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Biological and Biochemical Evidence for an Interaction Between Marek's Disease Herpesvirus and Avian Leukosis Virus *In Vivo*

(Epstein–Barr virus/lymphoproliferative disease/DNA·RNA hybridization/chickens/oncogenic RNA viruses)

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ABSTRACT **The DNA-containing Epstein–Barr herpesvirus has been implicated in the etiology of Burkitt's lymphoma, a malignant tumor of children in Africa. Recently, however, particles possessing four biochemical characteristics of RNA tumor viruses have also been identified in these tumors. The fact that both types of viruses are found suggests that an interaction between them may be playing a role in the etiology of Burkitt's lymphoma. To explore this possibility with a defined animal model, experiments were performed with the avian Marek's disease, a malignant lymphoproliferative disease associated with a herpesvirus. Controlled contact studies demonstrated that tumorigenesis in a line of isolator-derived, barrier-sustained, specific pathogen-free chickens requires exposure to both the Marek's disease herpesvirus and an avian leukosis virus, Rous-associated virus, type 2. Exposure to either agent alone did not result in tumors. Molecular hybridization experiments to cytoplasmic RNA from similarly contact-exposed conventional Cornell S-line chickens provided further evidence for the occurrence of an interaction between Marek's disease herpesvirus and the avian leukosis virus.**

The detection of viral-specific RNA in animal tumors by means of molecular hybridization (1) has been a useful tool in relating the etiology of these tumors to oncogenic RNA viruses (2, 3). Recently, radioactive DNA complementary to the RNA of the Rauscher murine leukemia virus was shown to hybridize with the RNA from murine and human leukemias (4), lymphomas (5), and sarcomas (6). Further, radioactive DNA complementary to the RNA of the mouse mammary tumor virus hybridized specifically with the RNA from murine mammary tumors (3) and from human malignant breast tumors (7).

Burkitt's disease, a malignant lymphoma first described among African children, has been linked by means of seroepidemiology (8–10), electron microscopy (11), and molecular hybridization (12) to the DNA-containing Epstein–Barr herpesvirus. Recent molecular hybridization studies have shown, however, that Burkitt's tumors contain RNA related to the RNA of murine leukemia virus (13), and also contain particles that band in sucrose gradients at 1.16–1.19 g/ml and possess both 60–70S RNA and RNA-instructed DNA polymerase (14). These are three features diagnostic of the RNA tumor viruses. These findings raised the possibility of an interaction between Epstein–Barr virus and an oncogenic RNA virus in Burkitt's tumors.

It was thus desirable to perform similar experiments under controlled conditions with another lymphoproliferative disease with which a herpes-like DNA virus has been associated and which is amenable to both *in vivo* and *in vitro* studies. A herpesvirus has been shown to play a role in the etiology of Marek's disease, a lymphoproliferative disease of fowl (15–18). This avian system is particularly appropriate since other types of viral information may participate with Marek's disease herpesvirus (MDHV) in tumorigenesis. Previous studies (19) have revealed that an interaction occurs between MDHV and an avian leukosis virus, Rous-associated virus type 2 (RAV-2), *in vitro*. That report showed that MDHV superinfection of chicken-embryo fibroblast cultures, previously infected with RAV-2, resulted in a reduction of MDHV focus formation and an increase in RAV-2 complement-fixing antigen.

An attempt was made in these experiments to simulate the conditions of natural MDHV infection by exposing untreated birds to others that had been previously infected by inoculation. In the experiments described here, none of the tissues examined came from birds directly inoculated with either MDHV or RAV-2. In all cases, samples were derived exclusively from either unexposed controls or chickens exposed by contact to birds inoculated with either RAV-2 or MDHV or both viruses. The results demonstrate that a line of isolator-derived, barrier-sustained specific pathogen-free (LSI-SPF) chickens develop Marek's disease tumors associated with a high mortality rate when exposed to birds infected with both MDHV and RAV-2. However, no tumors develop, nor does mortality occur, upon exposure to either virus alone. Further, we show by molecular hybridization that tissues of conventional Cornell S-line chickens exposed to both MDHV and RAV-2 or MDHV alone contain greater amounts of avian leukosis virus-related RNA than tissues of unexposed chickens or those exposed to RAV-2 alone. The relevance of the enhanced tumor response and increase in avian leukosis virus-related RNA to a possible interaction between the herpesvirus and an RNA tumor virus is discussed.

