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Augmentation of Retrovirus-Induced Lymphoid Leukosis by Marek's Disease Herpesviruses in White Leghorn Chickens

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Our objective was to determine whether the cell-associated herpesvirus vaccines used in chickens to control Marek's disease tumors can augment development of lymphoid leukosis (LL) induced by exogenous avian leukosis virus (ALV). Various single or mixed Marek's disease vaccines were inoculated at day 1, and ALV was injected at 1 to 10 days, with chickens of several experimental or commercial strains. Development of LL was monitored at 16 to 48 weeks in various experiments. In several strains of chickens we repeatedly found that the widely used serotype 3 turkey herpesvirus vaccine did not augment LL in comparison with unvaccinated controls. However, LL development and incidence were prominently augmented in several chicken strains vaccinated with serotype 2 vaccines, used alone or as mixtures with other serotypes. In one chicken strain, augmentation was demonstrated after natural exposure to ALV or serotype 2 Marek's disease virus viremic shedder chickens. Augmentation of LL by virulent or attenuated Marek's disease viruses of serotype 1 was intermediate in effect. Serotype 2 Marek's disease virus augmentation of LL was prominent in three laboratory lines and one commercial strain of White Leghorns, but it was not observed in an LL-resistant laboratory line or four commercial strains susceptible to ALV infection. Chickens developed similar levels of viremia and neutralizing antibodies to ALV regardless of the presence of augmentation of LL, suggesting that the mechanism of enhanced LL did not result from differences in susceptibility or immune response to ALV. We postulate that the serotype 2 herpesviruses may augment LL through one of several possible influences on bursal cells that are subsequently transformed by exogenous ALV.

There are two major neoplasms in chickens, and each is induced by a different class of viruses. One of these, Marek's disease (MD), is characterized by high mortality of chickens, with tumors of the viscera at less than 8 weeks of age, and became a problem in the United States in the 1950's (9). After intense study a herpesvirus termed Marek's disease virus (MDV) was associated with the tumors and shown to be the etiologic agent (4, 10, 40). MDV isolates were categorized into three classes based on serological characteristics. The pathogenic strains (serotype 1), nononcogenic MDVs from chickens (serotype 2), and the related nononcogenic turkey herpesvirus (HVT) (serotype 3) (46) were all serologically distinct (41). Monoclonal antibodies have been developed that distinguish between the three types (23). Shortly after MD herpesviruses were isolated, some serotype 1 attenuated (11) and serotype 3 (28) viruses were developed as successfull cell-associated vaccines against MD; these were the first successful vaccines used worldwide to combat tumors in any species (9). Later, when more virulent forms of MDV were described (48), the serotype 2 viruses were also developed and used, mainly as bivalent vaccines (7, 42).

The second major neoplasm in chickens is termed lymphoid leukosis (LL) (32). Retroviruses termed avian leukosis viruses (ALV), which also serve as helper viruses to defective Rous sarcoma viruses (33, 34), were shown to determine LL (3) as well as other forms of leukosis (3, 32). LL was a major cause of mortality to the poultry industry, causing B-cell lymphomas in chickens generally over 5 months of age (32).

Some studies have questioned the ability of ALV to cause LL or MDV to cause MD or have concluded there is an interaction between the two (20, 22, 29). Other studies with chickens confined in specific-pathogen-free isolators and infected with purified virus have definitely established an independent oncogenic potential for ALV and MDV (6, 45). However, the latter and subsequent vaccine studies have not included groups infected with controlled levels of different ALVs and different classes of MD viruses to assess whether one may augment tumors initiated by the other. Moreover, in one field test of bivalent MD vaccines two of five commercial strains vaccinated with or contact exposed to the bivalent vaccine developed a high level of LL mortality (47). This and other field and laboratory observations led us to test whether MD herpesviruses could augment LL development.

In this study, chickens vaccinated with different serotypes of MD virus and inoculated with exogenous ALV were studied for the duration of ALV viremia, the development of neutralizing antibodies to ALV, and the onset of LL development. An augmentation of LL was seen in chickens of two strains receiving one bivalent vaccine. Therefore, additional experiments were conducted by using that vaccination procedure on chickens of several different strains. Also, chickens in one LL-augmentable strain were vaccinated with combinations of several additional isolates of the three MDV serotypes, and some were exposed to MDV or ALV by infection from hatchmates.

