-
	280	+++	+	+	-	-	-
LL	033	-	-	-	+++	++	+
	034	-	-	-	+++	+	+
RE (BL)	029	-	-	-	+++	++	+
	246	-	-	-	+	-	-

All the monoclonals were relatively successful in differentiating T from B lymphoma types, but some monoclonals were better than the others. It appeared from these studies that CT3 and CB1 gave the most consistent and intense staining within the T-cell and B-cell antibody

groups, respectively.

Confirmatory studies on MD versus RE (NBL) lymphomas.

A panel of four antibodies were screened against several MD and RE (NBL) tumors to determine which of these would be the most useful for differentiation (see below):

Tumor	Bird	C-la	CTLA3	H19	11B119
MD	004	+++	+	++	-
	005	+++	+	+++	-
	006	+++	+	+	-
RE (NBL)	279	+++	++	-	++
	271	+++	+++	-	++

Although earlier data obtained by Cooper and Chen (unpublished) suggested that the C-la antibody did not stain RE (NBL) tumors, we could not confirm this observation. More work may be needed to confirm the specificity of the staining obtained with this antibody. However, several other antibodies appeared to have value for discrimination of these T-cell lymphomas. The CTLA3 antibody (CD8) stained a much higher proportion of RE lymphoma cells compared to MD. The H19 antibody (vs. pp38 antigen of MDV) stained a variable proportion of MD lymphoma cells. The staining seemed specific for MD, but the inconsistency was somewhat of a problem. More information is needed on the expression of pp38 in lymphoma cells before this can be considered a useful criterion. The 11B118 antibody (REV) was positive on a large number of lymphoma cells. Although its intensity was only average, it had good specificity and appeared to have potential use.

Confirmatory studies on MD versus RE nerve lesions.

Earlier work focused only on lymphomas. However, both MDV and REV infections may induce nerve enlargements characterized by the infiltration of mononuclear cells. Therefore, it was of interest to examine the cells contained in nerve lesions induced by the respective viruses. When stained with CT3 antibody, both types of lesions appeared similar, indicating that T-cells were prominent. However, CTLA3 antibody (CD8) stained a far greater proportion of cells in RE nerves compared to MD (as was also true for lymphomas). The H19 antibody was tested but did not stain either nerve type. However, the 11B118 antibody (REV) stained a large proportion of cells in RE nerves; It may be necessary to determine whether staining is

present in nerves of REV-infected birds lacking tumors or nerve lesions. Some of these observations are summarized below:

Tumor	Bird	CT3	CTLA3	11B118
MD	278	+++	-	-
	276	+++	+	-
	282	+++	+	-
RE (NBL)	286	+++	+++	++
	281	+++	+++	++
	274	+++	++	++

Confirmatory studies on MD and RE bursal atrophy.

Although not part of the recognized spectrum of neoplastic lesions, bursal and thymic atrophy are recognized sequelae to both MDV and REV infection. Such atrophy is commonly present in birds with tumors or nerve lesions. An effort was made to investigate any possible differences between the process of bursal atrophy in birds with MD or RE (NBL) tumors. Interestingly, the CT3 antibody was consistently negative on bursas from uninfected chickens, indicating that few if any T-cells were normally present in bursas of young chickens. However, bursas of MDV-infected showed considerably follicular degeneration with lymphocyte depletion and intrafollicular T-cell infiltration. In contrast, bursas of REV-infected birds contained variable proportions of T-cells within the follicles. The interfollicular pattern for MD is consistent with reports that bursal lymphomas, when present, are always interfollicular. The intrafollicular pattern for RE may be related to the presence of REV antigen within the bursal follicles, as evidenced by staining with 11B118 antibody.

Tumor	Bird	CT3	11B118
MD	278	++ (interfollicular)	-
	282	++ (interfollicular)	-
RE (NBL)	286	+ (intrafollicular)	++
	281	++ (intrafollicular)	++
Control	287	-	-
	288	-	-

Confirmatory studies on LL versus RE (BL) lymphomas.

Additional samples of both tumor types have been collected. In accord with earlier data, staining with CT3 and K-1 antibodies show that RE bursal lymphomas have more T-cell and macrophage infiltrations than LL lymphomas. The extent of the infiltration varies, and is never extensive. Perhaps this infiltration represents an immune response to the expressed viral antigens in the lymphoma. The tumors also can be distinguished by staining with the respective antiviral antibodies; 11B118 (REV) and 16AL42 (ALV).

Tumor	Bird	CT3	K-1	16AL42	11B118
LL	033	-	+/-	+++	-
	034	+/-	-	+++	-
	035	-	-	+++	-
RE (BL)	029	+	+	-	+++
	246	++	++	-	+++
	247	++	++	-	+++

Testing of mixed infection samples. Evaluation of samples from mixed infections (Table 24, groups D and E) using the six antibodies listed above (Table 26).

