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Study of the Lymphoproliferative Disease of Turkey and its Causative Virus

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ABSTRACT

The research covered various basic and applied aspects concerning the lymphoproliferative disease (LPD) and its agent the LPD virus (LPDV).

Studies on the pathogenicity established the equal susceptibility of 2 selected lines of turkeys whereas under field conditions a much higher morbidity was recorded in one of these lines. Chickens were found to be susceptible to experimental infection with LPDV, But ducks and geese were resistant. It was found that infection with LPDV results in a life long viraemia in almost all of the affected turkeys. The lymphoid and hematopoietic organs were found to be the target organs and the thymus derived cells, the target cells. The integration and the expression of LPDV in various organs have been studied. The role of bursa and thymus in LPD were studied and also the influence of the LPDV infection on the immunological system of the afflicted bird.

The study of the LPD virion covered the morphological aspects of the virus and of the infected cell by electron and optical microscopy, its structural viral specific and host derived proteins. The host of origin of the virus was investigated by trying to detect viral specific nucleic acid sequences in the cellular DNAs of 8 avian and mammalian species. By immunological analysis of the reverse transcriptase of LPDV and the representatives of 4 other groups of retroviruses it was established that LPDV represents a distinct retroviral group. Various routes of inoculation, sources of virus and storage conditions were compared.

The virus was successfully grown in vitro only for short periods of time and in low titers but its continuous propagation was not achieved.

Immunological aspects of the disease and the preparation of immunological reagents were investigated. For the immunological diagnosis of LPD two procedures were established to be satisfactory for the detection of LPDV viraemia: the agar gel precipitation test (AGPT) and ELISA.

Guidelines for the routine diagnosis of LPD and its differentiation from other diseases were elaborated and published.

REPORT

A. Study of the pathogenicity of LPD.

A₁. Susceptibility of various birds to LPD virus.

Under natural condition the disease is known to affect only turkey flocks. In order to establish whether domestic birds other than the turkey are susceptible to this infection and to find alternative experimental hosts for this virus, the susceptibility of chickens, ducks and geese to experimental inoculation with LPDV was tested.

Experimental design. The initial inoculation of the experimental birds was done with LPDV from infected turkey plasma (Fc 254 and LPDV-2) at the age stated for each experiment. The effect of the inoculation was established by collecting at various intervals post inoculation (p.i.) citrated blood samples and testing them for virus associated DNA-polymerase activity. For ducks and geese in which this inoculation gave negative results, no further passages were done. The chickens gave positive results at this inoculation and serial passages were performed. Each new passage was done with pooled plasma collected in the previous passage from birds having a high and persistent particle-associated DNA-polymerase activity in their blood. Two series of passages were done in chickens: in the first trial the birds were lost after three passages due to a technical failure of the ventilation of the isolators. In the second trial six passages were performed. It was previously shown (McDougall et al. 1978) that 4 weeks old turkeys inoculated with LPDV develop gross lesions in a higher proportion than those infected at one day of age. For a comparison of the age related susceptibility the age of the birds at inoculation varied from one to 28 days.

Turkey poults were used in some experiments as a control for the infectivity of the inoculated virus, and for reisolation assay of LPDV passed in chickens. In order to demonstrate that the particle-associated DNA-polymerase in the inoculated chickens was a result of a productive LPDV infection, molecular hybridization experiments were done between RNA extracted from high speed pellets of plasma or from organs of LPDV inoculated chickens and cDNA of LPDV, AMV and REV.

RESULTS

Experiments on ducks: Six one-day old and five 3.5 weeks old ducks were inoculated intra peritoneally (IP) with 0,5 ml LPDV (Fc254). In the younger group one bird died at 16 days p.i. Out of 16 blood samples collected and tested during the experiment at 2, 3.5 and 5.5 weeks p.i., only 3 samples gave borderline positive results (< 2 pmole) and each time the positive sample was from a different bird (Table 1). Among the ducks inoculated at 3.5 weeks

of age, one bird was positive at 2 weeks p.i. but negative at the next test. Also in the group of control uninoculated ducks, 4 out of 10 blood samples gave weak positive results and the bird positive at the first test was found negative 3.5 weeks later.

In contrast with these weak and sporadic results, the turkeys inoculated with the same material showed a high susceptibility to this virus as indicated by the proportion of positive results (19 out of 21 blood samples), the high level of the particle-associated DNA-polymerase activity, and the persistence of their viraemia throughout the duration of the experiment (Table 1).

None of the ducks showed any gross or microscopic LPD lesions. The plasma of all ducks and turkeys were negative for REV in the AGPT.

Experiments on geese

Ten one-day-old geese were inoculated IP with 0.5 ml LPDV-2 diluted 1/10. The control group was comprised of four birds which were separately reared. The response of geese to LPDV inoculation (Table 2) resembled that of ducks. Also here there was only a low level of particle associated DNA polymerase activity in two different geese, one at 2 weeks and another one at 4 week postinoculation. This again in contrast to the high sensitivity of turkeys inoculated with the same batch of virus. As in the previous experiment, no gross or microscopic LPDV specific lesions could be detected in the inoculated geese. All the birds used in this experiment were found negative for REV in the AGPT test.

Experiments on chickens:

First trial: Fifteen 1-day old chickens were inoculated with LPDV-2 (dilution 1/10, 0.2 ml IP). Uninoculated groups of birds were reared in isolation. The inoculated chickens were under observation for more than 6 months and their plasma was checked for particle-associated DNA polymerase and by AGPT for REV and MD. The results are presented in Table 3. Plasma collected and pooled from the inoculated chickens in which viraemia was detected by the DNA polymerase test was used for performing the first passage. Three such successive passages were done, the first and the third passage on 2-day old chickens, the second passage on 3 weeks old birds in order to test their susceptibility also at this age. The results are presented in Table 3.

Second trial: The first group of 5 chickens was inoculated as in the previous experiment with LPDV-2 at 2-days of age. Plasma collected from two of these birds at 10 weeks p.i. was used for the first passage. Six successive passages were done. At the third passage two groups of birds, one of 1 week the second of 4 weeks old birds were inoculated with the same pool of viraemic plasma collected from the second passage. The results of the second trial are presented in Table 4. The pattern of the results was similar to that observed in the first experiment.

As can be seen from data presented in tables 3 and 4, not all the chickens responded identically. At each inoculation some birds had high DNA polymerase activity from the first sampling (1 to 3 weeks p.i.) and remained positive throughout the experiment, others were positive at the first test but remained so only for a short period of 3 to 4 weeks, others were, and remained negative throughout the experiment. The range of DNA polymerase activity between the positive birds were quite large, varying from 1.1 to 400 pmole (^3H) TMP, and in all the passages it was noticed that birds which showed initially high readings, remained at a high level throughout the experiment. None of the birds which had negative or low readings at the first week p.i. became later strongly positive and only in a few of them a short and weak (< 2 pmole) DNA-polymerase was observed.

Gross LPD-like lesions such as enlarged spleen and/or liver and congested thymus were rare (2 out of 64 chickens examined). Microscopic lesions reminiscent of those described for LPD affected turkeys (McDougall et al 1978, Perk et al 1979, Ianconescu et al 1979) were seen in the spleen (20 birds), liver (15 birds), pancreas (10 birds) and thymus (8 birds). None of the uninoculated birds showed gross or microscopic lesions of LPD.

All the blood samples collected in both experiments from the inoculated and control birds gave negative results when tested for REV and MD in AGPT.

Control experiments for the specificity of LPDV infection in chickens.

RNA extracted from high speed pellets of chicken plasma (1st and 3rd passage of the first trial) was hybridized to (^3H) DNA probes complementary either to LPDV, AMV or REV. The results of this experiment showed that while the level of hybridization with LPDV (^3H) DNA reached 63%, the hybridization with either AMV or REV (^3H) DNAs, was never higher than 4%. A previous study (Gazit et al. 1982) aimed at determining the organotropism of LPDV, established the bone marrow, thymus, spleen and bursa Fabricius as the sites of in vivo LPDV replication. Hence, to demonstrate productive LPDV infection in the chickens, these organs were surveyed for the presence of LPDV specific viral expression. RNA was extracted from the bone marrow, thymus and bursa Fabricius of infected chickens (5th passage, second trial) as well as from normal chicken embryos (SPF). The extent of transcription of LPD - related viral RNA in the various tissues was determined by analysing its hybridization to LPDV (^3H) cDNA as a function of $C_r t$. As demonstrated in Fig. 1, LPDV (^3H) cDNA hybridized readily to cellular RNA from thymus and bursa Fabricius (maximum hybridization level of 57 %) as well as bone marrow (maximum hybridization level 34 %), but scarcely to normal cells RNA. In contrast, there was no hybridization between the various affected cell RNAs and the (^3H) cDNAs complementary to either AMV, REV or Moloney mouse leukemia virus (Mo-MuLV). Based on the ratio of $C_r t$ 1/2 of 70 S LPD viral RNA (6×10^{-2} mole. sec. liter $^{-1}$) to that of tissue RNA (1.5×10^{-1} mole. sec. liter $^{-1}$) for the thymus and bursa Fabricius, and 3×10^{-2} mole. sec. liter $^{-1}$ for the bone marrow) we could calculate that approximately 0.4% of cellular RNA from thymus and bursa Fabricius and 0.02% of bone marrow cellular RNA is LPDV specific. Assuming estimates of 5×10^{-12} μg of RNA

per viral genome and 8×10^{-6} μ g of total RNA per cell (DAVIS et al. 1977) it appears that the thymus and bursa Fabricius contains 6400 copies of LPD viral RNA per cell, while bone marrow cells contains 320 copies.

Another control experiment was to reisolate LPDV from the experimental host - the chicken, into the original host - the turkey. Plasma positive for particle-associated DNA polymerase from the 5th passage in chickens was inoculated IP to 3 weeks old turkeys reared from 1-day of age in isolation. The turkeys were tested at 3 and 5 weeks PI for viraemia by DNA polymerase test and at the end of the experiment at 5.5 weeks killed and examined for gross and microscopic lesions. The results (Table 5) showed that all 4 inoculated birds had high level of DNA polymerase activity; gross tumoral lesions were recorded in 2 birds, and the third one had typical microscopic LPD lesions in thymus, spleen, liver and pancreas.

DISCUSSIONS

The DNA polymerase test was used to explore the susceptibility of chickens, ducks and geese to LPDV. Ducks and geese were found practically unsuceptible to this virus. During the whole experimental period, only in 6 out of 54 blood samples collected from the inoculated ducks and geese, a low and sporadic DNA polymerase activity was detected.

The fact that none of these birds showed any persistance of the positive results as observed in the inoculated turkeys and chickens and the absence of any gross or microscopic lesions, induced us to consider a number of possibilities as: a) In both duck and geese the LPDV inoculation results only in a short, abortive viraemia unaccompanied by any pathogenicity. b) Weak DNA polymerase reactions are known to occur due to technical artifacts such as the presence of micro-clots which were not eliminated by the clarification centrifugations and which can absorb and retain the radioactive material. c) Another possibility which had to be considered especially in the case of ducks, were also among the control uninoculated birds, 4 out of 10 blood samples gave positive results, was the presence of an unidentified agent with DNA polymerase activity. More experiemnts are necessary to clarify these possibilities.

In contrast to these results, the chickens were found to be susceptible to LPVD inoculation. Out of a total of 103 inoculated chickens 72 gave positive results in the particle associated DNA polymerase test and remained so for shorter or longer periods, some of them for all the duration of the experiment (29 weeks in one experiment). The level of the DNA polymerase reaction in the inoculated chickens showed a wide range upto 100 to 400 times the arbitrary base line of 1000 CPM (1 pmole (3 H) TMP) per 1.5 ml plasma tested. Although that in each experimental group the birds were from the same hatch and were inoculated with the same material the results were rather heterogenous. In each group these were some birds which showed a rapid onset and high level of DNA polymerase in their plasma, other birds gave positive results only for

short periods p.i. and these results were usually low (1-3 pmole), and others were negative throughout the experimented period. These differences in reactivity suggested that there might be differences in susceptibility among individual chickens and further experiments employing pure generic lines of birds are necessary to elucidate this aspect.

The age of the birds at inoculation varied between 1 day and 4 weeks. Although the number of birds inoculated over 1 week of age is too small to allow sound conclusions, the results, as percentage and level of positiveness do indicate a drop of the susceptibility in older birds. This observation differs from that reported by McDougall et al. 1978, where 28 days old poultts were found more susceptible than those inoculated at 1 day of age.

The specificity of LPDV infection in chickens was proved in vitro by the high level of hybridization between LPDV (^3H) DNA and the RNA extracted from the high speed pellet of plasma, or from organs of infected chickens, and in vivo by reproduction of typical LPD in turkeys inoculated with infected chicken plasma. The incidence of gross or microscopic LPD lesions in chickens was rather low and less severe than in turkeys, but it is evident from our results that LPDV can infect and replicate in chickens, and even if its oncogenicity in this host is low, it may serve as a virus reservoir.

TABLE 1. Results of LPDV inoculation in ducks and turkeys.