MATERIALS AND METHODS

Chickens. Several lines congenic for the major histocompatibility *B*-complex have been developed in inbred line $15I_5$ at this laboratory. Chickens of the sublines 15.6-*2*, 15.7-*2* (homozygous for B^2B^2), and 15.P-*13* ($B^{13}B^{13}$) were used after four to five generations of backcrossing in $15I_5$ (38). In one experiment chickens of the 15.6-*2* and 15.7-*2* lines were

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TABLE 1. Influence of type of MD vaccine on LL tumors in experimental lines of RPRL WL chickens infected with RPL-42 ALV

Sex (age, wk)	Chicken line	Type of MD vaccine[a]	No. of chickens		No. of LL tumors[b]	% of LL tumors in lines[c]	
			Infected	At risk[d]		Separate	15.6-*2* and 15.P-*13*
Male (16)	15.6-*2*	HVT-SB1	35	34	7	20B	
	15.P-13	HVT-SB1	44	42	7	17B	18D
	6_3	HVT-SB1	30	29	0	OA	
	15.6-*2*	HVT	46	38	1	3A	
	15.P-13	HVT	35	30	0	OA	1C
Female (28)	15.6-*2*	HVT-SB1	41	37	33	89H	
	15.P-13	HVT-SB1	34	33	27	82H	86J
	6_3	HVT-SB1	50	48	0	0E	
	15.6-*2*	HVT	33	30	11	37F	
	15.P-13	HVT	43	36	22	61G	50I

[a] Chickens received 2,000 PFU of MD vaccine at day 1 and 10^3 IU of RPL-42 at 1 week. Chickens receiving HVT were housed in colony cages in a pen separate from those receiving HVT-SB1.

[b] Males with histologic evidence or females with gross or histologic evidence of LL at 16 and 28 weeks, respectively.

[c] (Number with LL/number at risk) × 100. Data from five hatches were pooled and statistically analyzed within sex, and figures in column followed by different letters differ significantly ($P < 0.05$).

[d] Excludes birds dying of nonspecific cause or other tumors, i.e., erythroblastosis or hemangioma.

pooled (used equally in each vaccine lot) and given a line designation 15.*B*[2]. Chickens of control inbred lines 6 subline 3 (6_3) and ($15I_5 \times 7_1$)F_1 were also used; these (H. A. Stone, U.S. Department of Agriculture Technical bulletin no. 1514, 1975) and chickens of the *B*-congenic lines (L. D. Bacon, A. M. Fadly, and L. B. Crittenden, Poultry Sci. **63**[Suppl. 1):58, 1984) are susceptible to infection with subgroup A ALV. Chickens from these lines were progeny of parents determined to be free of exposure to ALV, MDV (serotypes 1 and 2), and other pathogens by periodical serologic monitoring (14). Commercial chickens were hatched from fertile eggs provided by several breeders. One strain of feather-sexed White Leghorn (WL) chickens (designated A) in which LL was previously observed after bivalent vaccination (47) was used in experiments 2 and 3. Chickens from another feather-sexed WL strain (D), two rapid-feathering WL strains (B and C), and a female broiler breeder strain (E) were also used in experiment 3. All of the chickens (except line 6_3) were produced by hens vaccinated with HVT.

Viruses. Chickens were injected with 10^3 infectious units of ALV strain RPL-42 (18) (kindly supplied by L. B. Crittenden) into the jugular vein (experiments 1 and 2) or peritoneal cavity (experiment 3). RPL-42 is an uncloned field isolate containing only subgroup A virus that has been passed several times on C/E cells and is known to produce a variety of neoplasms depending on the dose of the virus, age at exposure or inoculation, and strain of chicken (17, 19). Some chickens in experiment 3 were infected by contact exposure at hatch to 1-day-old $15I_5 \times 7_1$ shedder chickens that had been inoculated in the yolk sac as 7-day embryos with RPL-42 (12, 19). Cloacal swabs and plasma samples for virus and antibody tests were collected (12) and stored in a nitrogen vapor storage tank. Several assays were used to detect exogenous ALV. Briefly, C/E chicken embryo fibroblasts (resistant to endogenous viruses) were infected with samples as described by Crittenden et al. (12). Plates were frozen and thawed, and samples were collected from the supernatant after 9 days of culture and assayed for the presence of ALV group-specific antigen by using the enzyme-linked immunosorbent assay of Smith et al. (39). Each culture supernatant was run in duplicate, and cultures were considered ALV positive if both supernatants had absorbance readings more than 0.2 units above the readings of control supernatants, which were cultures inoculated with material containing no exogenous ALV and tested on the same enzyme-linked immunosorbent assay plate. Neutralization tests for antibody to subgroup A ALV were conducted by incubating 0.1 ml of a 1:5 dilution of heat-inactivated plasma with an equal amount of appropriately diluted Rous sarcoma virus before infection of chicken embryo fibroblasts (31). A 90% or greater reduction in focus-forming units in this assay indicates the presence of antibody to ALV.

In most experiments, the chickens were protected against MD by immunization at 1 day of age with a total of 2,000 PFU of the FC126 strain of HVT (46), a serotype 2 isolate, SB1 (35), or a mixture containing equal portions of HVT and SB1, grown in chicken embryo fibroblasts, and used as a cell-associated preparation. Additional MD herpesviruses used in one experiment included serotype 3 virus AC16 (46), a serotype 2 virus 301/B (43), attenuated serotype 1 viruses CV1988/C (16) and Md11/75C/R2 (R2) (44), and the virulent serotype 1 viruses JM102W (37) and GA-22 (30). The vaccine lots used were free of exogenous ALV based on the tests described above.

Pathology. All birds that died and all survivors of the ALV challenge were necropsied. Tissues were examined histologically where macroscopic evidence of tumors was uncertain.