Thus far, reactions of these antibodies with the D1 group samples appears to indicate the tumors are typical MD lymphomas (as expected). REV and ALV appear to replicate in the MD lymphoma cells and nearly every tumor cell seemed to be infected. This is a new finding, but did not seem to interfere with the diagnosis. The staining with H19 antibody (pp38) was variable, but when positive was useful in confirming a diagnosis of MD. When the pp38 test was negative, it was difficult to prove whether the tumor was MD or NBL.

Reactions of these antibodies with the D2 samples appears to indicate the tumors are typical LL lymphomas (as expected). Although these birds were also infected with two different MDVs, this did not seem to interfere with the diagnosis. No staining with H19 (pp38) antibody was observed. Too few samples from the D3 and D4 groups have been tested to permit conclusions. Several of the samples lacked readily identifiable tumors in those tissues collected for frozen sections. Probably the choice of tissues to be saved was not always optimum. It seems to be important to have tissues where tumor cells can be readily differentiated from normal cells. Nodular tumors are best. Spleens are generally not good, as

the differentiation is difficult.

Summary and Conclusions

- A. The battery of seven antibodies seems to provide sufficient information for diagnosis of MD and LL tumors, even in the presence of other viruses. However, firm diagnoses are complicated when REV is present due to the lack of tumor-specific markers.
- B. As determined previously, the H19 (pp38) antibody reacts with some but not all MD lymphomas. The conditions influencing pp38 expression in MD tumors is being studied in another phase.
- C. ALV and REV appear to replicate well in MD lymphoma cells as indicated by antigens demonstrated by histochemistry. This finding complicates differential diagnosis based on virus-specific characteristics such as virus isolation, staining with anti-viral sera, and virus-specific PCR.
- D. It is important in histochemical diagnosis to select a tissue where tumor cells can be distinguished from normal cellular components. Focal tumors are best. Tumors in tissues such as heart, liver, kidney, gonad and nerves are good. Tumors in tissues such as bursa may be good, providing that the tumor is large or occupies discrete follicles. Tumors in tissues such as spleen or thymus are difficult and should be avoided.
- E. It appears that the best differential diagnosis will require detection of both tumor-specific markers and viral antigens. However, mixed infections appear to present special problems. A diagnostic algorithm is being developed, but may require at least some additional testing.

Table 26: Histochemistry on tumors from mixed infections

Virus	Group	Bird	Age Days	Tiss	Observed Tumor	Expt	CD3 CT3	CE1 CP1	CD8 CTLA3	MDV H19	REV 118/118	ALV 6AL42	Diagnosis	Remarks
RR1B	D1	131	35	hea	+	T5311	++++	-	++	++	++++	++++	MD	
RPL40	D1	131	36	gon	+	T5311	++++	-	+	+	++	++++	MD	
SNV	D1	133	36	liv	+	T5311	++	-	+	++	+++	++	MD	
	D1	134	36	gon	+	T5312	++++	-	+	-	++	+++	MD/NBL	
	D1	136	36	liv	+	T5312	++	-	+	-	++	-	MD/NBL	
	D1	132	36	hea										
	D1	132	36	spl										
RPL40	D2	163	104	liv	+	T5312	-	++++	+	-	-	++++	LL	
R2/23	D2	163	104	spl	+	T5312	+	++++	+	-	?	++++	LL	
SD-1	D2	163	104	bur	+	T5312	+	++++	+	-	-	++++	LL	
	D2	167	110	spl	+	T5314	+	++++	+	-	-	++++	LL	
	D2	167	110	bur	+	T5314	+	++++	-	-	-	++++	LL	
	D2	173	114	liv	+	T5314	+	++++	+	-	-	++++	LL	
	D2	174	114	bur	+	T5330	-	++++	+	-	-	++++	LL	
	D2	174	114	liv	+	T5330	-	++++	+	-	-	++++	LL	
	D2	184	124	spl	+	T5314	-	+++	-	-	-	++++	LL	
SNV	D3	218	74	liv	+	T5333/36	++	-	+	-	++++	++	MD/NBL	
R2/23	D3	222	78	hea	+	T5333/36	++	-	++	-	++++	++++	MD/NBL	
RPL40	D3	243	130	bur	+	T5335	-	++++	+	-	++++	++++	LL/BL	
	D3	191	50		-									
	D3	229	84		-									
	D3	145	46		-									
SNV	D4	137	58	pro	+	T5338	+++	+	++	-	++	+++	MD/NBL	scattered lymphs, tumor?
R2/23	D4	138	58	vag	+	T5336	?	-	+	-	-	?		minimal tissue, few lymphs
	D4	192	128	bur	+	T5336	++	++++	+	-	?	?		
	D4	211	147	liv	+	T5338	-	++++	+	-	++	?	BL	good tumor
	D4	211	147	bur	+	T5338	-	++++	+	-	++	?	BL	good tumor
	D4	159	97	liv	-	T5336								no visible tumor in liver