<u>Inoculated birds</u> (age at inoculation)	No.	DNA	POLYMERASE (pmole (^3H) TMP incorporated)	
			2 weeks ^a	3½ weeks 5½ weeks
DUCKS (1 day)	6	1/6 ^b c (1.30)	1/5 (1.10)	1/5 (1.94)
DUCKS (3½ weeks)	5	1/5 (1.85)	0/5 -	ND ^d
TURKEYS (2 days)	7	5/7 (1.91)	7/7 (4.37)	7/7 (8.19)
<u>Uninoculated birds</u>				
DUCKS	5	1/5 (1.51)	ND	3/5 (1.40)
TURKEYS	5	0/5 -	0/5 -	0/5 -

a. Time p.i. when samples were collected.

b. Number of birds with positive DNA polymerase (1 pmole (^3H)TMP)/number of birds tested.

c. Geometric mean of DNA polymerase results of the positive birds (pmole(^3H) TMP).

TABLE 2. Results of LPDV inoculation in geese and turkeys.

<u>Inoculated birds</u>		DNA POLYMERASE (pmole (³ H) TMP incorporated)			
(age at inoculation)	No.	2-3 weeks ^a	4-5 weeks	6-7 weeks	17-29 weeks
GEESE (1 day)	10	1/10 ^b (1.16) ^c	1/10 (1.35)	0/8 -	ND ^d
TURKEYS (3 weeks)	9	9/9 (9.34)	ND	8/9 (24.66)	7/8 (110.06)

Uninoculated birds

GEESE	4	0/4 -	0/4 -	0/4 -	ND
TURKEYS	5	0/5 -	ND	0/5 -	0/5

a,b,c,d same as for Table 1.

TABLE 3. Results of LPDV inoculation in chickens. First Trial.

<u>Inoculated birds</u>		DNA POLYMERASE (pmole (³ H) TMP incorporated).				
(age at inoculation)	No.	2-3 weeks ^a	4-5 weeks	6-7 weeks	9-11 weeks	17-29 weeks
<u>Primary inoculation.</u> (1 day)	15	13/15 ^b (5.89)	7/12 (4.51)	4/12 (8.92)	5/11 (3.72)	1/9 (12.66)
<u>1st passage</u> (2 days)	8	5/8 (10.52)	5/8 (10.54)	3/5 (6.64)	1/5 (32.74)	1/4 (36.20)
<u>2nd passage</u> (3 weeks)	10	4/10 (3.08)	5/10 (3.25)	3/10 (2.12)	1/8 (12.29)	1/4 (26.30)
<u>3rd passage</u> (2 days)	16	ND ^d	3/16 (2.57)	3/16 (11.21)	3/10 (21.36)	ND
<u>Uninoculated controls</u> (pooled)	28	0/28	0/24	0/16	0/12	ND

a,b,c and d same as for Table 1.

TABLE 4. Results of LPDV inoculation in chickens. Second trial.

Inoculated birds (age at inoculation)	No.	DNA POLYMERASE (pmole (³ H) TMP incorporated)				
		1-2 weeks ^a	3-4 weeks	5-7 weeks	9-11 weeks	15-19 weeks
Primary inoculation. (2 days)	5	1/5 ^b (1.37) ^c	2/5 (3.56)	2/5 (38.34)	2/5 (17.27)	ND ^d
1st passage. (4 days)	5	4/5 (9.45)	3/3 (11.22)	2/3 (14.29)	2/2 (6.27)	ND
2nd passage. (3 days)	9	7/9 (17.88)	ND	5/7 (4.24)	ND	ND
3rd passage (7 days)	8	ND	6/8 (37.95)	2/8 (18.92)	3/8 (48.29)	2/6 (82.64)
3rd passage (4 weeks)	10	1/10 (4.06)	5/10 (3.07)	5/9 (2.41)	3/9 (2.40)	1/4 (1.97)
4th passage (3 days)	9	ND	6/9 (12.18)	5/8 (9.44)	4/8 (33.08)	3/7 (21.97)
5th passage (3 days)	10	ND	9/10 (48.57)	9/10 (35.85)	10/10 (37.99)	9/10 (49.03)
6th passage (1 week)	14	10/14 (3.92)	8/14 (6.54)	6/12 (14.39)	5/11 (24.36)	4/9 (9.98)
Uninoculated controls (pooled)	45	0/28	0/39	0/45	0/24	0/12

a,b,c,d same as for Table 1.

TABLE 5. Results of inoculation of turkeys with chicken plasma from the 5th passage of LPDV in chickens.

	DNA POLYMERASE (pmole (³ H) TMP incorporated)		LPD lesions at 5 weeks p.i.	
	3 weeks p.i.	5 weeks p.i.	Gross	Microscopic
Turkeys (3 weeks)	4/4 ^b (24.34) ^c	4/4 (21.71)	2/4	3/4

b,c same as for Table 1.

Legend to Figure 1.

Detection of LPDV specific nucleic acid sequences in cellular RNA of LPDV inoculated chickens. Association kinetic analysis between LPDV-(³H)cdNA and LPDV-70S RNA (o), cellular RNA extracted from the thymus (▲), bursa of Fabricius (■), and bone marrow (●) of LPDV-inoculated chicken, or from normal chicken embryos (x); association kinetic analysis between REV-(³H)cdNA (□), AMV-(³H)cdNA (Δ), Mo-MuLV-(³H)cdNA (◇) and cellular RNA extracted from the thymus of LPDV inoculated chicken.

THE PERSISTENCE OF VIRAEMIA DURING THE LYMPHOPROLIFERATIVE
DISEASE INFECTION.

Plasma from LPD affected turkeys was found to be infective when inoculated into healthy turkeys.

By testing the particle associated DNA polymerase of this retrovirus in an exogenous reverse transcriptase assay the persistence and level of viraemia were studied. It was found that in both naturally and experimentally infected turkeys most of the birds became viraemic and remained so practically for all their life.

A commercial turkey flock of 1500 birds with cases of leukotic-like tumours was examined when the birds were 18 weeks old. According to the information obtained from the flock owner and the field veterinarian a mortality around 1.5% per week had been recorded in the previous 5 to 7 weeks. Citrated blood samples were collected from 18 randomly selected turkeys which were identified with wing band numbers and tested for virus-associated DNA polymerase. The flock remained under observation for 6 weeks and during this time the same 18 birds were bled three times. In order to facilitate the observation 8 of the wing banded turkeys were bought and reared at the laboratory on the floor, in the same room till the age of 35 weeks.

Experimental birds: The results to be presented in this work were collected from experiments in which turkeys were inoculated with LPDV for various purposes. The observation period of the experiments ranged from 3 to 18 months.

RESULTS

Seven out of 18 blood samples collected at 18 weeks of age from the field turkeys gave positive results for particle-associated DNA polymerase (Table 1). At the second test performed at 21 weeks of age, two birds (8 and 9) both weakly positive in the previous test became negative but two others (1 and 2) became positive. In the third test at 24 weeks, no additional positive or negative cases were observed. Eight turkeys, 4 positive and 4 negative were reared together at the laboratory for 11 more weeks. Of the 4 positive birds (1 - 4) one bird was found negative when tested at 30 and 35 weeks of age, the other 3 remained positive till the end of the experiment at the age of 35 weeks. No mortality was recorded among the 18 banded turkeys during the observation period (from 18 to 35 weeks of age). One out of four positive turkeys reared at the laboratory had at post mortem examination gross and microscopic LPD lesions, the other three microscopic lesions only. Although

the 4 negative birds were in contact with the viraemic turkeys throughout the 35 weeks of life all gave negative results during the experiment (results not shown). All blood samples were negative for REV antigen or antibody at all examinations.

Experimentally inoculated birds : Twelve 1-day-old poults were inoculated ip with 0.5 ml plasma from a naturally LPDV infected turkey. Two poults died in the first 5 days pi from undetermined causes. Five birds remained uninoculated and were reared in a separate room as controls. Blood samples were collected first at 4 weeks pi and thereafter at various intervals upto 53 weeks pi. The results of the DNA polymerase test presented in Table 2 show that 7 out of 10 inoculated birds were viraemic at 4 weeks pi and remained positive throughout the experiment. The uninoculated birds were negative in the DNA polymerase assay and all the birds were negative for REV all the time.

Twentyfour 3 weeks old turkeys were inoculated ip with 0.5 ml LPDV (dilution 1/20). During the observation period 4 birds died, 11 were sacrificed for organ collection for various experiments and 9 were still alive when this paper was written, and serving as a source of viraemic plasma, one and a half years pi. The results of the DNA polymerase tests for these 9 birds are presented in Table 3. From the 24 inoculated birds only two failed to develop viraemia and were eliminated at 8 weeks pi. During the observation period variations in the level of the particle associated DNA polymerase were noticed for every bird but none which was positive initially became negative. Out of 5 uninoculated turkeys from the same batch which were reared in the same room as contact controls 3 became viraemic at the ages of 9, 21 and 35 weeks respectively and the other two remained negative for 49 weeks when they were eliminated.

Gross LPD lesions were found in 3 out of 4 birds that died and in 5 out of 11 birds sacrificed during the experiment. Microscopic LPD lesions were present in 4 of the inoculated birds in which gross lesions were not found. None of the uninoculated birds reared in isolation had any gross or microscopic LPD lesions. All the blood samples collected during the experiment were tested for REV and were negative.

The results demonstrate that the viraemia in LPD ~~is~~ a consistant and ~~is~~ persistent, in most of the cases lasting practically for all the life time. No difference was observed in the persistance of viraemia between poults infected at one day or at 3 weeks of age and even ~~in a~~ ^{the} few turkeys which became infected by contact with inoculated birds and were detected as viraemic at 9, 11 and 35 weeks of age remained viraemic for more than one year. As reported also in other papers (Ianconescu et al 1981) no correlation was found between the level of viraemia and either the presence or the severity of the lesions.

(8c)

TABLE 1. Results of particle-associated DNA polymerase in plasma of naturally LPDV infected turkeys.

Bird No.	Age in weeks						LPD lesions	
	18	21	24	26	30	35	Gross	Micro
1	840 ^a	18300	71300	148600	124340	556420	-	+
2	850	10080	14430	76590	53480	86420	+	+
3	9650	85760	103420	397680	145300	12890	-	+
4	13440	6780	5120	1780	400	750	-	+
5	9540	56720	32790	ND ^b	ND	ND		ND
6	18470	100650	51790	ND	ND	ND		ND
7	134430	667800	438070	ND	ND	ND		ND
8	1250	710	390	ND	ND	ND		ND
9	3370	420	370	ND	ND	ND		ND

a. Counts/minute (CPM) per 1.5 ml plasma pellet.

b. Not done.

TABLE 2. Results of particle associated DNA Polymerase in plasma of experimentally inoculated 1-day-old turkeys.

Bird No.	Weeks post inoculation						LPD lesions	
	4	10	15	29	34	53	Gross	Micro.
1	10350 ^a	12760	121500	8540	15500	133470	-	ND ^b
2	2590	198460	63400	11320	7490	66520	-	ND
3	22470	93620	106880	78600	17420	142970	-	+
4	11690	31240	11440	7940	7300	21080	-	ND
5	8920	34540	53870	114370	25220	126570	-	+
6	28560	143640	82300	19830	7720	4650	-	+
7	10320	26400	46280	10260	8140	45200	-	ND
8	230	440	380	390	720	650	-	-
9	420	360	420	540	480	540	-	-
10	360	290	420	340	420	430	-	-

a, b same as in Table 1.

TABLE 3. Results of particle-associated DNA polymerase in plasma of experimentally inoculated 3 weeks old turkeys.

Bird No.	Weeks post inoculation							
	3	6	18	24	32	49	57	77
1	64500 ^a	57140	51050	63480	53840	53780	69550	47850
2	74340	40590	586340	107580	424590	729420	173480	724670
3	4590	6240	36530	35540	126730	124750	187440	94420
4	4080	13230	48580	86310	67590	ND	94640	116890
5	53430	37650	146150	232240	540320	587600	136640	945380
6	1460	13080	73250	56870	42180	68500	72090	47670
7	7310	7300	5930	19920	18370	13170	10470	15800
8	4430	13180	18980	114510	106900	88930	168630	143400
9	22480	21260	38460	142940	185350	226360	508720	312750
Uninoculated contact turkeys.								
10	480	2340	314340	219350	468760	512680	348720	498730
11	390	520	1590	12860	32990	29820	34690	39620
12	550	460	620	960	14200	68330	78480	99730
13	520	480	380	470	520	390	ND ^b	ND
14	390	410	520	630	480	530	ND	ND

a, b, same as in Table 1.

TABLE 4. Results of particle-associated DNA polymerase in plasma of chickens inoculated at 1 week of age with LPDV.