Statistical analysis. Tumor incidence, antibody, and virus percentage data were analyzed by the chi-square method. Median survival times for groups were estimated by the nomographic method of Litchfield (24).

RESULTS

Augmentation of LL tumors by HVT-SB1 bivalent MD vaccine in susceptible WL chickens. (i) Experiment 1. Chickens of two lines susceptible and one line resistant to development of LL tumors were obtained from five hatches. Chickens were vaccinated against MD with HVT or HVT-SB1 and housed separately; all chickens were inoculated intravenously with RPL-42 at 1 week of age. Males were killed at 16 weeks before the onset of LL mortality. Eighteen percent of the males of the two susceptible lines vaccinated with HVT-SB1 had LL tumors in the bursa, in contrast to 1% in similar HVT vaccinated males ($P < 0.05$; Table 1). This same association was seen in susceptible females at 28 weeks; 86% of the HVT-SB1 vaccinees had died off or had

TABLE 2. Influence of type of MD vaccine on ALV viremia and antibody in RPRL chickens infected with RPL-42 leukosis virus[a]

Chicken line	Type of MD vaccine	16 weeks (males and females)		22 weeks (surviving females)			
		No. of birds	Antibody plasma (%)[b]	No. of birds	Virus isolation (%)[c]		Antibody plasma (%)
					Plasma	Cloaca	
15.6-*2*	HVT-SB1	72	17B	14	100A	100A	21AB
15.P-*13*	HVT-SB1	76	14B	13	100A	100A	0A
6_3	HVT-SB1	76	14B	50	100A	100A	36B
15.6-*2*	HVT	66	9AB	30	100A	100A	30B
15.P-*13*	HVT	72	4A	37	100A	100A	16AB

[a] Chickens received 2,000 PFU of MD vaccine at day 1 and 10^3 IU of RPL-42 at 1 week. Percentages within columns followed by different letters differ significantly ($P < 0.05$).

[b] (Number of plasma samples with neutralizing antibody to RPL-42/number of chickens tested of that group) × 100.

[c] Each sample was cultured on C/E cells; results are given as (number of culture supernatants containing ALV group-specific antigen detected by enzyme-linked immunosorbent assay/number of samples tested) × 100.

LL tumors at the termination of the study, whereas 50% of those receiving HVT had LL ($P < 0.05$). In females receiving HVT there was a significant difference in LL tumor development between the 15.6-*2* and 15.P-*13* *B* congenic lines (37 and 61%, respectively, with LL; $P < 0.05$). Resistant 6_3 males and females did not develop LL tumors after HVT-SB1 vaccination.

The ability of strain RPL-42 to produce viremia and the ability of the chickens to develop neutralizing antibodies are presented in Table 2. At 16 and 22 weeks, virus was detected in cloacal swabs or plasma samples of essentially all birds. There was no consistent difference within line in the frequency of birds with ALV antibody between groups vaccinated with HVT and HVT-SB1. Virus isolations were done on a subsample of chickens to verify that the HVT vaccinees were not inadvertently exposed to serotype 2 (SB1) MDV (see footnotes to Table 4 for methods). Serotype 2 MDV was undetected in HVT vaccinees but was present in one of four HVT-SB1 vaccinees.

(ii) Experiment 2. Chickens of the susceptible 15.B^2 *B* congenic lines and of ALV-susceptible commercial WL strain A were selected to confirm experiment 1. The commercial-strain females and chickens of both sexes in the 15.B^2 line were observed for LL through 48 weeks of age (Table 3 and Fig. 1). The results of two hatches were similar, and the data were pooled for analysis. In the 15.B^2 chickens inoculated with RPL-42, the HVT-vaccinated and nonvaccinated groups did not differ in final LL loss (56 and 70%, respectively), whereas 98% of the HVT-SB1 vaccinees had LL ($P < 0.05$). At earlier ages, the group differences were even more dramatic (Fig. 1), and this was verified in computation of the median survival time. The chickens vaccinated with HVT-SB1 had a median survival time before LL of 155 days, in contrast to 230 or 290 days in unvaccinated or HVT-vaccinated 15.B^2 chickens. The commercial-strain females inoculated with RPL-42 and vaccinated with HVT also did not differ in LL development from RPL-42-exposed unvaccinated controls (59 and 48%, respectively), whereas 84% of the HVT-SB1 vaccinees had LL ($P < 0.05$). Again, the differences were greatest at earlier ages (Fig. 1), and the MST before LL in the HVT-SB1 vaccinees was 165 days in contrast to 290 or 330 days in HVT-vaccinated and unvaccinated commercial strain A females. An unvaccinated control lot of commercial strain A that received no RPL-42 had 17% LL; this was attributed to uncontrolled maternal transmission of ALV, which occasionally occurs in this strain.