NO.18	E1	187	41 spl	+	T5338	-	-	-	+++	-	NBL?	lg cell foci spleen, tumor?
SNV	E1	188	41 spl	+	T5338	+++	-	-	+	+++	MD?	diffuse lg cells spleen, tu
	E1	200	49 spl	+	T5338	-	-	-	-	++	NBL?	diffuse lg cells spleen, tu
	E1	201	49 spl	+	T5338	+++	-	++	-	+++	MD/NBL	good tumor
	E1	201	49 bur	+	T5338	+++	-	-	-	+++	MD/NBL	interfollicular tumor cells
	E1	221	70 spl	+								
	E1	221	70 bur	-								

RPL40	E2	146	72 spl									
SNV	E2	146	72 liv									
	E2	146	72 bur									
	E2	147	72 spl	+								
	E2	147	72 liv	+								
	E2	147	72 vag	+								
	E2	155	90 bur	-								
	E2	155	90 spl	+								
	E2	158	97 spl	+								
	E2	158	97 int	+								
	E2	162	103 kid	+								

RPL40	E3	166	spl	+								
SA	E3	166	bur	-								
	E3	176	liv	+								
	E3	176	spl	+								
	E3	178	spl	-								
	E3	178	gon	+								
	E3	180	bur	+								
	E3	180	spl	-								
	E3	216	hea	+								
	E3	216	bur	+								

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Description of cooperation

The ADOL supplied the KVI with monoclonal antibodies to REV and primers for REV-PCR during the first year of the project. Dr. Malkinson visited ADOL in September 1993 for coordinating the submission of the first annual report and discussions on the continuation of the project. Dr. Richard Witter from ADOL received valuable information from KVI on the status of REV infection in Israel. This information guided efforts in ADOL to develop appropriate diagnostic tools.

During the second year of the project an additional shipment of REV diagnostic reagents was received from ADOL. This included primers for ALV. Dr R. L. Witter visited Mertyn Malkinson and Irit Davidson at KVI in April 1994. Two on-site visits to farms with outbreaks of RE lymphomas were made.

During the third year of the project Dr. Irit Davidson visited ADOL on January 1995 and presented a seminar on the vertical transmission of REV and differential diagnosis by PCR. Fruitful discussions on future plans with Drs. Witter, Fadly, Smith and others were conducted. Dr. Davidson and Dr. Witter met for project-associated presentations at the workshop "Molecular Biology of Marek's disease virus" held in January 1995 in St. Petersburg, Florida. During the third year prototype REV strains were received by KVI for comparison with the Israeli REV isolates.

This project was built on an established fruitful collaborations between ADOL and KVI on the study of avian oncogenic viruses, ALV, MDV and now, REV. The collaboration started in 1981 with BARD project US-296-80 on LLV and continued with projects I-644-83 and I-1266-87 on MDV to the present project on REV.

Evaluation of Research Achievements

Erradication program – chickens: The data accumulated from rigorous testing of the grandparent (light) and greatgrandparent (broiler) breeding flocks indicate that while the former lines are free from REV infection, there appears to be a major problem associated with one foundation flocks. By selection of REV antibody free birds from that flock, REV infection was eliminated. The PBU are quite convinced about breeding only from REV negative females. To perform an effective eradication program, birds have to be housed in individual cages, and due to that opportunity the clean-up project was successful. The F1 generation was seronegative by ELISA.

Infectivity experiments – chickens: The results of this extensive experiment have some information concerning the pathogenesis of REV infection. The biological properties of the Israeli isolate, and in particular its tolerogenicity, places the virus in an unique situation regarding its effect on the immunocompetence of the bird. The experiment also sheds additional light on the degree of pathogenicity of wild-type nondefective REVs affecting commercial flocks. The persistence of the virus under field conditions appears to be as important economically as its ability to be vertically transmitted through the fertile egg and its eventual impact on the developing immune system of the embryo and hatched chick. The incidence of neoplasia in this experiment was quite low, while long-term morbidity and mortality without tumor formation were much higher.

Protection of progeny from horizontal infection. Much time and effort were involved in tracing the source and mode of transmission of the two episodes affecting the laying pullets. Our results indicate that the chicks were exposed early in their lifetime to the virus and that the parent flock played a critical role in its transmission. The exact nature of the contaminating event remains to be determined by close surveillance of the farm in future years.