Bird No.	Weeks post inoculation				
	2	4	7	11	17
1	690 ^a	1040	ND ^b	ND	ND
2	2930	470	450	520	360
3	3340	1550	1090	ND	ND
4	470	350	470	420	460
5	790	760	400	650	ND
6	8380	24170	ND	ND	ND
7	15750	27270	104530	70260	38600
8	1090	330	420	560	540
9	1840	6860	22270	15720	12110
10	1100	490	450	560	530
11	670	460	450	560	410
12	43650	35250	22470	38160	13640
13	3670	2010	26790	8260	1560
14	1910	60990	5830	24640	ND

a, b as in Table 1.

In chickens experimentally inoculated with LPDV the viraemia was also persistent although the proportion of birds with a short transient viraemia seemed to be higher than in turkeys.

No method for detecting LPDV antibody has yet been found and it was therefore not possible to establish if this persistent viraemia is due to a lack of a specific immunological response or whether it can persist even in the presence of antibody.

The persistence of LPDV viraemia and its high level even in the absence of any apparent tumoural disease can be of importance for the consumers of the meat of such birds. At the same time it creates conditions for a continuous shedding of this virus both horizontally and vertically. These aspects deserve attention and have yet to be studied.

A₂ Target organs and target cells.

a. Biochemical aspects.

Inoculation of LPDV into 3-week-old turkeys results in lymphoproliferative involvement of the spleen, thymus and pancreas within 2 weeks post infection. In this rapid onset of the leukemic process, LPDV resembles the highly oncogenic avian acute leukemia viruses rather than the lymphatic leukemia viruses which provoke the disease after a prolonged incubation of months or even years.

Further insight into this viral neoplastic disease requires the resolution of several crucial questions, to wit: Which of among the many afflicted organs is the first to be attached by the virus, And if there is such a primary target, what is the route by which the virus spreads to other organs or tissues? Does LPDV replication occur in every afflicted organ? Are the target organs for virus replication and virus transformation identical? The present paper provides answers to some of these questions.

The organotropism of LPDV was established from the extent of virus replication within various organs as well as from tumor formation in turkeys. The high level of LPDV specific RNA sequences detected in lymphoid organs (thymus, bursa of Fabricius and spleen), as compared to the very low levels of LPDV expression in other organs such as liver, pancreas, brain and muscle, pointed to the lymphoid tissue as the target for LPDV infection (Fig.1).

FIGURE 1 - Distribution of LPDV-specific RNA sequences in various organs of infected turkeys. LPDV (³H)cDNA was hybridized at different Crt values to cytoplasmic RNA from two turkeys, obtained two months following their inoculation. Each point represents the percentage of hybridization obtained with the RNA extracted from the individual organs of each turkey. Solid lines are the average Crt curves for each organ from the total number of turkeys examined. Symbols: thymus, T (●); bursa of Fabricius, B (○); spleen, S (□); liver L (■); pancreas, P (▲); brain, Br (△); and muscle, M (◆).

Figure 1.

Further experiments were performed to systematically trace the kinetics of virus replication within different organs at various times following LPDV inoculation. (Fig.2 and 3). It was found that the bone marrow was the first organ to be infected, and subsequently, virus replication extended to the thymus, spleen and bursa of Fabricius.

FIGURE 2 - Distribution of LPDV-specific RNA sequences in various turkey organs at different times post infection. LPDV (^3H)cDNA was hybridized at different Crt values to homologous 70S RNA (A), or to cytoplasmic RNA extracted from the various organs on days 1-4(B), 5(C), 10(D), 15(E), 20(F), or 25(G) post inoculation. 70S RNA was extracted from purified LPDV virions. Cellular RNA was extracted from thymus, T(●), bursa of Fabricius, B(○), spleen, (S) (□), liver, L(■), pancreas, P (▲), brain, Br(△), muscle, M(◆), and skin, S (◇). Points and lines in this Figure represent the same as in Figure 1.

FIGURE 3 - Distribution of LPDV RNA sequences in the bone marrow and thymus of infected turkeys. LPDV (³H) cDNA was hybridized at different Crt values to cytoplasmic RNA extracted from bone marrow, B.M. (◇), or thymus, T (●), at days 3 (A), and 5(B) post inoculation. Points and lines have the same significance as in Figure 1.

When the same organs of the infected birds were examined microscopically and histologically at different periods following inoculation for tumor formation (Table 3), the organs established as clinically involved were the spleen, thymus, pancreas and later, the liver too. No tumorous involvement could be detected in the bursa of Fabricius.

The absence of any evidence for tumorous involvement of the bursa of Fabricius hints at the possibility that the exertion of LPDV oncogenic effects is confined principally to T cells or to their progenitors.

Regulation of LPDV organotropism

As has been shown, the bone marrow and then the lymphoid organs are targets for virus replication. Such a pattern of organ specificity might result from the capacity of LPDV to infect only lymphoid cells. Alternatively, such organotropism can be regulated at the level of transcription; that is to say, LPDV virions can possibly absorb, penetrate and integrate into chromosomal DNA of several types of cells, but only in lymphoid cells the integrated provirus can be expressed.

The study was designed to establish whether LPDV organotropism is controlled at the level of integration or at the level of transcription. To this end, cellular DNAs were extracted from various organs at different times following inoculation. The amount of LPDV genomes integrated within cellular DNAs was quantitated by studying the kinetics of annealing of radio-labelled LPDV cDNA with unlabelled cellular DNAs (Fig. 4, 5).

TABLE 3 - Gross and Histopathological Lesions of LPD in infected Turkey

ORGAN	Gross lesions					Histopathology				
	At various days after infection					At various days after following infection				
	1-5 d	10 d	15 d	20 d	25 d	1-5 d	10 d	15 d	20 d	25 d
Spleen	0/25	0/5	1/5(1+) ¹	2/5(1.5)	4/5(1.5+)	0/25	1/5(1+)	3/5(1.3+)	4/5(1+)	2/5(1+)
Thymus	0/25	0/5	2/5(1+)	1/5(1+)	2/5(1+)	0/25	0/5	2/5(1+)	1/5(1+)	5/5(1.4+)
Bursa	0/25	0/5	0/5	0/5	0/5	0/25	0/5	0/5	0/5	0/5
Pancreas	0/25	0/5	1/5(1+)	3/5(1.3+)	3/5(1+)	0/25	0/5	4/5(1+)	2/5(1.5+)	3/5(1.7+)
Liver	0/25	0/5	0/5	1/5(1+)	1/5(1+)	0/25	0/5	0/5	1/5(1+)	1/5(0.5+)
Muscle	0/25	0/5	0/5	0/5	0/5	0/25	0/5	0/5	0/5	0/5
Skin	0/25	0/5	0/5	0/5	0/5	0/25	0/5	0/5	0/5	0/5

¹The numbers given in parenthesis represent the mean degrees of damage as graded from 1+ to 3+ on the basis of gross or histopathological appearance of lesions.

FIGURE 4- Association kinetics of LPDV cDNA with cellular DNAs of target and non-target organs. LPDV (^3H)cDNA was hybridized at different Cot values to cellular DNAs extracted from various organs on the 3rd (A), 5th (B), 10th (C), 15th (D), 20th (E) and 25th (F) day post inoculation. Each point represents the percentage of hybridization obtained with the DNA extracted from the individual organs of each turkey. Solid lines are the average Cot curves for each organ from the total number of turkeys examined. Symbols: thymus, T; spleen, S; bursa of Fabricius, BF; bone marrow, BM; liver, L; pancreas, P; skin, S; brain, Br; muscle, M.

FIGURE 5 - Number of LPDV DNA and RNA copies in target and non-target organs. The number of LPDV DNA copies per haploid genome were computed as described in text from the hybridization data presented in Figure 4. The number of LPDV RNA copies per cell were calculated from the percentage of LPDV-specific sequences in the various organs as presented in a previous report (Gazit et al., 1982), based on the estimates

The outcome of this study revealed three major points: a) The level of integrated LPD proviruses within the DNA of lymphatic organs was higher than in the nonlymphatic ones. b) The primary organs in which new-integrated LPDV information was detected, were identical to those that were established as the first organs transcribing this viral information. c) The accumulation of integrated viral copies alongwith the time following infection, coincided with the increasing levels of LPDV specific RNA sequences. Taken together, these data indicate that LPDV organotropism is regulated at the level of integration, i.e. LPD proviruses can integrate only into the DNA of lymphoid cells, whereas cells of non-lymphatic origin seem to restrict LPDV productive infection at any one of the stages prior to integration.

The integration pattern of LPDV

Among the lymphoid organs, established as targets for LPDV replication, the thymus was found to contain the highest level of integrated LPDV proviruses. In contrast, the spleen and especially the bursa of Fabricius were found to contain low levels of integrated viral sequences. These low levels of viral sequences could stem from the infection of a low number of cells in the spleen and bursa of Fabricius or from the integration of few viral copies within the cell DNA of these organs.

A study was thus designed to establish the difference in the number of viral copies present in the various target organs. This was performed by cleavage of cell DNA with restriction enzymes, and determination of the number of LPDV viral copies by the Southern blotting technique, followed by hybridization with ³²P-labelled LPDV cDNA. The first set of experiments were conducted to identify the restriction enzymes that do not cleave within LPDV genome. Among the enzymes tested, several were found to cut the viral genomes more than twice (Sal I, Eco R_I, Bam HI, Kpm I, Bgl II, Hind III, Bal I, Sac I, Hpa I), while other two enzymes (Pvu II and Pst I) cut the genome once at each end, rendering all these enzymes unuseful for our study. Only the restriction enzyme Cla I did not cut within the viral genome. Thus, this enzyme was further used to study the number of integration sites of LPDV-nucleic acid copies. Cell DNA was extracted from the various lymphoid organs, and cleaved with Cla I. Following electrophoresis, blotting and hybridization to LPDV-³²P cDNA, it was found that LPDV integrated in multiple sites within the various organs. There was no difference in the distribution of the number of integration sites among the various organs, thus indicating that the low levels of integrated viral sequences in the cell DNA of the spleen and bursa of Fabricius result from the fact that a lower number of cells are infected in these organs.

The multiple sites of integration in the tumoral cells of the thymus and spleen show that the tumors induced by LPDV are not monoclonal. This in contrast to the monoclonality of tumors induced by chronic leukemia viruses in which the transformation is not a direct effect. Hence, these data suggest the existence of 'onc' gene within LPDV genome.

A₂b. The role of bursa of Fabricius and thymus in LPDV infection.

The effect of neonatal surgical and chemical bursectomy on LPD pathogenesis and viremia

Since LPD was accompanied by marked hypergammaglobulinemia (IgG, 7S) and since microscopic examinations of LPD lesions often revealed abundance of plasma cells not only around, but also within the lesions, it was thought that the lymphoid tumor cells may be B (bursa-descendent) cells. To test this hypothesis surgical or chemical bursectomy were performed after hatching. Chemical bursectomy was performed by daily injections of 4 mg of cyclophosphamide (CY) given for 4 days. Three and half weeks after this treatment turkeys were inoculated with LPDV. Turkeys were killed 4 or 9½ weeks after inoculation, spleen and bursa were wet weighed and organs were processed for histopathologic evaluation. Also, virus-associated plasma reverse transcriptase activity was determined at 3, 6 and 9 weeks after inoculation with LPDV. Results of this experiment (Tables 4-6) showed that LPD lesions could develop even in surgically and in chemically bursectomized turkeys and that viremia was much higher in CY-treated turkeys. Since many of the turkeys neonatally treated with CY were actually shown to be almost agammaglobulinemic (Table 7) and to lack humoral immune response towards 2 antigens, which showed that they were indeed deficient of B cells, we concluded that LPD can develop even in the absence of B cells and that most probably the target organ for both virus replication and for transformation are T cells.

TABLE 4. Corrected wet weights of spleen and bursa obtained from turkey poultts which were chemically or surgically bursectomized after hatching, and experimentally infected with LPD virus at 4 weeks

Experimental group ^a	Corrected organ weight (g/kg)			
	Weeks following infection			
	4		7½	
	Spleen	Bursa	Spleen	Bursa
Control - uninfected	1.21±0.19 ^b	1.10±0.14	1.38±0.16	0.97±0.09
Control - infected	2.55±0.60 ^c	1.50±0.05	1.50±0.13	1.14±0.12
CY ^e -uninfected	0.51±0.08 ^d	0.35±0.23 ^d	1.06±0.05	0.28±0.03 ^d
CY - infected	0.98±0.13 ^{cd}	0.31±0.03 ^d	1.01±0.09 ^d	0.34±0.03 ^d
Bx ^f -uninfected	1.02±0.11	-	1.49±0.24	-
Bx-infected	1.12±0.05 ^d	-	1.49±0.13	-

^aThere were 6-8 animals/group

^b Mean ±SE

^cSignificantly different from the corresponding uninfected groups (p<0.05)

^dSignificantly different from the corresponding non-bursectomized group (p<0.05).

^eCY = Chemical burnectomy by treatment with cyclophosphamide.

^fBX = surgical bursectomy.