Tests to confirm the presence of herpesviruses and exogenous ALVs in experiment 2 are summarized in Table 4. The herpesvirus tests on a sample of birds confirmed that unvaccinated chickens lacked serotype 2 and 3 herpesviruses and that those vaccinated with HVT lacked serotype 2 (SB1) herpesvirus. The RPL-42 virus induced prolonged viremia in line 15.B^2, since all of the inoculated chickens had ALV in cloacal swab or plasma samples at 18 weeks. However, at 18 weeks proportionately fewer chickens receiving HVT-SB1 had antibody to ALV than HVT or unvaccinated controls ($P < 0.05$). All of the commercial strain A chickens tested at 4 weeks after inoculation with RPL-42 had ALV in the blood. At 18 weeks nearly all females had ALV in swab samples, and about half of the blood samples were positive regardless of the type of MD vaccination. However, in contrast to 15.B^2 chickens, more strain A chickens vaccinated with HVT-SB1 had ALV antibody than did HVT-vaccinated chickens, and unvaccinated chickens were intermediate ($P < 0.05$).

Important results were seen in the control lot of commercial strain A chickens not inoculated with RPL-42. Of 59 chickens tested at hatch, 2 had ALV in the blood (data not shown); this represented vertically transmitted virus. At 4 weeks 40% of 30 chickens tested had ALV in blood samples, and by 18 weeks 94% of the chickens had antibody to ALV (Table 4), indicating that horizontal transmission of the ALV had occurred. Ultimately, 17% died of LL (see above).

Augmentation of LL by HVT-SB1 is dependent on the WL chicken strain. The results of experiment 2 indicated that commercial WL strain A was highly susceptible to the augmentation of LL tumors by the HVT-SB1 vaccine. Therefore, in experiment 3 we tested strain A and three additional commercial WL strains and a commercial broiler breeder line as well as a standard $(15I_5 \times 7_1)F_1$ experimental line for LL augmentation after MD vaccination. For each strain, one lot received HVT and one lot received HVT-SB1 vaccine; the chickens of both lots were inoculated with RPL-42 at hatch and kept in separate isolators. The chickens in the WL A strain were killed at 27 weeks of age (Table 5). A control group receiving no ALV or MD vaccine failed to develop tumors, whereas chickens inoculated with RPL-42 and receiving no MD vaccine or HVT vaccine had significant low levels of LL (24 and 21%, respectively). Compared with these groups, chickens receiving SB1 or HVT-SB1 had more LL tumors (50 and 76%, respectively; $P < 0.05$). The latter groups had similar survival times. In two other control lots for WL A, the chickens were infected with RPL-42 by contact with shedder chickens; again the HVT-vaccinated