MATERIALS AND METHODS

Viruses. (*a*) Cell-free MDHV was prepared in day-old isolator-hatched RPL 100 × 7 chickens interperitoneally injected with 0.2 ml of a strain of MDHV from the GA isolate (20) (supplied by Dr. B. R. Burmester, USDA, East Lansing, Mich.) cloned in duck embryo fibroblasts. 4 Weeks later, the chickens showing the symptomatology and pathology characteristic of Marek's disease were used as donors for skin and

Abbreviations: MDHV, Marek's disease herpesvirus; RAV-2, Rous-associated virus type 2, an avian leukosis virus; AMV, avian myeloblastosis virus; LSI-SPF, a line of isolator-derived, barrier sustained, specific pathogen-free; RSV(RAV-2), Rous sarcoma leukosis virus.

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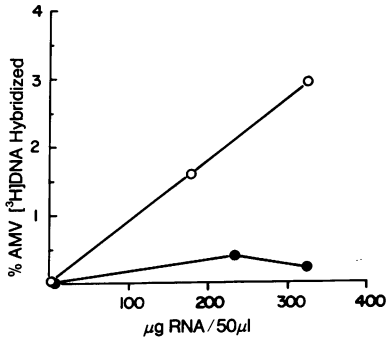

FIG. 1. Comparison of annealing reactions, at various RNA concentrations, between AMV-[³H]DNA and cytoplasmic RNA from livers of individual chickens exposed to MDHV and RAV-2 or to RAV-2 alone. The individual annealing reactions were analyzed by Cs_2SO_4 density centrifugation and the percentage of DNA hybridized was determined by the [³H]DNA, after correction for background, banding in the RNA region (between densities 1.62 and 1.69 g/ml) of the gradients. O, Liver from chickens exposed to MDHV and RAV-2; ●, Liver from chickens exposed only to RAV-2.

feather follicle epithelium for virus extraction. A 10% homogenate was prepared in modified SPA stabilizer (21) diluent containing 5 μg of Fungizone, 500 U of penicillin, 500 μg of streptomycin, 10 μg of tylocine, and 10 μg of gentamicin, per ml. The homogenates were quick-frozen and stored at −70°. 7 Days later, the preparation was thawed at 30°, and chilled 15-ml aliquots were sonicated at 80 W for four 30-sec bursts with 1-min intervals. The sonicate was clarified by centrifugation at 2000 rpm (1000 × *g*) at 4° for 20 min, dispensed in vials, frozen and stored at −70°. The MDHV preparations were tested for the presence of avian leukosis viruses by the procedures of Sarma *et al.* (22) and Rubin (23) and for adventitious avian viruses and microbial contaminants (24). All tests were negative.

(*b*) RAV-2 was prepared in chick-embryo fibroblast cultures of the C/O phenotype, and the virus-containing tissue culture fluids (supplied by Dr. E. H. Bernstein, University Laboratories, Highland Park, N.J.) were stored at −70°. Tests for resistance-inducing factor were conducted with Rous sarcoma-leukosis virus [RSV(RAV-2)], and appropriate phenotypes of chick-embryo fibroblast cultures. The results of the tests showed the presence of subgroup B avian leukosis virus. Electron microscopic examination (performed by Dr. H. Hirumi, Boyce Thompson Institute, Yonkers, N.Y.) revealed only virus particles of the C-type to be present in the RAV-2 pool.

Chickens. MDHV-susceptible Cornell S-line chickens (supplied by Dr. R. Cole, Cornell University, Ithaca, N.Y.) were used as donors for tissues used in the hybridization studies. LSI-SPF chickens were also employed to demonstrate viral interaction by symptomatology and tumor response. The derivation of these chickens and their responses to MDHV are described elsewhere (Frankel, J. W., Prickett, C. O., Farrow, W. & Groupé, V., manuscript in preparation). In all experiments each group contained 25 or more chickens. Symptomatology and mortality were recorded daily. Chickens that died during the first 6 days of the experiments were routinely discarded as nonspecific. All chickens were subjected to complete necropsy and representative tissues were collected for histological examination.