TABLE 5. Virus-associated reverse transcriptase activity determined in the plasma of turkeys inoculated with LPD virus following neonatal surgical and chemical bursectomy

Experimental group	Weeks following infection		
	3	6	9
Control, uninfected ^d	0.8 ^a 0/8 ^b	1.6 1/7	2.5 1/5
Control, infected	4.9 14/17	4.4 10/12	16.2 9/9
CY, uninfected ^d	0.4 0/8	0.4 0/3	ND
CY, infected	23.8 ^c 15/17	101.1 ^c 10/10	95.1 ^c 6/6
Bx, uninfected ^d	0.5 0/9	1.0 1/4	ND
Bx, infected	10.6 18/21	8.4 12/13	16.4 8/8

^a pmoles of (³H)-TMP incorporated; geometric mean.

^b Number of viraemic animals/number of animals examined.

^c Significantly different from other infected groups ($P \leq .05$), by the nonparametric Mann-Whitney ranking test (one-tailed).

ND = not determined.

^d The uninoculated birds were reared in contact with the infected group.

Table 6. Summary of histopathologic findings, plasma viral-associated reverse transcriptase activity, serum Y-globulin level and serum agglutinins to SRBC and B. abortus in selected neonatal CY-treated turkey poult experimentally infected with LPDV and their uninfected controls^a

Treatment	Poult	Microscopic lesions	Plasma viral-associated reverse-transcriptase (3H)-TMP pmoles incorporated :			Serum γ-globulins, % of total protein ^b	Log ₂ titre agglutinating serum Ab ^c following 2nd immunization with	
			Weeks after infection				SRBC	B.abortus
			3	6	9			
Infected	1	+	80.3	302.9	-	- 0	1	3
CY-treated	2	+	9.2	21.7	-	1.6	1	3
	3	+	155.2	332.8	-	- 0	1	3
	4	+	53.3	208.4	162.0	- 0	1	4
	5	+	6.2	44.4	529.0	1.7	1	2
	6	-	23.3	27.7	-	2.8	2	1
	7	-	44.0	121.3	60.2	7.3	1	4
	Non-infected	8	-	.5	.3	-	- 0	1
CY-treated	9	-	.4		-	- 0	1	3
	10	-	.3	.2	-	19.8	1	1
	11	-	.3	.4	-	5.0	1	1

^aAnimals were injected 4 times with 4 mg of CY after hatching. They were infected with LPDV at 3½ weeks, immunized with both antigens at 5 and 6 weeks p.i. and killed 9½ weeks p.i.

^bSerum Y-globulins were 22.2 ± 1.9 percent of total serum protein in 8 intact controls.

^cAgglutinin titers of ≤ 3 are considered negative. Positive titers determined in intact infected and non-infected controls are illustrated in Text-figure 2.

Table 7. The effect of neonatal chemical and surgical bursectomy and of infection with LPD virus on serum γ -globulins level

Experimental group	Serum γ -globulins (g/100 ml):			
	Weeks following infection ^a			
	0	2	4	7 $\frac{1}{2}$ ^b
Control, uninfected	.39 $\pm$.06	.37 $\pm$.06	.36 $\pm$.09	.96 $\pm$.10
Control, infected	-	.60 $\pm$.07 ^c	.87 $\pm$.17 ^c	.84 $\pm$.08
CY, uninfected	.08 $\pm$.01 ^d	.07 $\pm$.02 ^d	.17 $\pm$.15 ^d	.19 $\pm$.13 ^d
CY, infected	-	.16 $\pm$.05 ^d	.24 $\pm$.17 ^d	.19 $\pm$.06 ^d
Bx, uninfected	.36 $\pm$.02	.32 $\pm$.05	.41 $\pm$.08	.62 $\pm$.08
Bx, infected	-	.66 $\pm$.16 ^c	.71 $\pm$.08 ^c	.95 $\pm$.07 ^c

^a Turkeys were infected with LPD virus at 3 $\frac{1}{2}$ weeks. There were 6 to 8 animals group.

^b Determined 7 days after 2nd immunization with SRBC and B. abortus

^c Significantly different from the corresponding uninfected group ($p \leq .05$).

^d Significantly different from the corresponding control group and the surgically bursectomized group. ($p \leq .05$)

The effect of bursectomy and of LPD virus infection on the humoral immunone response of turkeys.

Turkeys of the experiment described above were injected intraperitoneally (i.p.) with sheep red blood cells and with killed *Brucella abortus* organisms at 5 and 6 weeks after infection with LPDV. Agglutinin titers were determined 7 days after first and second challenge with antigens. Results of this analysis revealed a marked inhibition of antibody production in CY-treated turkeys but not in surgically bursectomized turkeys. Intact turkeys infected with LPDV did not reveal a decline in agglutinin titer; however, surgically bursectomized turkeys infected with LPDV showed a significantly decreased titer which was more pronounced after the first challenge, as compared to their bursectomized but non-infected controls. Three out of 12 and 4/14 bursectomized turkeys infected with LPDV did not produce antibodies to *B. abortus* and SRBC, respectively, even after the second challenge (figure 2). The effect of cyclophosphamide on the bursa in the above experiment was also confirmed by microscopic examinations of the bursa, which showed degeneration of the follicles, and by immunodiffusion and immunoelectrophoretic analyses of the serum which demonstrated the marked decrease or even absence of IgG in these turkeys.

The effect of niridazole on LPD pathogenesis and viremia

Two experiments were carried out using niridazole, a drug which suppresses T-cell functions in mammals and in chickens (Am. J. Vet. Res. 38:2013, 1977; Am. J. Vet. Res. 39:1858, 1978). In the 1st experiment a sub-lethal dose was used: 50 mg/kg were given by gavage in 4 consecutive days to turkeys at the age of 4½ weeks. Three days after cessation of this treatment turkeys were inoculated with plasma containing LPD virus (high reverse transcriptase activity). There were 4 experimental groups : 1) Intact control; 2) niridazole-treated controls; 3) LPDV-inoculated; 4) niridazole-treated turkeys inoculated with LPDV. Virus-associated reverse transcriptase activity was determined in the plasma of all turkeys 3½, 5½ and 8½ weeks after inoculation of the virus. Randomly selected turkeys of the 4 experimental groups were killed 13 weeks after inoculation with LPDV.

The blastogenic response of peripheral blood lymphocytes to mitogens, phytohemagglutinin (PHA) and Concanavalin A (Con A), was determined in all turkeys 1, 3 and 11 weeks after inoculation with LPD virus. Results of this experiment showed that the dose of niridazole used was not effective - it did not decrease the response of peripheral blood lymphocytes to mitogens. LPD pathogenesis and the viremia were not affected by niridazole. However, this experiment revealed a marked effect of inoculation with LPDV on the mitogenic response of lymphocytes (Table 8).

TABLE 8. Mitogenic response of peripheral blood lymphocytes of turkeys after in vivo treatment with niridazole and inoculation with LPD virus.

Experimental group	Weeks after infection	PHA (c.p.m.)	ConA (c.p.m.)
Intact control	1	91,017	87,420
	3	98,588	140,697
	11	129,903	59,146
Niridazole ^a	1	114,487	131,905
	3	102,570	131,485
	11	150,882	82,268
LPD virus	1	16,337 ^b	29,333 ^b
	3	90,889	134,869
	11	42,140 ^b	52,607
LPD virus + niridazole	1	42,754 ^b	38,494 ^b
	3	78,726	77,980
	11	62,489	48,983

^aNiridazole was given to 4 week old turkeys 50 mg/kg bodyweight on 4 consecutive days.

^bSignificantly different from the non-inoculated group. The non-parametric Mann-Whitney ranking test (one-tailed) was employed.

A second experiment was performed using higher doses of niridazole. After testing the toxicity of 3x200 mg/kg and 3x100 mg/kg the latter regimen was chosen, as it exhibited an LD₅₀ effect while the higher dose killed all the turkeys. Again, 4 experimental groups were used as above. All turkeys were weighed before and at various time intervals after treatment with niridazole. Virus-associated reverse transcriptase activity was determined 2, 4 and 8 weeks after inoculation with the virus.

Mitogenic response of peripheral blood lymphocytes was tested 1, 2, 4 and 8 weeks after inoculation with LPDV. Results of this experiment showed that niridazole caused a temporary suppression of the mitogenic response of lymphocytes. As mentioned, the regimen used was highly toxic, killing about 50% of the treated turkeys and the survivors exhibited a significant decrease in body weight, which partially recovered with time. Viremia was not depressed by treatment with niridazole but, rather, increased. Mitogenic response of peripheral blood lymphocytes was markedly decreased in turkeys which were inoculated with the virus. This effect was dramatic at 2 and 4 weeks p.i. but was less pronounced at 8 weeks p.i. (Table 9). These 2 experiments showed that toxic, nearly lethal doses of niridazole can depress the mitogenic response of lymphocytes (as a measure of T-cell activity) but that non-lethal though high dose did not have such

Table 9. Mitogenic response of peripheral blood lymphocytes of turkeys after in vivo treatment with niridazole and inoculation with LPD virus

Experimental group	Weeks post - infection	PHA (c.p.m.)	Con A (c.p.m.)
Intact control	1	170,682	157,987
	2	98,107	100,530
	4	120,150	128,136
	8	60,112	171,762
Niridazole ^a	1	109,373	108,643
	2	45,532	104,225
	4	103,827	79,422
	8	89,103	136,309
LPD virus	1	137,154	121,822
	2	8,395 ^b	10,032 ^b
	4	24,734 ^b	13,632 ^b
	8	90,025	132,023
LPD virus + niridazole	1	90,134	73,992
	2	7,688 ^b	5,640 ^b
	4	9,458 ^b	9,635 ^b
	8	42,615	55,868

^a Niridazole was given to 4 week old turkeys 100 mg/kg on 3 consecutive days.

^b Significantly different from the non-inoculated group ($P \leq 0.05$).

an effect. When effective in this respect, the drug enhanced virus-associated reverse transcriptase activity in the plasma. Thus, inhibition of T-cell activity prior to inoculation with virus did not interfere with its infectivity to lymphocytes and its replicative activity. Niridazole does not kill T cells but, rather, acts on the cell membrane and alters functional aspects. Even if T cells are indeed the target for LPDV replication and for transformation, treatment with niridazole prior to inoculation with virus could have no effect on viral activities, as apparent from the last experiment mentioned. Histopathologic examination performed on these turkeys 9 weeks p.i. demonstrated LPD lesions in the pancreas, spleen and thymus - organs which are usually most affected in LPD of turkeys. That inoculation with LPDV depresses the response of lymphocytes to mitogens upto 11 weeks p.i. is important, since beside direct mortality due to LPD in commercial flocks, the possible harmful effects of LPDV on the defence mechanisms are usually overlooked.

In few experiments we have observed a decline in growth rate of turkeys which were inoculated with LPDV at the age of 4 weeks. This effect lasted as long as 2 months after inoculation (experiments were usually stopped at this time) and ranged from 10 to 30% decrease in body weight. This and the inhibitory effects of the virus on immune mechanisms in turkeys, which may well increase their susceptibility to common pathogens present in the flock, make the eradication of LPD an important goal.

Enumerating of T-lymphocytes in LPD lesions using the histochemical demonstration of a-naphthyl acetate esterase

As mentioned above, (See section A2a) kinetic analysis for the presence of virally coded nucleic acids in the organs of turkeys performed 5, 10, 15, 20 and 25 days after inoculation with LPD virus, revealed the thymus as the most affected organ; the spleen, bursa and pancreas were less affected (in that decreasing order) and other tissues (striated muscle, skin and brain) were only minimally infected. This pointed to T lymphocytes as main targets for virus replication and, possibly, also for transformation. We therefore, tried to examine LPD lesions in various organs using acid a-naphthyl acetate esterase (ANAE), as a marker for T cells. This method was shown by others to be indicative of the presence of T cells in lymphomas in man, and to differentiate between B and T cells in lymphoid organs of chickens (Laboratory Investigation 39:40, 1978; Avian Disease 24:886, 1979). Turkeys which were inoculated with the virus at 4 weeks were killed 8 weeks p.i. Thymus, spleen, bursa and pancreas were fixed with buffered formol-sucrose for 1 day at 4°C, treated for another day with Holt's penetrating solution and then frozen. Frozen sections were prepared by cryostat and analyzed for ANAE activity (Avian Dis. 24:886, 1979), including the necessary controls (sections treated with stain only, etc.). These slides were compared to sections of the same organs which were prepared by cryostat and by the routine methods for microscopic examination, and stained with hematoxylin and eosin. Results of this experiment showed that LPD lesions in the pancreas

consisted of macrophages, which stained heavily for ANAE, of 10 to 20% T cells (containing 1 or 2 conspicuous granules of the stain), and about 70% lymphocytes which were negative for ANAE. LPD lesions in the spleen and the thymus were more variable in the percentage of ANAE positive and negative lymphocytes. We interpreted these results as pointing to the lesions being composed mainly of B cells or of relatively immature lymphocytes which could not be related to either B or T lines of differentiation. We are currently employing the ANAE method to tissue sections prepared from chicken leukosis and Marek's disease in order to clarify further the significance of this approach in identifying lymphocytes in avian lymphomas and especially in LPD. Fluorescent rabbit anti-chicken IgG is currently used by us in order to characterize lymphocytes in LPD lesions.