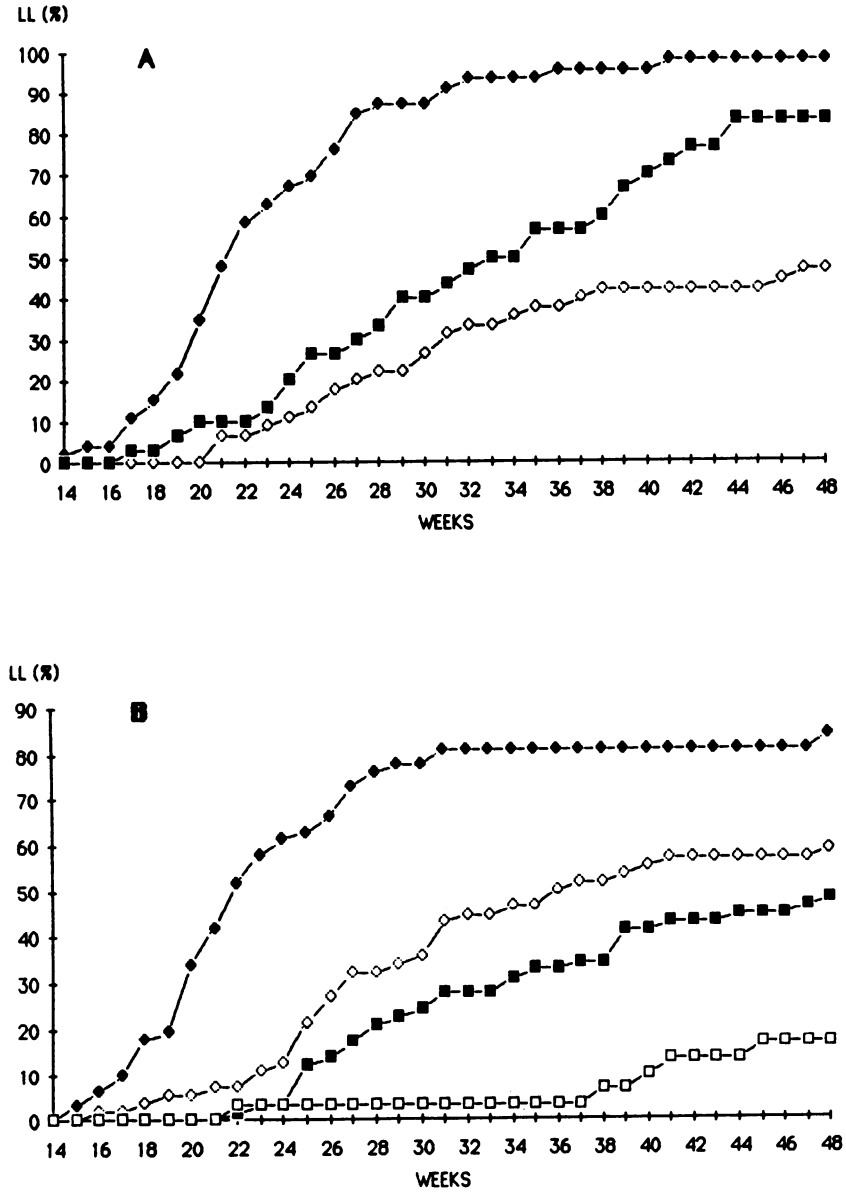

FIG. 1. Influence of type of MD vaccine on survival time before LL mortality in $15.B^2$ (A) or commercial strain A (B) WL chickens infected with RPL-42 ALV at 10 or 1 day(s) of age, respectively. Symbols: ◆, HVT-SB1, RPL-42 infected; ◇, HVT, RPL-42 infected; ■, no MD vaccine, RPL-42 infected; □, no MD vaccine, no RPL-42, control. The experiment was terminated at 48 weeks. Details on birds at risk and vaccines are given in Table 3.

chickens developed less LL than did HVT-SB1 vaccinees (30 versus 72%; $P < 0.05$).

In the WL strains B, C, and D, there was no significant difference on LL development between the HVT-vaccinated lot and the HVT-SB1-vaccinated lot. These lots had from 6 to 22% LL, although kept to 34 weeks. About half of the broiler birds had nonspecific mortality, but none developed LL. The experimental $(15I_5 \times 7_1)F_1$ susceptible control line differed in LL response depending on the use of HVT or HVT-SB1 vaccine (81 versus 100%; $P < 0.05$), and prominent differences in the median survival time before LL were noted (124 versus 199 days; $P < 0.05$).

Relationship of MD herpesvirus serotype to the augmentation of retrovirus-induced LL. The previous experiments indicated that an MD vaccine containing SB1 in combination with HVT augmented the development of LL in susceptible strains in contrast to HVT vaccination. Therefore we screened the ability of several serotype 3-, 2-, and 1-virulent or 1-attenuated vaccines alone or in mixtures to augment tumors in the susceptible commercial WL strain A (Table 6). This work was done concurrently with that in Table 5, and therefore some control lots are identical. Of two control lots that received no RPL-42, one not vaccinated for MD lacked LL, whereas one that received HVT-SB1 had 6% LL. Four of 10 chickens subsequently tested from this lot had exogenous ALV that was presumably due to vertically transmitted ALV. The remaining lots were injected with RPL-42 at hatch, and 24% of those receiving no MD vaccination had LL. This level of LL was similar to that seen in lots receiving monovalent serotype 3 MD vaccination with the standard

TABLE 3. Influence of type of MD vaccine on LL in 48-week-old hens of susceptible commercial strain A or experimental WL lines infected with RPL-42 ALV

Chicken line	RPL-42 given on day[a]	Type of MD vaccine[b]	No. of chickens		LL tumors			
					MST[e]		No.	%[f]
			ALV infected[c]	At risk[d]	Days	Limits		
$15I_5 \cdot B^2$	10	HVT	47	45	290C	244–345	25	56A
	10	HVT-SB1	50	46	155A	145–165	45	98B
	10	None	42	30	230B	203–261	21	70A
Commercial strain A	1	HVT	67	56	290E	243–346	33	59C
		HVT-SB1	70	62	165D	155–176	52	84D
	1	None	67	58	330E	273–399	28	48C
	Not given	None	34	30			5	17

[a] RPL-42 (10^3 IU) was injected intravenously.
[b] Vaccine (2,000 PFU in 0.1 ml) was injected intraperitoneally at the time of hatch.
[c] In 15.*B* all chickens were studied. In the commercial line the males were killed at 4 weeks, and the number of females studied is given.
[d] Excludes birds dying of nonspecific cause before 21 weeks or with other tumors, i.e., erythroblastosis or hemangioma. Chickens of each line, vaccine, or hatch group were in one small canopy isolator to 22 weeks; then the HVT-SB1 groups were in cages in one pen, and other birds were in cages in another pen. Data from two hatches were combined for analysis.
[e] MST, Median survival time within 95% confidence limits. Values within columns followed by different letters differ significantly ($P < 0.05$).
[f] Number with LL/number at risk) × 100. Statistical analysis was done for data of both hatch groups within each line, and values within columns followed by different letters differ significantly ($P < 0.05$).

FC126 isolate or the AC16 isolate (21 or 41% LL, respectively). In contrast, monovalent serotype 2 isolates SB1 and 301/B both augmented the level of LL (50 and 67%, respectively). The monovalent serotype 1-attenuated isolates CVI988/C and R2 produced similar results, but only the latter had significant augmentation (41 and 50%, respectively). High degrees of augmentation were seen when either of the serotype 2 isolates was used in a bivalent vaccine with FC126 (76 and 88% LL), or in a trivalent vaccine with FC126 and R2 (77 and 72% LL). Finally, when two serotype 1 virulent MDVs were used at day 7 in chickens vaccinated at day 1 with FC126 some MD developed, but in the survivors there was moderate LL without significant augmentation (45 and 46% LL), similar to that seen with monovalent 1-attenuated virus.