Experimental Design. 3-Day old S-line and LSI-SPF chickens were inoculated interperitoneally with about 10^3 focus-forming units of MDHV and housed in Building H. At bimonthly intervals thereafter, similarly inoculated chickens were introduced. At these times, uninoculated chicks were also placed in the same facility. Inoculated and contact-exposed chickens were maintained in this MDHV monocontaminated environment and symptomatology and tumor incidence observed. Building S was used as a RAV-2 monocontaminated facility to contain RAV-2-inoculated and contact-exposed S-line and LSI-SPF chickens; new samples of *both groups* were introduced bimonthly. Building HS constituted an environment contaminated with both MDHV and RAV-2. The methods used to introduce birds infected with both MDHV and RAV-2 and to infect LSI-SPF and Cornell S-line chickens by contact exposure were identical to those used in the monocontaminated environments. Building A housed untreated S-line chickens. Building 1, a specially constructed biocontainment facility, was used to maintain untreated LSI-SPF chickens.

Histopathology. Tissues used for hybridization studies were examined histologically. The tissues were fixed in Petrunkevich paranitrophenol (25) and stained with hematoxylin–eosin.

Preparation of Cytoplasmic RNA. Contact-exposed and control S-line chickens were housed in the same environments utilized for the biological studies, except that the

TABLE 1. *Cumulative mortality and tumor incidence among untreated LSI-SPF and Cornell S-line chickens exposed for 60 days to RAV-2, MDHV, and MDHV + RAV-2 environments*

Virus exposure	Response							
	LSI-SPF				Cornell S-line			
	No. dead/ total	Percent	No. tumors*/ total	Percent	No. dead/ total	Percent	No. tumors*/ total	Percent
RAV-2	0/49	0	0/49	0	0/29	0	0/29	0
MDHV	0/40	0	0/40	0	8/45	18	45/45	100
RAV-2 + MDHV	47/58	81	58/58	100	53/60	88	60/60	100
None	0/55	0	0/55	0	0/25	0	0/25	0

* Presence of lymphoid tumors in neural and/or visceral tissues.

group exposed to MDHV alone was maintained in an isolator. Representative tissues from the different exposure groups were obtained 6–7 weeks after initial exposure. The tissues were disrupted in a Silverson homogenizer at 4° in 30 ml of 5% sucrose in Tris–saline–Mg buffer [0.01 M Tris·HCl–0.15 M NaCl–0.002 M $MgCl_2$ (pH 7.4)]. The suspension was centrifuged at 20,000 × *g* for 10 min at 4°. The supernatant fluid was then layered onto 20 ml of 25% sucrose in the same buffer and centrifuged for 180 min at 180,000 × *g* in a 60 Ti rotor (Spinco). The pellet, consisting of monosomes and polysomes, was resuspended in the buffer with 0.5% sodium dodecyl sulfate, and the RNA was extracted twice with an equal volume of phenol–cresol (pH 8.0). The nucleic acid was then precipitated from the aqueous phase by the addition of two volumes of ethanol and one-tenth volume of 4 M LiCl. The cytoplasmic RNA was redissolved in a 1:1 mixture of formamide and 3 mM ethylenediaminetetraacetate (EDTA).

Preparation of Viral DNA Product. RSV(RAV-2), kindly supplied by Dr. Bernstein, University Laboratories, and avian myeloblastosis virus (AMV), kindly supplied by Dr. J. W. Beard, Life Sciences Research Laboratories, Life Sciences, Inc., were purified and concentrated as previously described (26). The endogenous [^{3}H]DNA product homologous to RSV(RAV-2) RNA or AMV RNA was synthesized in the following manner: 1 ml of reaction mixture, incubated at 37° for 180 min, contained 100 μg of protein of purified virus, 50 μmol of Tris·HCl (pH.8.3), 40 μmol of KCl, 6 μmol of $MgCl_2$, 2.5 μmol of dithiothreitol, 0.00125% Nonidet P-40, 100 μmol of each of dGTP, dATP, dCTP, and 5 × 10^4 pmol of [^{3}H]dTTP (8000 cpm/pmol). After the addition of 0.5% sodium dodecyl sulfate and extraction with an equal volume of phenol–cresol, the [^{3}H]DNA product was purified by Sephadex G-50 chromatography and treated with 0.5 M NaOH at 43° for 24 hr to hydrolyze any RNA present. The [^{3}H]DNA product was then precipitated in 2 volumes of ethanol and subsequently dissolved in 3 mM EDTA with 75% formamide.