A₃ Age and turkey strain susceptibility.

Lymphoproliferative disease of turkeys has been recognised in Israel since 1976 (Ianconescu et al., 1979). Although in this country two strains of turkeys (A and B) are reared in approximately equal numbers, it was found that out of 10 field outbreaks of LPD between 1977 and 1979, nine were in strain A and only one in strain B.

In order to determine whether this difference in incidence was due to a genetically controlled susceptibility or to some fortuitous epizootiological situation, a number of experiments was performed.

Susceptibility of 3-week-old strain A and B turkeys to LPDV infection.

Groups of seven 3-week-old poults of each strain were inoculated intraperitoneally (ip) with 0.5 ml of a 1:10 dilution of the liver and spleen homogenate. Three birds of each strain served as uninoculated controls and were housed in a separate room. Infection was monitored by examination of plasma samples taken at intervals post inoculation (pi) for the presence of virus-associated reverse transcriptase. The experiment was terminated 30 weeks pi.

The results are presented in Text-fig. 1. In both strains a viraemic state was detected as early as 3 weeks pi. The level of viral reverse transcriptase reached a peak at 3 weeks in strain A and then slowly declined, whereas in strain B the peak was not reached until 15 weeks pi. At 30 weeks pi four out of five strain A and three out of four strain B turkeys were still viraemic.

One strain B turkey died at 5 weeks pi and it had typical gross lesions of LPD and none of the strain A birds died during the experiment. Two birds from each group were sacrificed at 8 weeks of age; three of them had only minor microscopic lesions of LPD, the fourth, a strain A bird had an enlarged spleen, congested thymus and mottled pancreas. Only minor lymphoid infiltrations or regressing lesions were observed in the turkeys sacrificed at the end of the experiment.

The plasma samples collected from uninoculated birds did not contain particle-associated reverse transcriptase activity, and the tissue sections examined at the end of the experiment were found to be free of lesions of LPD. All of the inoculated and control birds were negative for the presence of REV precipitogens or precipitating antibody..

Susceptibility of 1-day-old strain A and B turkeys to LPDV infection.

Nine 1-day-old turkeys of strain A and six 1-day-old turkeys of strain B were each inoculated ip with 0.5 ml. plasma from the field case of LPD, and examined as described in the previous experiment.

The results are summarised in Text-fig. 2, and are similar for the two strains of turkey. Viral-associated reverse transcriptase was detected in all the

Figure 1. Reverse transcriptase activity in the plasma of two strains of turkey inoculated intraperitoneally with lymphoproliferative disease virus at 3 weeks of age and controls sampled at intervals post infection. Solid vertical lines = strain A and broken vertical lines = strain B poults. The curves represent the geometric means for strain A poults (0—0) and strain B poults (0---0).

Figure 2. Reverse transcriptase activity in the plasma of two strains of turkey inoculated intraperitoneally with lymphoproliferative disease virus at 1 day of age and controls sampled at intervals post infection. Solid vertical lines - strain A and broken vertical lines = strain B poults. The curves represent the geometric means for strain A poults (0—0) and strain B poults (0---0).

inoculated birds from 4 to 15 weeks pi. At the termination of the experiment at 19 weeks pi three out of nine strain A turkeys and three out of four strain B turkeys were still viraemic, but the geometric mean values were much reduced compared with the values for 15 weeks pi.

Birds of both strains killed at the end of the experiment 19 weeks pi had minor histological lesions of LPD in the pancreas, spleen and liver. None of the birds had gross lesions of LPD.

The uninoculated turkeys were negative for particle-associated reverse transcriptase as well as for the presence of typical lesions of LPD. No evidence for infection with REV was found by the AGPT and histopathology in inoculated or control turkeys.

The low incidence of outbreaks of LPD observed in turkey flocks of strain B compared with strain A suggested that strain B was genetically resistant to infection with LPDV. To examine this assumption, turkeys of both strains were experimentally infected with LPDV. Because susceptibility to the development of LPD has been reported to be related to age at infection (Biggs et al, 1974; McDougall et al., 1978), the experiments were conducted in 1-day-old and 3-weeks-old birds.

In view of our previous observation (Yaniv et al., 1979) that LPD lesions are preceded by a retrovirus viraemia, which can be detected by the presence of virus-associated reverse transcriptase activity, the major parameter for ~~the~~ following the development of the infection in this study was the detection and level of this activity in the plasma. No obvious difference in the RT activity in the plasma of the two strains of turkeys was observed, except that for the poults infected at 3 weeks of age the level of viraemia reached a peak in strain A 12 weeks earlier than in strain B. The significance of this requires further investigation.

Although the number of inoculated birds was too small for definite conclusions to be drawn, the absence of a difference between the two strains in the incidence and severity of the lesions of LPD suggests similarity of susceptibility to the disease.

Analysis of major protein fractions of the serum in turkeys with LPD

This was analyzed in turkeys inoculated with LPDV as well as in sick turkeys obtained from farms with LPD breakouts. LPD was accompanied by a significant decrease in serum albumin level and by a very dramatic increase in the γ -globulin fraction which was shown (by ultracentrifugation, polyacrylamide gel electrophoresis, and by the immuno-diffusion technique) to be the IgG (7S) fraction (Table 1). Since transferrin moves with the B- and Y-globulins in paper electrophoresis and it is significantly altered in some avian diseases (i.e., reticuloendotheliosis), its level was also determined in both naturally

TABLE 1.— Paper electrophoretic pattern of serum proteins determined in turkey poultts infected with LPDV and in healthy controls

Treatment	Total protein (g/100 ml)	Albumin (g/100 ml)	Globulins (g/100 ml)					Albumin/Globulins
			α_1	α_2	α_3	β	γ	
3 wks:								
Control	2.93±0.02 ^a	1.55±0.08	0.24±0.03	0.34±0.03	0.14±0.02	0.38±0.02	0.28±0.03	1.20±0.08
n = 8								
Infected	3.04±0.07	1.32±0.04	0.15±0.04	0.34±0.04	0.11±0.02	0.50±0.02 ^c	0.62±0.08 ^c	0.82±0.04 ^c
n = 43	(104%) ^b	(85%)				(132%)	(22%)	(68%)
6 wks:								
Control	2.79±0.06	1.70±0.07	0.16±0.03	0.25±0.03	0.08±0.01	0.37±0.04	0.23±0.04	1.65±0.02
n = 8								
Infected	3.44±0.03 ^c	1.36±0.06 ^c	0.17±0.04	0.33±0.05	0.12±0.02	0.52±0.03	0.93±0.03 ^c	0.84±0.09 ^c
n = 21	(123%)	(80%)				(140%)	(404%)	(51%)

^aNumbers are mean ± SE

^bNumbers in parentheses are percent of control value

^cSignificantly different from control (P < 0.05).

and experimentally LPD-infected turkeys but transferrin level in the serum was found to be unaffected by LPD (Table 2). A summary of the frequency of microscopic and macroscopic lesions and serum proteins changes after experimental infection is shown in Table 3. Follow-up of serum γ -globulins and virus-associated reverse transcriptase activity in the plasma of field cases of LPD is shown in figure 1, showing that viremia was significant at 3 weeks p.i. and persisted for many weeks, and that the hypergammaglobulinemia also prevailed for long periods.

Determination of plasma and tissue total acid and alkaline phosphatase activity in turkeys inoculated with LPD virus

Since phosphatases are often used to monitor disease in man and animals, the level of these enzymes was determined in turkeys which were experimentally infected with LPD. It was found that serum acid phosphate activity was 27% decreased at 7 weeks (but not at 4 weeks) after inoculation of 4 week old turkeys with LPDV, and that plasma alkaline phosphatase was 21 and 18% decreased at 4 and 7 weeks p.i., respectively (Table 4). Total acid phosphatase activity in the spleen and the pancreas was not different from controls at 7 weeks p.i. Total alkaline phosphatase activity in the pancreas was 18% decreased in infected turkeys, while activity in the spleen was unaltered (Table 5). Since phosphatases activity in the plasma was determined by Sigma kit originally designed for clinical laboratories, its reliability was also examined by checking the activities in males, females and egg-laying female turkeys. It was found that the older turkeys had much lower alkaline phosphatase activity as compared to young turkeys, this age effect being well known for man and for avian species. Laying females had 2 to 3 fold increase in serum alkaline phosphatase as compared to males of the same age and breed. The significance of the decreased levels of serum phosphatases in turkeys inflicted by LPD is not clear yet. It may reflect inhibition of processes taking place in the bone marrow, pancreas, intestine or other organs of the tumor-bearing turkeys.

TABLE 2.— Determination of serum total iron, serum unsaturated iron-binding capacity (UIBC) and calculated total iron-binding capacity (TIBC) of control turkey poultts and those experimentally infected with LPD virus.

Treatment	Total iron ($\mu\text{g}/100\text{ ml}$)	UIBC ($\mu\text{g}/100\text{ ml}$)	TIBC ($\mu\text{g}/100\text{ ml}$)
3 wks:			
Control n = 8	153.3 $\pm$ 12.1 ^a	137.4 $\pm$ 18.1	290.7 $\pm$ 18.6
Infected n = 10	100.5 $\pm$ 16.2	155.4 $\pm$ 11.5	255.9 $\pm$ 16.1
6 wks:			
Control n = 7	101.6 $\pm$ 12.9	216.4 $\pm$ 14.1	318.0 $\pm$ 15.0
Infected n = 13	120.2 $\pm$ 10.5	224.4 $\pm$ 12.6	344.6 $\pm$ 10.4

^aNumbers are mean $\pm$ SE

TABLE 3.— Summary of parameters determined in turkeys following experimental infection with LPD virus

Treatment	Time, p.i.	Plasma reverse transcriptase activity	Hypergammaglobulinemia		Lesions ^a	
			Paper electrophoresis	PAGE ^b	Macroscopic	Microscopic
Infected	3 weeks	8.10 ± 1.62 44/50(88%)	20/41(49%)	23/28(82%)	9/29(31%)	22/29(76%)
	6 weeks	62.70 ± 14.30 21/23(91%)	13/22(59%)	16/23(70%)	10/23(43%)	21/22(95%)
	3 weeks	0.35 ± .05	0/8	0/8	0/8	0/8
Controls	6 weeks	0.31 ± 0.03	0/8	0/8	0/7	0/7

^aGross and microscopic lesions were usually detected in the pancreas, spleen and thymus; they were more rarely detected in the liver, kidney and gonads.

^bIntensity of staining in the IgG band was estimated; 1+ to 6+ ranks were arbitrarily given, as observed independently by 3 individuals. Intensity of 3+ or more was considered significantly elevated.

^cFor details of assay see text. Means ± SE is given

^dNumber of animals which demonstrated significant changes/total number of animals examined. An increase of more than 2 x S.D. above normal level was considered significant in analyses.

FIGURE 1. Analysis of virus-associated reverse transcriptase activity in the plasma, and of serum gammaglobulin concentration in 5 turkeys naturally infected with LPD virus (presented individually- $\circ \bullet \Delta \nabla \square$) and in 6 uninfected controls ($\blacksquare$; mean + SE). Clinical onset of LPD in this flock (with few mortalities) presumably occurred at the age of 17 to 18 weeks.

TABLE 4. Serum total protein and total acid and alkaline phosphatase determinations in turkey poultts experimentally infected with LPD virus.

Experimental group	Total protein gr/100 ml	Acid phosphatase (Sigma units / ml)	Alkaline phosphatas
4 weeks:			
Control (7) ^{a/}	3.68 ± .13 ^{b/}	.685 ± .178	48.50 ± 7.43
infected A (11) ^{c/}	4.42 ± .77 ^{d/}	.760 ± .178	38.10 ± 10.18 ^{d/}
" B (13)	4.50 ± .99	.762 ± .279	39.50 ± 8.72 ^{d/}
7 weeks:			
control (7)	3.91 ± .13	2.12 ± .32	47.14 ± 4.77
infected A (10)	4.11 ± 1.38	1.53 ± .39 ^{d/}	41.10 ± 6.35
" B (13)	4.27 ± 1.48	1.56 ± .32 ^{d/}	35.82 ± 9.90 ^{d/}

a/ The number of animals examined is given in parenthesis.

b/ Numbers are mean ± S.D.

c/ Four weeks old turkey poultts were inoculated with infective (LPD virus) mate obtained from Dr. P.M. Biggs (Houghton Poultry Research Station, England) (group A) or with highly viraemic plasma obtained from a turkey inflicted with LPD (field case) in Israel.

d/ Significantly different from the corresponding controls ($P \leq .05$).

Table 5. Total acid and alkaline phosphatase activity in the spleen and pancreas and total protein/organ wet weight determined in turkey poultts 7 weeks after experimental infection with LPD virus.