Several additional lots were studied to determine whether the method of presentation or quantity of SB1 would influence the augmentation of LL tumors (Table 7). One lot of hatched RPL-42-infected chickens grown with chickens shedding SB1 had a higher percentage of LL than did chickens of another lot where the SB1 was injected (91 versus 50% LL; $P < 0.05$). The chickens exposed by contact also had a shorter survival time. In two other bivalent vaccine lots, both receiving 1,000 PFU of FC126, the LL-augmenting ability of 100 and 1,000 PFU of inoculated SB1 was compared. Both lots had 76% LL, indicating that only small amounts of SB1 are needed to augment LL. The one receiving only 100 PFU had a shorter median survival time (156 versus 172 days; $P < 0.05$).

Pathology. Characteristics of LL lymphomas from chick-

TABLE 4. Tests for viruses and anti-ALV antibodies in WL chickens receiving different MD vaccines before infection with RPL-42 (experiment 2)

Chicken line	RPL-42 day[a]	Type of MD vaccine[b]	Serotype in buffy coat cells[c] (no. positive/no. tested)		ALV viremia in females[d]					ALV antibody at 18 wk in females[e] (%)
					4 wk (blood)		18 wk			
							Cloacal swab		Plasma (%)	
			II	III	No.	(%)	No.	(%)		
$15.B^2$	10	HVT	0/21	17/21	20	100	43	100	100	37B
	10	HVT-SB1	1/17	13/17	20	100	36	100	100	17A
	10	None	0/14	0/14	20	100	32	100	100	50B
Commercial line A	1	HVT	0/19	19/19	20	100	52	94	57	68C
	1	HVT-SB1	11/20	19/20	20	100	55	86	42	85D
	1	None	0/20	0/20	20	100	61	100	36	70CD
	None	None	ND	ND	30	40	32	61	9	94

[a] RPL-42 (10^3 IU) was injected intravenously. One control group was left uninjected, but 2 of 59 chicks were inadvertantly shown to contain virus transmitted from the hen based on tests at hatch.
[b] Vaccine (2,000 PCU in 0.1 ml) was intraperitoneally at the time of hatch.
[c] Blood was sampled from 10 males of each hatch group, and the results were pooled. Buffy coat cells (2×10^6) were cultured on duck embryo fibroblasts. On day 10, cultures were transferred to plates with cover slips. On day 15, cover slips were flooded with monoclonal antibody specific for serotype II or III herpesvirus. Indirect fluorescent antibody tests were made on each cover slip in duplicate. Fibroblasts receiving no buffy coat cells were negative. ND, Not done.
[d] The number of samples cultured on C/E cells is given followed by the number of supernatants containing gs. Percentages are given as (antigen detected by ELISA/number of samples tested) × 100.
[e] Number of plasma samples with neutralizing antibody to RPL-42/number of chickens of that group) × 100. Data within lines were analyzed, and values followed by different letters differ significantly ($P < 0.05$).

TABLE 5. Augmentation of LL tumors by MD vaccination is dependent on the strain of WL chickens infected with RPL-42 ALV

Chicken line	RPL-42 on day 1[a]	Type of MD vaccine[b]	No. of chickens at risk[c]	LL tumors			
				MST[d]		No.	%[e]
				Days	Limits		
WL A	None	None	30	NA		0	0A
	Injected	None	29	NA		7	24B
	Injected	HVT	29	NA		6	21B
	Injected	HVT-SB1	34	172AB	160–184	26	76D
	Injected	SB1	32	191B	168–216	16	50C
	Contact	HVT	30	NA		9	30B
	Contact	HVT-SB1	32	166A	153–180	23	72D
WL B	Injected	HVT	31	NA		2	6E
	Injected	HVT-SB1	31	NA		7	22E
WL C	Injected	HVT	31	NA		4	13F
	Injected	HVT-SB1	27	NA		5	18F
WL D	Injected	HVT	30	NA		4	13G
	Injected	HVT-SB1	29	NA		6	21G
Broiler strain E	Injected	HVT	18	NA		0	0J
	Injected	HVT-SB1	16	NA		0	0J
$(15I_5 \times 7_1)F_1$ control	Injected	HVT	32	199D	180–219	26	81H
	Injected	HVT-SB1	28	124C	120–128	28	100I

[a] RPL-42 (10^3 IU) was injected intraperitoneally at the time of hatch. Two lots of WL A were infected by contact exposure to four chickens that were shedding ALV after inoculation of RPL-42 on day 13 after embryogenesis.

[b] Vaccine (2,000 PFU in 0.1 ml) was injected intraperitoneally at the time of hatch.

[c] Excludes the 35 chicks started dying of nonspecific causes before 26 weeks of age or that had other tumors, i.e., hemangioma. Initially, all chickens of both sexes of each line were in one isolator. All WL A birds were killed at 27 weeks. For other lines at 23 weeks the groups were moved to colony cages in one pen, and the HVT-SB1 groups were moved to cages in another pen. Chickens in both pens were killed at 34 weeks.