Hybridization Technique. Molecular hybridizations were carried out in 50 μl of reaction mixtures containing 50% formamide in 0.4 M NaCl. An average of 350 μg of cytoplasmic RNA was hybridized to 2000 cpm of RSV(RAV-2) [^{3}H]DNA product that had been denatured by preincubation at 80° for 10 min in 75% formamide and subsequent quick-chilling. The annealing reaction was incubated for 18 hr at 37°, mixed with 11 ml of half-saturated Cs_2SO_4 (initial density = 1.52 g/ml) and centrifuged at 44,000 rpm for 60 hr at 15° in a 50 Ti rotor. Fractions of 0.4 ml were collected from the bottom of the gradient, and assayed for trichloroacetic acid-precipitable radioactivity.

RESULTS

Mortality and Tumor Response. As shown in Table 1, neither tumors nor mortality were seen during 60 days of observation among LSI-SPF chickens exposed to either RAV-2 or MDHV-inoculated birds. Similarly, unexposed control birds did not show any evidence of disease. By contrast, untreated LSI-SPF chickens, exposed simultaneously to both RAV-2 and MDHV, responded with high mortality (81%) and tumor incidence (100%). Gross and microscopic examination of tissues from these affected chickens revealed the massive visceral and neural lymphocytic infiltration characteristic of Marek's disease (27).

TABLE 2. *Mean percent* [^{3}H]*RSV(RAV-2) DNA hybridized*

Virus exposure	All organs	Liver	Kidney	Spleen	Thymus	Proventriculus
RAV-2	2.80	3.54	2.97	2.35	1.30	2.63
MDHV	5.17*	5.69	5.72	3.22	6.79	0.86
RAV-2 + MDHV	6.40*	6.77	9.46	4.15	11.70	2.15
None	1.60	0.80	1.70	1.84	2.85	1.72

* No statistical difference could be demonstrated between these two groups by Student's *t*-test for unpaired samples. However, both of these groups (MDHV and RAV-2 + MDHV) were significantly different ($P < 0.005$ or better) from the unexposed or RAV-2-exposed groups.

To complement these findings, the data in Table 1 show a significant increase in mortality (88%) among the Cornell S-line chickens exposed to *both* MDHV and RAV-2 as compared to MDHV (18%) or RAV-2 (0%) alone. Gross and microscopic lesions characteristic of Marek's disease were observed in the liver, kidney, spleen, and thymus derived from all chickens showing Marek's disease symptomatology.

Molecular Hybridization. Because of the enhanced response of LSI-SPF and Cornell S-line chickens exposed to both RAV-2 and MDHV, as compared with birds contact-infected with either virus alone, tissues from Cornell S-line chickens were screened for the presence of avian leukosis virus-specific RNA. The hybridization data obtained from representative tissues are presented for each of four different exposure groups (Table 2). The extent of the annealing between RSV(RAV 2) [^{3}H]DNA and cytoplasmic RNA extracted from tissues is indicated by the arithmetic mean percent hybridization. A significantly higher level of RSV (RAV-2)-specific RNA was observed in tissues of birds contact-exposed to MDHV and RAV-2 simultaneously or to MDHV alone than was observed in the corresponding tissues of unexposed birds or birds contact-exposed to RAV-2 alone. The most striking differences were observed in the thymus. The proventriculus of birds used in these experiments showed little gross histological evidence of involvement, and in agreement with this, no significant differences in content of RAV-2-specific RNA were observed in this organ.