	Control	Infected ^{a/}
Acid phosphatase: ^{b/}		
spleen	10.69 ± .57 ^{c/}	9.97 ± 2.32
pancreas	2.95 ± .25	2.58 ± .16
Alkaline phosphatase: ^{d/}		
spleen	72.3 ± 9.2	73.4 ± 3.62
pancreas	48.1 ± 10.0	39.6 ± 5.40
Protein/wet weight (mg/100mg)		
spleen	16.23 ± .94	17.29 ± 1.50
pancreas	19.46 ± 1.91	20.75 ± 1.08

a/ Randomly selected from experimentally infected group B mentioned in table 5.

b/ μ moles of p-nitrophenyl released/mg protein/1 hour at 37°C.

c/ Numbers are mean ± S.D. There were 5 turkeys in each group.

d/ μ moles of p-nitrophenyl released/mg protein/1 hour at 37°C.

A₄ Inoculation routes, sources of infective materials and storage conditions.

a. Routes of inoculation. Groups of 5 three weeks old poultts were inoculated with viraemic LPDV turkey plasma intraperitoneally (IP), intravenously (IV), intramuscularly (IM) and intrachealy (IT). The result of the inoculation were assayed by the presence of LPDV in the plasma at 4 weeks pi in the DNA polymerase test (Table 1).

Table 1

No. of birds /group	Route of inoculation			
	IP	IM	IV	IT
5	5/5 ^a	1/5	3/5	2/5

a. Number of positive birds/number tested.

These results indicate that the most severe route of inoculation was IP but that the virus is infective also by the other tested routes. The fact that two out of 5 birds inoculated IT become infected indicate that even if this route is not as efficient as the IP one, the natural infection may occur by this way.

b. Sources of infective materials. Viraemic plasma, buffy coat, and 10% homogenates of thymus, spleen and bone marrow from an LPDV infected turkey were prepared and stored for one week at -70°. Groups of 3 weeks old turkeys, 5 birds/group, were inoculated IP 0.5 ml/bird, and 3 weeks pi the birds were tested for viraemia by DNA polymerase test (Table 2).

Table 2

No. birds /group	Inoculated material				
	Plasma	Buffy coat	Thymus	Spleen	Bone marrow
5	5/5 ^a	3/5	2/5	3/5	2/5

a. Number of positive birds/number tested.

No attempt to titrate these materials was done but plasma appears to be the best choice of infective material due to the possibility to obtain it without killing the donor and by the simplicity in its preparation and storage.

c. Storage conditions: A pool of citrated plasma collected from LPD turkeys was divided in glass sealed ampoules and stored at -20°, -70° and in liquid nitrogen. Samples of this material were tested for their activity by DNA polymerase test (Table 3).

Table 3

Time of storage	Temperature of storage		
	-20°	-70°	-196°
o (fresh)	141.2 ^a	139.4	140.2
2 weeks	140.3	141.1	138.8
6 months	68.2	138.3	141.6
20 months	4.3	96.4	139.7

a. Thousands of CPM/100 μ l

These results indicate that for short storage periods -20° is enough, but for longer periods liquid nitrogen or at least -70° are necessary.

During the experiments concerning the propagation of LPDV in cell cultures the stability of this virus at 37° was tested by inoculating it in a flask containing cell culture medium with 15% fatal calf serum but without cells. After 24 and 48 hours incubation, 4.5 ml medium was ^{ultra}centrifugated and the pellet tested for DNA polymerase activity. At 24h the activity dropped at 40% and at 48h only 5% of the initial activity was present.

B. Epiornithological studies.

B₁ Incidence of LPD. It was not possible to determine precisely the incidence of LPD due to the fact that according to the veterinary regulation LPD is not a disease in which every case has to be reported. Usually in field conditions not all the dead turkeys are examined and only if the mortality becomes significantly higher than the "normal" mortality the dead birds are brought for examination at the regional poultry diseases laboratories.

During the period of this research project 9 cases of LPD outbreaks were studied. In contrast with the observation done in 1977-1979, when out of 10 studied outbreaks 9 were in BUT flocks and only one in Nicholas turkeys, from 1980 until 1982 a third of the studied outbreaks were in Nicholas birds. All three outbreaks in Nicholas turkeys occurred in farms where in the same premises BUT turkeys were reared previously. Only in one case the farmer could tell us that among those BUT turkeys there were a few cases of birds with "big spleen" - probably LPD. The number of BUT and Nicholas flocks reared in Israel are practically equal and usually in a farm the same kind of birds are reared all the time. The increased incidence in the LPD morbidity in the Nicholas turkeys confirms our results (see A₃) that there is no difference in the susceptibility to LPD among these 2 strains of turkeys and that the difference in morbidity observed before 1979 was due to epiornithological conditions such as the import of the disease by the BUT turkeys from England where the disease appeared first. As previously reported, the age at which the LPD mortality was first recorded was around 9-10 weeks followed by a peak around 14-16 weeks dropping rather sharply after that. In one case only in a laying flock a prolonged course of the disease was observed but it was found that this flock had a double infection with both REV and LPDV.

Blood samples were collected from 22 random flocks at the slaughterhouse - total 248 blood samples - and tested for LPDV viraemia by DNA polymerase. Positive results were obtained from 74 out of 103 blood samples originating from 9 flocks. None of the carcasses from these flocks were condemned on the processing line due to any tumoral condition. In two other occasions it was possible to be present at the slaughterhouse when turkeys from flocks in which cases of LPD were recorded were processed. Out of 680 birds examined on the processing line only in two birds gross lesions were seen (one with enlarged spleen and mottled pancreas, the second one with congested thymus and mottled pancreas). Out of 36 blood samples collected from these turkeys before they were slaughtered 21 gave positive results in the DNA polymerase test. These results show that the incidence of LPDV infection is much higher than the tumoral manifested disease and at the same time indicates that in the flocks where the disease takes a manifested tumoral form part of the turkeys die with tumors during the acute phase (9-18 weeks of age) but probably in some birds the tumors are regressing. In contrast

with the low incidence of the tumors, the proportion of birds which remain viraemic is rather high.

No significant difference was found between LPD affected and negative flocks when their blood samples were tested for antibody against NDV (HI), hemorrhagic enteritis (AGPT), and Mycoplasma Galisepticum (slide agglutination).

B₂. Horizontal transmission. In 3 experiments uninoculated turkeys were reared in the same room or isolator with LPDV inoculated birds. The birds were checked periodically for LPDV viraemia with the DNA polymerase test. From a total of 22 such contact control birds 9 (41%) were detected as viraemic after 4 till 35 weeks of cohabitation with the inoculated turkeys. The number of birds was too small to determine if there is any difference in the horizontal transmission between groups in contact from 1 day or 3 weeks of age. It was not possible to accomplish the ornithological inquiry concerning the vertical transmission by checking the parent flock of the LPD affected birds due to organizational reasons. It was also not possible to obtain eggs from viraemic turkey hens from commercial flocks because the farmers refused to let us do the necessary selection i.e. to bleed the hens for selecting these viraemic and to separate them in cages for eggs identification. From the laboratory reared turkeys only a limited number of eggs were available and these eggs were used part for preparation of cell cultures (see C₃) part were hatched. None of the 6 poults hatched from these eggs was found viraemic but from such a number of birds no conclusion can be reached.

DNA extracted from spermatozoa obtained from a viraemic sir was tested by molecular hybridization with LPDV cDNA for homologous sequences to those specific to the viral genome. A positive reaction (43% hybridization) was obtained proving the integration of the viral genome in the DNA of the spermatozoa. No experiment for demonstrating the infectivity of the semen was done but this result emphasizes the possibility of vertical transmission of the LPDV.

C. Study of the LPD virus.

C₁ Morphogenesis of the virus.

a. Electron microscopy. Turkeys inoculated at the age of 4 weeks with LPDV developed lesions in the thymus, spleen and pancreas, and occasionally in the liver, gonads, kidneys and heart. Microscopic evidence for LPD was present starting 15 days after inoculation. LPD lesions consisted of pleomorphic population of lymphoid cells (lymphoblasts, lymphocytes, reticulum, plasma and fusiform giant cells). Examination by transmittance E.M. confirmed the lymphoid characteristics of the cells and demonstrated their irregular shape, the abundance of polyribosomes, and the presence of deep clefts and of nuclear bodies in some cells. Virus particles morphologically characteristic of Retroviridae and resembling murine oncornavirus C-particles were detected in tissues with LPD tumor involvement. Stages of virus formation were identified and particles budding from the cell membrane were characterized (showing crescent-shaped cores of 70-80 nm in diameter and an inner layer). Also, extracellular virus particles, 100-120 nm in diameter, were observed in these tissues. High speed pellets prepared from pooled plasma of LPD-bearing turkeys revealed also the presence of virus particles (mature and immature forms) typical for C-type retroviruses.

C₂ Biochemical study of the LPDV

C₂a. Structural proteins of LPDV.

The composition of LPDV structural polypeptides was analyzed by agarose gel chromatography (Fig.1) and SAS-PAGE (Fig.2,3). These analyses established the presence of nine proteins with apparent molecular weights of 76000, 42000, 31000, 28000, 20000, 15000, 14000, 12000 and 8500 daltons. Of these, the 76000-dalton polypeptide was found to be glycosylated (Fig.4).

In experiments entailing radio labelling of external protein, the 76000-dalton polypeptide was established as the major envelope glycoprotein of LPD virions (Fig.5). The 20,000-dalton protein too, is probably an envelope-constituent. The majority of the 76,000-dalton protein was found disulfide-linked to a small amount of 28000 and 42000 dalton proteins. A minor part of p³¹, too, appeared as disulfide-linked complexes (Fig.6).

Immunological, as well as enzymatic assays, were employed to identify the virus-coded proteins, and to distinguish them from contaminate proteins contributed by the host. Thus, by immunoprecipitation of LPDV protein with antinormal turkey plasma proteins and by immunoprecipitation of normal turkey proteins by anti LPDV serum, the majority of gp76, a part of p28 and p42 were established as host contributed proteins (Fig.7).

By immunoprecipitation with anti-actin-serum, the p42 protein was identified as cellular actin (Fig.8).

Fig. 1. Analysis of ¹²⁵I-labeled LPDV polypeptides by agarose gel chromatography in 6M GuHCl. (A) Purified disrupted ¹²⁵I-labeled virus was dissociated with 6M GuHCl, loaded onto a 100 x 1.5 cm sepharose 4B column and eluted with 6M GuHCl. Fractions (1 ml) were collected and the radioactivity was measured. The column was calibrated by protein markers - bovine serum albumin (68,000), alcohol dehydrogenase (37,000), and cytochrome C (11,700). (B) Peak protein fractions, as monitored by radioactivity were pooled, dialysed, lyophilized, and 4000 cpm from each pool were subjected to 10% SDS-PAGE. Lanes a through f correspond to peaks a through f of the GuHCl column illustrated in A. As reference, approximately 16,000 cpm of ¹²⁵I-labeled LPDV polypeptides were parallelly electrophoresed (lane V).

Fig.2. Analysis of ^{125}I -labeled viral polypeptides by 10% SDS-PAGE.

lane a - Mo-MuLV

lane b - ASV

lane c - LPDV

lane d - REV

Fig.3. Analysis of unlabeled viral polypeptides on 7.5% (A), 10% (B), 15% (C) SDS - PAGE, and of ^{125}I -labeled viral polypeptides on 17% SDS-PAGE (D).

lane a - molecular weight protein markers; lane b - MoMuLV; lane c - ASV; lane d - LPDV; lane e - REV.

Fig. 4. Analysis of viral glycoproteins by SDS-PAGE. Purified disrupted unlabeled virions of MoMULV (a), ASV (b), LPDV (c) and REV (d), were electroforesed on 10% SDS-PAGE and stained by PAS technique.

Fig. 5. Identification of external proteins. LPDV disrupted (a) or intact (b) virions were ^{125}I -radiolabeled and electrophoresed on 10% SDS-PAGE.

Fig. 6. SDS-PAGE analysis of LPDV polypeptides under nonreducing conditions. (A) ^{125}I -labeled polypeptide (50000 cpm) were disrupted with 10% SDS at 100° for 3 min. in the presence of ME (a), in the absence of ME (b), or in the absence of ME but in the presence of 0.05M iodoacetamide (c). (B) ^{125}I -labeled LPDV polypeptides (1.7×10^7 cpm) were disrupted in the absence of ME and electrophoresed on a preparative 10% SDS-PAGE. Following autoradiography the 4 bands of the high molecular weight polypeptides (bands a', b', c', d') were excised from gel, crushed and proteins were eluted and samples (20.000 cpm) were electrophoresed separately on parallel lanes in nonreducing conditions in (in the absence of ME and in the presence of iodoacetamide).

Fig. 7

Immunological identification of virus coded LPDV polypeptides.