[d] MST, Median survival time within 95% confidence limits. Values within columns with different letters differ significantly ($P < 0.05$). NA, Not applicable (<50% LL).

[e] (Number with LL/number at risk) × 100. Statistical analysis was done to compare the vaccine groups within each chicken line, and values with different letters differ significantly ($P < 0.05$).

ens vaccinated with serotype 3 virus or nonvaccinated chickens were compared with those of chickens vaccinated with serotype 1 or 2 viruses in experiment 3 (data not shown). Gross bursal involvement was observed in 92 to 98% of tumor-bearing chickens and was not affected by the vaccine type. However, lymphomas in chickens vaccinated with serotype 2 viruses had a higher rate of metastasis to other ograns (94% versus 76 or 62%; $P < 0.05$) and a lower frequency of focal liver tumors (40% versus 66 or 75%; $P < 0.05$), compared with other groups. This is consistent with the earlier onset and higher frequency of LL in serotype 2 vaccinees. No consistent microscopic differences were noted among lymphomas in different vaccine groups.

DISCUSSION

In this report, we show that serotype 2 MD herpesvirus strongly augments LL induced by ALV infection. The augmentation was demonstrated in WL strains that were relatively susceptible to LL development but did not occur at significant levels in relatively resistant strains. Commercial chickens are frequently exposed to or vaccinated with serotype 2 MDVs. Based on the data presented here, the use of serotype 2 vaccines in LL-susceptible egg-type or breeder chickens that may be exposed to exogenous ALV has the potential to increase LL losses; therefore, their use should be avoided or undertaken with caution. This precaution may be unwarranted for chickens of strains that are known to be resistant to LL induction. However, it is possible the augmentation could also reach significant levels in resistant strains under other conditions.

In the LL-susceptible strains two serotype 2 MD herpesviruses both gave the augmentation effect when given alone or in concert with serotype 3- or 1-attenuated, vaccine MDVs. Pronounced augmentation also occurred when 1/10 of the normal vaccine dose was used and, of practical significance, when the ALV exposure came from shedder chickens, or when the serotype 2 MDV was introduced by shedder chickens. Use of the serotype 1-attenuated or virulent viruses generally resulted in an intermediate augmentation that was occasionally statistically significant in these limited experiments. In chicken strains susceptible to this phenomenon, the best methods for eliminating the problem of LL augmentation involve the use of HVT vaccine or the prevention of exposure to exogenous ALV.

A prominent augmentation of LL was seen in serotype 2 MDV-vaccinated ALV-infected chickens of three laboratory lines and one commercial strain of WLs. However, augmentation was not significant in a selected resistant laboratory line or three other commercial WL strains or a broiler strain. Each of these strains was shown to be over 90% susceptible to infection with subgroup A ALV (unpublished data). The commercial strains in experiment 3 had undergone selection for eradication of ALV, and at hatching 30 chickens of each line were tested for ALV. All strains were free of ALV except for one augmentation-resistant strain, where 8% of the chickens were viremic (data not shown). Tests for maternal antibodies to ALV were not done in experiment 3, but levels were probably low except for the strain with 8% viremia. Thus, the genetic (strain) differences in tumor augmentation do not appear to result from variability in

TABLE 6. Variability of three serotypes of MD herpesvirus to augment LL tumors in susceptible commercial line A of WL chickens infected with RPL-42 ALV

RPL-42 on day 1[a]	MD herpesvirus[b]		No. started:		LL tumors				
					MST[d]		No.	%[e]	
	Type	Isolate	With MD	At risk[c]	Days	Limits		Alone	Pooled
None*	None		0	30	NA		0	0A	
None	3 + 2	FC126 + SB1	0	34	NA		2	6AB	
Yes*	None		0	29	NA		7	24CD	24A
Yes*	3	FC126	0	29	NA		6	21BC	
Yes	3	AC16	0	29	NA		12	41CDE	31A
Yes*	2	SB1	0	32	191DE	168–216	16	50EFG	
Yes	2	301/B	0	33	167BC	148–188	22	67FGH	58BC
Yes	1-att	CV1988/C	0	29	NA		12	41CDE	
Yes	1-att	R2	1	26	195E	181–210	13	50EFG	45AB
Yes*	3 + 2	FC126 + SB1	0	34	172CD	160–184	26	76H	
Yes	3 + 2	FC + 301B/1	0	25	148A	136–161	22	88H	81D
Yes	3 + 2 + 1	FC + SB1 + R2	0	31	159ABC	142–178	23	77H	
Yes	3 + 2 + 1	FC + 301 + R2	0	29	159ABC	144–173	21	72GH	73C
Yes	3 + 1-vir	FC126 + JM102W	9	22	NA		10	45DEF	
Yes	3 + 1-vir	FC126 + GA/22	3	26	NA		12	46DEF	46AB