Statistical analyses utilizing Student's *t*-test for unpaired samples were performed on the percent hybridization data for each of the four groups. The results indicate no significant difference between hybridization in the MDHV-exposed group and the MDHV + RAV-2-exposed group. However, the percent hybridization of both the MDHV-exposed and MDHV + RAV-2-exposed groups differed significantly ($P < 0.005$ or better in each case) from that of unexposed or RAV-2-exposed chickens.

Hybridization studies in our laboratory have demonstrated at least a 40% homology between the RNAs of RSV(RAV-2) and AMV. AMV [^{3}H]DNA product complementary to AMV 60–70S RNA was used as an additional probe to detect leukosis virus-specific RNA in the tissues from these S-line chickens. [^{3}H]AMV DNA was annealed with increasing concentrations of cytoplasmic RNA from livers of chickens

contact-exposed to both MDHV and RAV-2 or to RAV-2 alone. Fig. 1 shows that less than 0.5% of the input AMV [^{3}H]DNA is hybridized to the cytoplasmic RNA from the RAV-2 contact-exposed chickens. A 6-fold increase in the amount of hybridization was observed when [^{3}H]AMV DNA product was complexed to the cytoplasmic RNA from the chicken contact-exposed to both MDHV and RAV-2. Comparable results were obtained with either the AMV or the RSV(RAV-2) DNA probe.

DISCUSSION

These biological and molecular hybridization results are evidence that under the conditions of these experiments, an interaction occurs between Marek's disease herpesvirus and an avian leukosis virus *in vivo*. LSI-SPF and conventional Cornell S-line chickens showed a significant enhancement in tumor and mortality responses after infection by horizontal transmission of both MDHV and RAV-2, as compared to birds similarly infected with either virus alone. Further evidence for the occurrence of an interaction between MDHV and RAV-2 is suggested, since significantly higher levels of leukosis virus-specific RNA were observed in tissues from birds infected with MDHV alone or MDHV and RAV-2 than in the corresponding tissues from chickens exposed to RAV-2 or from unexposed chickens.

The biological and biochemical findings may be interpreted in several ways. The enhanced expression of leukosis-specific RNA in tissues of birds exposed by contact to both viruses may be due to an increase in DNA synthesis induced by MDHV infection (29). This may account for an increase in the expression of an ubiquitous passenger leukosis virus unrelated to the pathogenesis of the disease. However, the absence of a clinical response among LSI-SPF chickens exposed to MDHV alone, together with the failure to detect infectious avian leukosis virus in this stock, do not favor this interpretation.

It is of interest to note that the coexistence of herpes-like particles and particles resembling C-type viruses in the same layer of feather follicle epithelium has been demonstrated by electron microscopy (Hirumi, H., Prickett, C. O., Maramorosch, K. & Frankel, J. W., manuscript in preparation). The possible cocarcinogenic roles of MDHV and RAV-2 may be explained by immunologic interactions. Thus, it has been shown that MDHV and avian leukosis viruses may be immunosuppressive under the proper conditions (28, 30), and thus the oncogenic potential of the other agent may be enhanced. On the other hand, the presence of both MDHV and RAV-2 may be reqiured for tumorigenesis for completely different reasons.

The demonstration of an interaction between a DNA and RNA virus in tumorigenesis is relevant to the study of Burkitt's lymphoma in man. Seroepidemiology, electron microscopy, and biochemical studies have all implicated Epstein–Barr herpesvirus involvement in this disease. Recent investigations have shown that Burkitt's tumors contain RNA that is related to that of the murine Rauscher virus, an agent known to be leukemogenic in the mouse (13). These tumors also contain particles with a density of 1.16–1.19 g/ml that encapsulate RNA-instructed DNA polymerase and 63–70S RNA (14). These features are characteristic of the animal tumor viruses.

Once elucidated, the mechanism(s) of interaction between DNA and RNA viruses may aid in the interpretation of the role of Epstein–Barr virus in the nonneoplastic infectious mononucleosis, in which cells of the patient lack the RNA particles (14), and in the etiology of Burkitt's lymphoma, in which patients' tissues contain the particles (14).

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