Economic impact of the infection. Useful data have been collected on the effect that the virus

exerts on the economic performance of the laying pullets and the g.g.p. broiler flock.

Considering the pathology of these flocks, most of the damage takes the form of mortality of the birds, either before, or after they come into lay. In contrast, the broiler breeder flock appears not to have been affected by the infection. Because of the complexity of the situation regarding the "REV-high risk farm", no conclusions could be reached as to whether the parent flocks had performed badly in any way. Flock records did not reveal abnormally high mortality. In many other flocks with neoplastic or immunosuppression disorders where REV was diagnosed by PCR and ELISA, economic losses were due to the mortality of affected birds. In experimental infections of chick embryos we have shown that REV infection can cause a reduction in the hatchability rate of day-old chicks and to defective development of embryos and both parameters can lead to decreased production in hatcheries.

Differential diagnosis. A most valuable lesson to be learned from the study of the outbreaks of RE lymphomas in the laying flocks was the contribution of PCR as a diagnostic method in differentiating the several causes of tumors. Our results indicated that PCR could be effectively used on whole blood DNA or tumor DNA from relatively few birds in an affected flock. Likewise, good correlation was found between the PCR and ELISA at the flock level.

The data on histochemistry are promising for differentiation of tumors or tumor groups. Techniques for differentiating T and B lymphomas seem to be in hand. Several approaches have been identified for differentiating between MD and RE (NBL) lymphomas, nerve lesions and bursal atrophy and between LL and RE (BL) lymphomas.

Some additional data of interest has been obtained and may prompt followup studies on ontogeny of tumor development. Further study of the expression of pp38 antigen in MD lymphomas as revealed by staining with the H19 antibody may lead to a useful diagnostic tool, as well as a better understanding of the role of pp38 in MD oncogenesis. We have also noticed that not all LL or RE (BL) tumors appear to have identical cell types. Reasons for this

observation, if confirmed, would be important.

List of Project-Associated Publications and Communications

1. Davidson I., Borowski A., Perl S. and Malkinson M. (1995). Use of the Polymerase chain reaction for the diagnosis of natural infection of chickens and turkeys with Marek's disease virus and reticuloendotheliosis virus Avian Pathology 24: 69-94.
2. Davison I., Smith E. J., Perl S. and Malkinson M. (1995). PCR diagnosis of Marek's disease, reticuloendotheliosis and lymphoid leukosis in chickens and turkeys. In: PCR Protocols for diagnosis of Human and Animal virus diseases. Eds.: Y Becker and G. Darai. Suppl. of Frontiers of Virology, Vol. 1., pp. 543-553, Springer-Verlag, Berlin, Heidelberg, Germany.
- 3.. Davidson I. and Malkinson M.(1995). Natural and experimental mixed infections of chickens with Marek's disease and reticuloendotheliosis virus. Current Development in the Molecular Biology of Marek's disease virus workshop. St. Petersburg, Florida, USA, January 6-8.
4. Davidson I. (1995) Rapid identification of avian oncogenic viruses by PCR. Trinational Animal Health Project (TARHP). 4th Scientific Workshop and Symposium, Tiberias, Israel, April 23-27.
5. Davidson I., Yang H., Borowsky A. and Malkinson M. (1995) Vertical transmission of reticuloendotheliosis virus in adult chickens. Annual meeting of the Israeli Society for Microbiology, Technion, Haifa.
6. Davidson I. and Malkinson M. (1995) Natural and experimental mixed infections of chickens with Marek's disease and reticuloendotheliosis virus. Annual meeting of the Israeli Society for Microbiology, Technion, Haifa.

7. Malkinson M. (1995) The epidemiology of reticuloendotheliosis in Israeli poultry. Trinational Animal Health Project (TARHP). 4th Scientific Workshop and Symposium, Tiberias, Israel, April 23–27.
8. Davidson I and M Malkinson (1995) Rapid identification of avian oncogenic viruses (Abst). Progress in Clinical Virology, 1995 Joint Meeting, Prague, Czech Republic. Sept. 10–14, p.353.
9. Davidson I, H Yang, RL Witter and M Malkinson (1996) The immunodominant proteins of reticuloendotheliosis virus. Vet Microbiol. In Press.
10. Davidson I, and M Malkinson (1996). A non–radiactive method for identifying enzyme–amplified products of the reticuloendotheliosis proviral env and LTR genes using psoralen–biotin labelled probes. J. Virol. Methods, IN Press.
11. Davidson I., E. J. Smith, S Perl and M Malkinson (1996) Application of the polymerase chain reaction for the diagnosis of natural infections of chickens and turkeys with avian oncogenic viruses. Is. J. Vet. Med., In Press.