[a] RPL-42 (10^3 IU) was injected intraperitoneally at the time of hatch. The data with asterisks (*) are also in Table 5.
[b] Virus (2,000 PFU in 0.1 ml) was injected intraperitoneally at the time hatch, except the 1-attenuated virulent virus, which was injected at day 7. Equal quantities of virus were used in mixtures. att, Attenuated; vir, virulent.
[c] Excludes chickens dying of nonspecific causes before 26 weeks of age or other tumors, i.e., hemangiomas and nephroblastomas and MD. Chickens of both sexes of each MD herpesvirus group were in separate isolators until they were killed at 27 weeks.
[d] MST, Median survival time within 95% confidence limits. Values within the column followed by different letters differ significantly ($P < 0.05$). NA, Not applicable (<50% LL).
[e] (Number with LL/number at risk) × 100. Statistical analysis was done to compare the different herpesvirus groups; values within columns followed by different letters differ significantly ($P < 0.05$). Serotypes were tested separately (alone) or pooled.

susceptibility or congenital exposure to ALV but must relate to host genes that regulate cellular function or perhaps endogenous retroviruses (13).

LL is known to result from transformation of B cells in the bursa by ALV, generally involving activation of the c-*myc* oncogene (21, 27; reviewed in reference 13). There are several possible mechanisms whereby serotype 2 MD viruses may augment this process. From an immunological standpoint, there may be antigens on serotype 2 MD herpesviruses that render ALV more tolerogenic, thus providing a greater chance for ALV transformation after prolonged ALV viremia. The data on antibody and viremia in experiments 1 and 2 gave no consistent evidence for this hypothesis. It appeared that all chickens, with or without various herpesviruses, developed a relatively long-term ALV viremia; this result is consistent with previous observations with RPL-42 (17).

It is also possible the serotype 2 MD herpesvirus may alter or interact with major histocompatibility complex antigens in a way that results in less immune competence to an ALV transformed cell. The major histocompatibility complex influences disease resistance, including resistance to MD (1), and in experiment 1 in this paper we noted that *B*-congenic 15.6-*2* birds had less LL than did 15.P-*13* birds after vaccination with HVT (but not after vaccination with SB1-HVT). However, there is little evidence for immune resistance to LL (2, 13).

Another explanation for the augmentation evolves from evidence that serotype 2 and 3 MD herpesviruses are not cytolytic for bursal cells, as are serotype 1 isolates (5) but that all serotypes cause a pronounced splenomegaly about 1 week after injection. The bursa of Fabricius, the progenitor of B-cells in chickens, is minimally enlarged by serotype 2 and 3 viruses, but the splenomegaly is greatly reduced in

TABLE 7. Capacity of different doses or exposures of SB-1 MD virus to augment LL tumors in susceptible commercial strain A chickens infected with RPL-42 ALV

RPL-42 on day 1[a]	MD herpesvirus[b]			No. at risk[c]	LL tumors			
					MST[d]		No.	%[e]
	Type	Isolate	PFU		Days	Limits		
Yes*	2	SB1	2,000	32	191B	168–216	16	50D
Yes	2	SB1	contact	32	156A	145–168	29	91E
Yes	3 + 2	FC126 + SB1	1,000 + 1,000	34	172B	160–184	26	76E
Yes	3 + 2	FC126 + SB1	1,000 + 100	33	156A	148–166	25	76E

[a] RPL-42 (10^3 IU) was injected intraperitoneally at the time of hatch. The data from the lot with an asterisk (*) are also in Table 6.
[b] Total PFU of virus in 0.1 ml injected intraperitoneally at the time of hatch. In one lot four chickens shedding SB1 were used to expose 35 chickens.
[c] Excludes 35 chicks that died of nonspecific causes before 26 weeks of age or that had other tumors, i.e., hemangiomas and nephroblastomas. Chickens of both sexes of each MD herpesvirus group were in separate isolators until they were killed at 27 weeks.
[d] MST, median survival time within 95% confidence limits. Values within the column followed by different letters differ significantly ($P < 0.05$).
[e] (Number with LL/number at risk) × 100. Statistical analysis was done to compare the different herpesvirus groups; values within the column followed by different letter differ significantly ($P < 0.05$).

bursectomized chickens (36). It is possible that the hyperplasia in B cells after vaccination or infection with the serotype 2 MDV differs from that resulting from HVT and results in a higher frequency of transformation in the B cells of retrovirus-infected birds. Calnek et al. (8) have shown that viral internal antigens that are an indication of herpesvirus genome turn-on are expressed in cultured spleen cells much more frequently and in higher numbers when the cells are from SB1-infected as opposed to HVT-infected chickens. They also present indirect evidence that B cells are infected with SB1 in contrast to HVT, and they conclude that latent infections with HVT differ qualitatively as well as quantitatively from those with SB1.

In conclusion, we have demonstrated that ALV-induced LL is augmented by serotype 2 MD herpesvirus given alone or in concert with serotype 3- or 1-attenuated vaccine MDVs in LL-susceptible strains of chickens. The mechanism(s) determining this phenomenon may involve B-cell hyperplasia and could be comparable to one of those operating to determine similar herpesvirus-retrovirus augmentation syndromes in humans (15, 25, 26).

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