(A) Immunoprecipitation of LPDV polypeptides by antiserum elicited against normal turkey proteins. Lane a - ^{125}I -labeled LPDV polypeptides (100,000 cpm); lane b - ^{125}I -labeled polypeptides (100,000 cpm) immunoprecipitated by anti normal turkey serum. (B) Immunoprecipitation of normal turkey proteins by anti LPDV serum. Normal turkey plasma, concentrated 4-fold against polyethylene glycol, was ^{125}I -radiolabeled and electrophoresed (100,000 cpm) before (a), or after (b) immunoprecipitation by anti LPDV serum. ^{125}I -labeled 100,000 g-pellet of normal turkey plasma (100,000 cpm) electrophoresed before (c), or after (d) immunoprecipitation with anti LPDV serum. ^{125}I -labeled normal turkey cell extract (100,000 cpm) electrophoresed before (e) or after (f) immunoprecipitation with anti LPDV serum. (C) identification of virus-coded polypeptides by immunoprecipitation with preabsorbed anti LPDV serum. Anti LPDV serum was preabsorbed with normal turkey proteins according to Avrameas and Ternynck (1969). ^{125}I -labeled normal turkey plasma proteins before (a), or after immunoprecipitation with anti LPDV serum (b), or with preabsorbed anti LPDV serum (c). ^{125}I -labeled LPDV proteins before (d), or after immunoprecipitation with anti LPDV serum (e), or with preabsorbed anti LPDV serum (f).

The virus-encoded components of gp76, and of p28 were identified by peptide mapping using partial proteolysis with V-8 protease (Fig.9 A,B). Employing peptide mapping it was also established that there is no structural relatedness between p31 and p28 (Fig.9,C).

Figure 8.

Identification of LPDV-associated p42 as host-contributed actin. ^{125}I -labeled LPDV polypeptides before (a), or after (b) immunoprecipitation with anti actin serum. ^{125}I -labeled pure actin before (c), or after (d) immunoprecipitation with anti actin serum.

Figure 9.

V8 protease cleavage profiles of LPDV polypeptides. Radiolabeling, protein elution from gels, and V8 protease digestion with 50 ug/ml of protease, were performed. Proteolysis products were analyzed on 17% SDS-PAGE. (A) V8 proteolysis of gp76 obtained from normal turkey plasma (a), or from LPD virions (b). (B) V8 proteolysis of p28 obtained from normal turkey plasma (a), or from LPD virions (b). (C) V8 proteolysis of viral p31 (a), or p28 (b).

C₂b

Search for the origin of LPDV

To establish the animal of origin of LPDV, we have adopted the approach of searching the presence of LPDV specific nucleic acid sequences in cellular DNAs of various animal species. The molecular hybridization assays conducted thus far between (³H) cDNA of LPDV and cellular DNAs (Table 2) revealed that LPDV does not originate either from the listed animal species, or from the turkey.

TABLE 2. Nucleic acid homology of LPD virus with cellular DNAs.

Cellular DNA	Maximum hybridization (%)	
	60°C	80°C
Turkey	9.7	5.5
Chicken	12.2	6.6
Mouse	11.7	5.5
Rat	15.7	9.1
Hamster	13.1	7.1
Guinea pig	11.1	6.5
Cat	12.1	6.2
Human	14.1	7.5

"LPD virus (³H) cDNA (0.05 ng) was annealed to a Cot of 10,000 mol.s/liter with the listed cellular DNAs as described in the text. After hybridization, the reaction mixtures were diluted in 2 ml of 0.12 M sodium phosphate buffer, pH 6.8, containing 0.4% sodium dodecylsulfate, and applied to a hydroxylapatite column at 60°C. Unhybridized DNA was eluted with 20 ml of the same buffer, and the hybrid was then melted in 5°C increments and eluted with 8 ml of buffer. After the 100°C elution, the column was washed with 0.4 M sodium phosphate buffer to remove any remaining hybrids. The percentage of hybridization at 60 or 80°C refers to the fraction of (³H)cDNA remaining hybridized when the column temperature was 60 or 80°C, respectively.

C₂c The relatedness of LPDV to other oncornaviruses.

Serological analysis of the reverse transcriptase of LPDV

On the basis of immunological analysis of the reverse transcriptase, several distinct classes of oncornaviruses can be identified - the avian leukosis-sarcoma viruses, the lower mammalian leukemia-sarcoma viruses, the primate leukemia-sarcoma viruses and the mouse mammary tumor virus.

Reverse transcriptase inhibition assays were performed using the IgG fraction of antisera directed against the reverse transcriptase of spleen necrosis virus (SNV)

(Fig.10), Rous associated virus - 61 (RSV-61) (Fig.11), Rauscher leukemia virus (RLV) (Fig.12), and Mason-Phizer monkey virus (MPMV) (Fig.13) as well as IgG from the respective normal animal (rabbit or goat) used for the preparation of antibodies. These IgGs were tested against the reverse transcriptases of the homologous or the related virus and against that of LPDV. The data obtained showed no cross-reactivity between the LPDV-reverse transcriptase and that of the tested viruses. Thus, consistent with the lack of genetic relatedness of LPDV to other oncornaviruses and its distinct polypeptide pattern, the antigenic determinants of its reverse transcriptase is also unique, indicating that LPDV belongs to a distinct retroviral group.

Fig. 10

Fig. 11

Fig. 12

Fig. 13

C₂d. Comparative study of the optimal requirements of reverse transcriptase reaction of LPDV and REV.

Differential diagnosis between LPDV and REV

A reverse transcriptase assay was applied as a diagnostic test for LPDV. The assay is able to detect LPDV in the blood of afflicted turkeys and to differentiate this virus from REV. The assay is based on the finding that the LPDV reverse transcriptase strongly prefers magnesium ions while the REV enzyme prefers manganese ions (Table 1). The assay is rapid, simple and requires only a small volume of blood.

Table 1. Lymphoproliferative disease (LPD) and reticuloendotheliosis (RE) diagnosis based on divalent cation requirement of the exogenous reverse transcriptase activity and on molecular hybridisation.

Turkey flocks	Specimen No.	Diagnosis by reverse transcriptase activity ^a				Diagnosis by molecular hybridisation assay ^b	
		Ct/min of incorporated (3H)TTP in the presence of		Divalent ^c cation requirement index	Diagnosis based on reverse transcriptase	% hybridisation of viral RNA with (3H) cDNA of	Diagnosis based on hybridisation
		Mg ²⁺	Mn ²⁺				
LPD-afflicted	1	179,474	76,130	2.3	LPD	55.3	LPD
	2	165,000	42,500	3.8	"	42.2	"
	3	5,998	972	6.1	"	21.8	"
	4	3,481	916	3.8	"	25.3	"
	5	2,556	655	3.9	"	26.7	"
	6	2,422	597	4.0	"	25.0	"
	7	2,345	994	2.3	"	26.5	"
	8	1,900	470	4.0	"	19.4	"
	9	1,593	796	2.0	"	17.5	"
	10	733	475	1.5	Negative	8.3	Negative
RE-afflicted	11	655	733	0.9	"	8.4	"
	12	34,581	296,548	0.1	"	9.1	RE
	13	25,836	70,003	0.3	"	11.2	"
	14	1,550	2,592	0.5	"	8.7	"
	15	1,125	2,247	0.5	"	7.5	"

^a Lymphoproliferative disease (LPD) virus was obtained from plasma of turkeys experimentally inoculated with spleen extracts from LPD cases. Reticuloendotheliosis virus strain T was grown in chick embryo fibroblasts. The viruses were purified by sucrose density gradient centrifugation. Viral pellets obtained from 2.5 ml of plasma were subjected to a DNA polymerase reaction for 30 min at 37°C.

^b Viral RNA was prepared as follows: Virus pellet from 5 ml of plasma was resuspended in 0.01 Tris-HCl pH 7.4 and lysed by adding SDS to a final concentration of 1%. NaCl (0.2M), and 0.25 mg/ml nuclease-free soluble RNA from yeast were added to the suspension which was then extracted twice with phenol-cresol solution. Following alcohol precipitation, viral

Table 1 contd/-

RNA₃ was dissolved in a small volume of 5mM EDTA, divided into two parts and each part was hybridised with (³H)cDNA of LPDV and REV. (³H)cDNA of LPDV and REV was synthesised in an endogenous reverse transcriptase reaction of purified viral particles as previously described. The fidelity of the synthesised DNA transcripts was established from the association kinetics analysis with their homologous 70S RNA and from the thermal stability of the hybrids. Hybridisation reactions between the viral RNAs and (³H)cDNA of LPDV or REV were conducted for 18 hours at 68°C and assayed by resistance to S1 nuclease.

$$= \frac{\text{Counts per min. of incorporated } (^3\text{H}) \text{ TMP in the presence of } \text{Mg}^{2+}}{\text{Counts per min. of incorporated } (^3\text{H}) \text{ TMP in the presence of } \text{Mn}^{2+}}$$

C₃. Propagation of LPDV in cell cultures.

The experimental design was to prepare cultures from :

- a. In vivo LPDV infected tissues such as thymus, spleen, bone marrow, buffy coat etc. collected from LPD birds or from potentially infected embryos (LPDV inoculated eggs and eggs laid by viraemic turkey hens).
- b. Various normal tissues and to try to infect them with LPDV by inoculation with viraemic plasma, virus pelleted from such plasma or by cocultivation with infected cells (thymus, spleen etc).

Different cell culture media were tested for improving the viability of the cells and various supplements were used to enhance the infectivity of LPDV for the cultivated cells (polybrene, DMSO, trypsin, DEAE etc).

The successful propagation of the virus in cultures was checked by observing the cultures for morphological changes (CPE) and by assaying the culture supernatant for particle-associated DNA polymerase. In the following part the experiments which gave negative results will be only mentioned and some details will be given only for those in which positive results were obtained.

1. Turkey eggs inoculated intravenously with LPDV. At 2 and 6 days pi the embryos and the yolk sac were collected and trypsinized. Control cultures were prepared in the same way from uninoculated eggs. The turkey embryo fibroblast (TEF) and the yolk sac (YS) cultures were observed for 12 days and then subcultured nine times (total time in culture : 90 days). No CPE was observed in the primary cultures or during the passages and the medium tested every second day for DNA polymerase remained negative throughout the experiment. This experiment was repeated thrice using also eggs from viraemic hens but also with negative results.

2. TEF prepared from normal eggs were inoculated with LPDV. The cells in suspension or as monolayer were treated with polybrene, DMSO, trypsin, DEAE, before and after inoculation and various cell culture media were tested (MEM, 199, Alpha-MEM, RPMI 1640) supplemented with 5-20% fetal calf serum (FCS) and 5-15% turkey or chicken serum. None of the 8 experiments done with such cultures gave any indication of growth of LPDV. The same experiment was done 4 times with chick embryo fibroblast cultures (CEF), with negative results too.

3. A number of cell lines were tested for LPDV propagation: SiRC, kitten lung, mink, FRH-L, HOS, bat lung, QT-6, TAHR and OFC. With each culture at least 3 blind passages were done after inoculation. All the results were negative. Various media and supplements tested or incubation of the cultures at 37° or 41° gave similar results.

4. As the target cells for LPDV are the T-cells (see A₂) a special attention was accorded to the assays to propagate this virus in such cell lines. Three lines of T-lymphocytes derived from Marek's disease lymphomas (MSB₁, HL₁ and HL₂) were obtained and inoculated with LPDV. A high DNA polymerase was detected in the HL₁ and HL₂ cultures inoculated with LPDV but also in the control cultures of these two lines. The presence in these cells of another retrovirus was suspected and a subtype A avian leukosis virus was found to be the contaminant agent (J. Payne personal communication). The MSB₁ inoculated cultures gave also positive results in the DNA polymerase test but this activity showed a continuous drop during the 8 to 14 days pi and disappeared completely afterward. An example of the results obtained with these cells :

DNA polymerase (CPM/4.5 ml medium ultracentrifugate).

	0 ^a	1	2	4	8	10	14	19	23	26
MSB ₁ + LPDV	40520*	13630	4790	3830	1170	2430	900	730	430	410
MSB ₁ cont.	420	510	360	430	520	380	410	430	500	390
LPDV in medium without cells.	41700	13700	1410	530	440	ND	ND	ND	ND	ND

a. Days in culture; 0: immediately after inoculation

* Only results showing 1000 CPM are considered as positive.

The cells were subcultured every 2 or 3 days. Various media or supplements did not improve the results. Addition of fresh cells to the culture resulted in rise of the DNA polymerase only for a short time. In no condition the propagation of LPDV in these cultures gave titers high enough to justify its utilisation for preparation of virus.

5. Cultures were prepared from LPDV infected turkeys organs known to contain the virus from the target organs experimnts (see A₂). Such organs as thymus, spleen, bone marrow, buffy coat, bursa of Fabricius were obtained from experimentally inoculated turkeys or from field cases of LPD. The tissues were minced and cultured either as fragments (Maitland type culture) or further dispersed by passing them through a nylon mesh. The fragment or the cell cultures were tested for optimal survival and multiplication in various cell culture media and supplements. The best results were obtained with medium Leibovitz and McCoy in equal parts supplemented with 10% triptose broth, 15% FCS, 5% chicken or turkey serum and 2 mercaptoethanol 10⁻⁵M + Na pyruvate. Results of such cultures are presented :

DNA polymerase (CPM/4.5 ml medium ultracentrifugate)

	Primary culture			1st passage			
	1st ^a	2nd	3rd	1st ^a	2nd	3rd	5th
Spleen fragments	2150	1700	790	630	580	460	ND
Spleen cells	1070	480	510	360	420	320	ND
Thymus fragments	12150	3420	500	730	510	840	ND
Thymus cells	5500	1920	600	460	480	390	ND
Bone marrow cells	5510	5200	1945	8480	5600	1920	640
Bursa of Fabricius	650	470	480	340	410	390	ND

a. Days in culture.

The highest DNA polymerase activity was found in the thymus fragments culture but dropped rapidly and disappeared on the third day in culture. The bone marrow (BM) culture had at the beginning a lower DNA polymerase activity but this persisted also in subculture for a total of 6 days. Only in BM cultures could an active cell proliferation be seen both in the cells floating in the supernatant and in those which were adherent to the culture flask. In the BM subculture, fibroblast-like cells appeared among the macrophage cells and in the subsequent cultures the fibroblasts become predominant. This experience was repeated thrice with similar results, and cultures prepared with chickens organs from LPD infected birds also gave comparable results. Cultures prepared from organs from normal birds gave constant negative results.

6. Based on the previous observations that cultures prepared from LPDV infected organs continued to produce virus for a short period after explanation (5) and that some virus replication occurred also in LPDV inoculated MSB₁ cells, it was tried to combine these cultures by cocultivation of MSB₁ cells with cells prepared from infected organs. Cultures containing only cells from infected organs with the same density as in the mixed cultures, or only MSB₁ cells were prepared as controls.

	DNA polymerase (CPM/4.5 ml medium ultracentrifugate)										
	Days in culture										
Thymus + MSB ₁	7730	1420	930	2760	750	570	ND	ND	ND	ND	ND
Thymus only	5670	2510	1400	900	ND	840	ND	ND	ND	ND	ND
Spleen + MSB ₁	2360	930	480	1120	1020	650	ND	ND	ND	ND	ND
Spleen only	2210	540	420	ND	960	1430	630	ND	ND	ND	ND
BM + MSB ₁	2860	2950	2510	2260	1350	990	560	ND	ND	ND	ND
BM only	4440	13840	3830	1920	1370	1410	2210	2060	1240	500	570

ND - Not done.

The results showed that LPDV persists longer and with a somewhat higher titer in cell cultures from infected organs than in those in which these cells were cocultivated with MSB₁ cells. Same kind of results were obtained with cells collected from LPDV infected chickens and with buffy coat cells.

7. In order to try if higher LPTV titers and its growth for longer periods can be obtained, fusion between MSB₁ cells and cells from infected organs was tried by treatment with polyethylene-glycol. The results were the same as with unfused cells.

8. Cultures in soft agar with cells from infected organs or with fragments of such organs and with cells inoculated in vitro were tried. Foci of proliferating cells were observed especially in BM cultures but none of these foci could be subcultivated more than twice or thrice and no LPDV could be detected in such cultures. The propagation of LPDV in cell cultures was a key objective of this project.

The possibility to obtain the virus in quantities and in a pure form by its growth in vitro was a necessary condition for the immunization experiments, the preparation of various antigens for diagnosis tests and attempts to prepare a vaccine. Although a big volume of work was consecrated to this problem only partial results were accomplished. The virus was cultivated only for short periods and in low titer but the objective to find a host cell culture able to produce quantities of virus was not reached.

Recently, lines of turkey T-cells were established (K.Nazerian) and it will be of great interest to try such cells for LPDV propagation. It will be tried also to stimulate and improve the growth of T-cells from LPDV infected organs with the T-cells growth factor such as Interleukin II.

D Immunological aspects

D₁ Preparation of reagents. The preparation of LPDV immune serum proved to be a very difficult task.

The first attempt tried was to select from the experimentally infected turkeys the birds in which the viraemia following the inoculation will drop and disappear assuming that this is a result of the immunization and appearance of humoral antibody. It was found that with very few exceptions the turkeys remain viraemic for more than a year and a half i.e. practically for all their life. No difference in this aspect was observed between birds inoculated at 1 day and those inoculated at an age when they were immunologically competent i.e. after 3 weeks or by contact transmission of the infection.

A number of attempts were made to immunize rabbits. They were injected with LPDV pelleted from turkey viraemic plasma or with density gradient (sucrose 20-55%) purified virus. Different immunization schedules were used with or without adjuvants (Freund complete or incomplete). Sera from these rabbits was tested in an ELISA assay and in agar-gel-precipitation test (AGPT) with viraemic and normal plasma. In these tests a very strong reaction with both the LPDV and normal plasma was observed. The absorption of the anti turkey antibody was tried by treating the rabbit sera with glutaraldehyde-insolubilized immunoabsorbent prepared with normal turkey plasma or turkey embryo tissues. When sufficient absorption to abolish the anti turkey reaction was done, the sera lost also their reactivity versus the LPDV containing materials. It is known that the retroviruses tend to include antigens contributed by the host and this could explain the reactions against normal turkey proteins. In order to avoid these difficulties LPDV was disrupted by treatment with GuHCl and its proteins separated by electrophoresis in SDS-PAGE. The p20, p31 and part of the p76 were identified as specific viral proteins and strips of SDS-PAGE containing these fractions were eluted and used for rabbit immunization. The sera obtained were tested in AGPT but failed to react either with LPDV (viraemic plasma or concentrated (pelleted) virus) or with normal turkey proteins. In the ELISA test (plates coated with rabbit gammaglobulin, reacted with viraemic LPDV plasma followed by the alkaline phosphatase conjugated to rabbit gammaglobulin) these sera gave unsatisfactory results, not being able to differentiate the LPDV containing turkey plasam from the normal plasma. It is possible that the quantity of purified proteins used was not big enough to elicit a good immunological reaction in the immunized rabbits.

In order to avoid the formation of anti turkeys antibody new attempts to immunize turkeys and chickens were done. Turkeys were injected thrice with LPDV disrupted by treatment with Nonidet P-40 or by Tween-Ether extraction and then inoculated with live LPDV as booster and also for testing their resistance to infection. All these birds become and remained viraemic for more than 4 months when they were eliminated.

In chicken a different approach was tented: During the experiments with LPDV in chickens it was observed that some of the inoculated birds become viraemic only for short periods. Such birds were reinjected twice with LPDV pelleted from chicken viraemic plasma and twice with the same material extracted with Tween-ether. None of these birds was found viraemic after the reinoculation of LPDV. The sera were tested in AGPT and ELISA with LPDV and control materials and satisfactory results were obtained. In the following section the methods used and the results will be presented.

D₂ Immunological reactions for LPDV identification.

Two immunological reactions were found satisfactory for the detection and identification of LPDV infection : AGPT and ELISA.

1. AGPT. Immune LPDV chicken serum was reacted in gel (1% Noble agar, 8% NaCl, pH 7.4) with normal turkey and chicken plasma, viraemic plasma from the same birds and concentrated by ultracentrifugation LPDV from turkey and chicken plasma. As control for the concentrated virus normal plasma from these birds was treated in the same way (4.5 ml plasma centrifugated for 30 min at 10^5 xg and the pellet resuspended in 0.3 ml TNE buffer pH 7.4). Clear precipitation lines were seen after 18 h incubation at 37° between the immune serum and the concentrated turkey and chicken plasma but not with the crude plasma from infected or normal birds, neither with concentrated normal plasma. Concentrated plasma from 3 out of 5 turkeys reared in contact with LPD turkeys but which were negative in the DNA polymerase test also gave precipitation lines in this test. It is possible that in some infected birds the LPDV is complexed with antibody or exist as an incomplete virus and in these forms it is devoid of DNA polymerase activity but reacts as LPDV antigen in AGPT. These possibilities were not yet investigated.

A serious drawback for the use of AGPT as a routine reaction for the detection of LPDV was the necessity to concentrate by ultracentrifugation the viraemic plasma in order to obtain a positive reaction. No other methods for concentrating the LPDV were tried at this stage.

2. ELISA. Standard Libro ELISA plates were coated with LPDV immune chicken gammaglobulin (ChGG) prepared by 3X precipitation with 35% ammonium sulphate and incubated at 4° for atleast 18 h. The reaction was performed by replenishing the wells with dilutions (1/3, 1/6, 1/12) of viraemic and normal plasma and incubation for 1 h at 37° , in the next step the well were reacted with ChGG conjugated to alkaline phosphatase (AP) or horseradish peroxidase (HPO) and reincubated 1 h at 37° then the substrate was added (p-Nitrophenyl phosphate) for AP conjugate, ABTC for HPO conjugate. The results read on a Dynach ELISA spectrophotometer.

The AP conjugate gave unsatisfactory results. The known positive samples gave often lower absorption readings than the negative controls. In order to find out the cause of these paradoxal results, known positive and negative plasma samples were serially diluted (1/2 to 1/64) on the plate and the substrate for the AP added. This experiment showed that turkey and chicken plasma have a strong AP activity and that activity is stronger in the normal than in the LPDV ...

this activity could mask the results of the immunologic reaction. Treatment of the plasma samples for 30 min at 56° for inactivation of the enzymatic activity gave unsatisfactory results, coagulation or precipitation occurring in many of them. By conjugating the same ChGG to HPO satisfactory results had been obtained. The same method as for the AP conjugate was used. The following dilutions of the various reactivities gave optimal results. ChGG (5 mg/ml solution) diluted 1/1000 (1 ug/well); plasma to be tested 1/3; ChGG-HPO conjugate 1/50. The results of turkey and chicken plasma from known negative flocks were negative (absorption 0.20 - 0.40) and also negative results were obtained with known lymphoid leukosis viraemic chicken plasma and viraemic REV chicken and turkey plasma. The absorption reading of the positive samples was 0.80 - 1.20 but not always a linear correlation was found between the intensity of the ELISA reaction and the results of the DNA-polymerase test. As in the AGPT reaction a number of contact turkeys gave positive reactions also in the ELISA test. Although the specificity of this ELISA assay with samples from experimental birds was found good, the test was not yet used for large field surveys, these results being reached only in the last month of the project. The test could be improved and made more accessible for the field laboratories first of all by the utilisation of a stronger immune serum. Preparation of this serum in chickens is difficult due to the limited available quantities of chicken LPDV viraemic plasma. The immunization of turkeys from which larger quantities of serum can be collected has to be tried again and the production of monoclonal antibody should be tested. Taking into consideration the persistent viraemia of the LPDV infected turkeys the immunological reactions for its diagnosis has to be directed for virus detection rather than for antibody.

Evaluation of the research achievement with respect to
the original research proposal.

All the main points of the research proposal were studied. The work done covers some basic aspects such as the origin and the relationship of LPDV to other retroviruses, its biochemical and immunologic structure, target organs and its integration and expression in the tumourally affected and unaffected organs. Other aspects studied included the pathogenicity for turkeys and other domestic birds, diagnostic methods, differentiation from REV using the reverse transcriptase test and humoral and enzymatic changes caused by this infection all of which have immediate practical importance. In most of the problems the study covered all of the planned points, in some of them additional aspects were included in the study based on the results obtained. A few points of the proposal were not studied due to organisational difficulties (aspects concerning the vertical transmission of the disease) or to lack of time and technical conditions (the susceptibility of some mammalian hosts to LPDV). The results obtained forced us in some cases to change the original plan and to redirect the work along new lines. It was found for example, that the LPDV infection is accompanied by a persistent viraemia and instead of a test aimed at antibody detection, an assay for virus detection was established. Positive results in this directions were obtained only in the last part of the project and field surveys planned for Israel and USA with these tests could not be accomplished.

In some of the problems studied in spite of much work done, not all the expected results were achieved. The propagation of LPDV in cell cultures was successful for only short periods of time and in spite of numerous trials with various cultures, nutritive media and conditions the continuous cultivation of the virus was not obtained.

The results of this project clarify many previously unknown aspects concerning the disease and its etiological agent, but new aspects deserving further studies surfaced: the difference between the high incidence of the infection and the relative low incidence of manifested tumoural disease; the possible regression of the tumour, the role of immunity in the disease and the persistence of the virus for such long periods.

Description and results of cooperation.

The main points of cooperation between the Israeli team and Prof. McCapes (Davis University Calif.) was the exchange of experience and materials concerning the turkey industry in Israel and USA and especially concerning the occurrence and incidence of leukotic diseases in turkeys. A large serological survey of turkey flocks in US was planned after a suitable test for detecting the LPDV infection will be established in order to check the presence of this virus in the US where the disease has not yet been diagnosed. Prof. McCapes visited our laboratories and a number of turkey flocks in Israel in 1980 and had the opportunity to see typical LPD cases and laboratory procedures for LPD and RE diagnosis. The Israeli principal investigator Dr. Ianconescu visited the laboratories at Davis Univ. and a number of turkey farms and slaughter houses, and together with Prof. McCapes collected samples for histopathology and serology. The planned survey for LPD in US turkey flocks was not done because positive results for the establishment of a suitable diagnostic test were obtained only during the last month of the project.

The cooperation will continue after the termination of this project: Dr. Ianconescu is going to spend a sabbatical year in the US working on LPD and RE with Prof. McCapes at Davis University and Dr. Witter at RPRL, East Lansing.

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