AVIAN LEUKOSIS

DEVELOPMENTS IN VETERINARY VIROLOGY

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AVIAN LEUKOSIS

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PREFACE

The intention of the series Developments in Veterinary Virology is to provide monographs dealing with the major animal viral diseases. Each volume will include the latest achievements in fundamental research and practical applications and should be readable for people from various disciplines and different backgrounds. The multi-author approach provides the best opportunity to keep each chapter at the highest level and makes the composition of the volumes manageable to the editors.

This monograph on Avian Leukosis presents comprehensive reviews on the recent history of avian retrovirus research, on epizootiological, virological, pathological aspects, on tumor induction, the immune response to avian retroviruses, virus-cell interactions and on techniques for diagnosis. The volume deals mainly with exogenous avian leukosis virus (ALV) infections, but one chapter is entirely devoted to endogenous avian leukemia virus. Molecular biology aspects are confined to various oncogenes and to lymphoma induction since retroviruses, including those specific for avian species, have recently been described in detail in the Cold Spring Harbor Laboratory series "Molecular Biology of Tumor Viruses". Two chapters are devoted to the practical application of insights obtained from avian leukosis research: influences of ALV infection on production performance and eradication procedures.

The study of avian retrovirus-induced neoplasia led to important developments in the understanding of the nature and development of various tumors in avian species and has also provided, as a result of comparative research, insight into the structure and biology of retroviruses, virus-cell interactions, oncogenes and neoplasia in general. Current knowledge of the viral etiology of tumors, oncogenes, in vitro transformation, defective genomes, helper viruses and endogenous viruses (to name only a few) is for a considerable part based on work performed in the avian system. The genetically defined resistance (or susceptibility) at the cellular level to the various viral subgroups has complicated avian leukemia research, but has at the same time made the research projects of greater scientific interest.

Seventeen authors, whose names are listed on a previous page, have contributed to this monograph. I gratefully acknowledge the willingness of all sixteen experts in the various subdisciplines to add a chapter to this volume. Their

pre-eminent cooperation in the joint effort was very encouraging. The final product attained a quality that probably no single one of us could have generated for the whole field on his own. The communication between various authors was of great help in avoiding overlaps between chapters or conflicting opinions expressed.

The illustrations were kindly prepared by Johan IJzerman, an amateur artist working in our laboratory as a technician. The cover design symbolizes the crucial moment in the epizootiology of ALV: congenital infection may have been established and horizontal spread of virus may start with great intensity.

Finally, it is my privilege to thank, on behalf of all the authors, all colleagues who assisted in scrutinizing the various chapters of this monograph. One or more manuscripts were kindly reviewed by Drs. T.J. Bagust, K. McCullough, A.J. van der Eb, J. Hadar, J. Ignjatovic, J.H. van Middelkoop, J.M. Phillips, H.G. Purchase and M.A. Wainberg, and, from my own laboratory, J.G. van Bekkum, R. de Bruin, G. Koch, R.J.M. Moormann and P.W. van Olm. The figures in chapter 2 were kindly computer-designed by Dr. H. Vreeswijk. The photographs in a number of chapters were remade by Mr. K. Dekker and Mr. A.P.M. van Alphen. Above all it is my pleasure to thank Mrs. Ria de Kok-Heuckeroth and Mrs. Jacqueline C. Hoogeveen-Hilhorst who mastered the word processor. This new administrative gadget created conditions in which several versions of all chapters could be typed by our administrative staff with increasing interest in the editing activities, and at the same time they provided significant editorial assistance.

I hope that the comprehensive information on avian leukosis virus infection in this monograph will be of value to research workers in various disciplines, to teachers and students, and also to veterinarians, poultrymen and vaccine manufacturers who need this information for practical applications.

G.F. de Boer.

AVIAN LEUKOSIS

1

A HISTORICAL REVIEW OF AVIAN RETROVIRUS RESEARCH

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1. THE ORIGINS OF AVIAN RETROVIRUS RESEARCH 1908-1945

Avian retrovirus research began with the first transmission of chicken leukemia in 1908 by Vilhelm Ellerman and Oluf Bang (1) who demonstrated that cell suspensions and cell-free filtrates from tissues of a chicken with myeloblastosis produced a similar disease in recipient birds. Over the next 12 years eight separate virus isolations were reported from Ellerman's laboratory and confirming experiments were described in independent investigations from three other laboratories (2). The fact that it was many years before this pioneering work

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gained the recognition it deserved, grew from the misunderstanding, in those early years, of the true nature of leukemia. Nearly 20 years were to pass until, in the late twenties, transplantation studies in mammalian systems established that leukemic cells behaved like cancer cells (3). Indeed, because of their viral etiology, scepticism as to the relevance of avian leukemias persisted at least until 1967, when one text referred to avian and murine leukemias as "leukaemoid reactions to infection" (4). Thus, the later studies of Peyton Rous with solid chicken tumors were to be much more influential in the early years of cancer virus research.

In 1911, Rous (5) reported passage with cell-free filtrates of the transplantable chicken sarcoma described by him a year earlier (6). It is clear that at first this result was viewed by Rous himself with suspicion. The initial attempts at filtration to establish the need for intact cells in tumor transmission were made using ordinary filter paper, a procedure that was known to prevent transplantation of mammalian tumors, and it was doubtless a surprise when tumors resulted. Increasingly rigorous tests by centrifugation, Berkefeld filtration and dessication (credited, in a footnote, to J.B. Murphy) all confirmed cell-free transmission of the tumor. Now convinced of the validity of his result, Rous anticipated the criticism that would follow, and set out, in a series of careful experiments, to confirm that the chicken sarcoma was indeed a malignant neoplasm and not merely an infectious granuloma. The criticism arose nevertheless and persisted in the face of evidence to the contrary, so that as late as 1933 Claude and Murphy were "Tentatively accepting the conclusion that fowl tumors are true neoplasms, ..." (7). Confirmation followed quickly with four virus isolations from Rous' laboratory by 1914 (8,9,10,11) and, in that same year, Fujinami and Inamoto (12) demonstrated filterability of a transplantable chicken tumor, first described by them in 1910.

Progress in the ensuing two decades was summarized in reviews by Claude and Murphy (7), Foulds (13) and Gye and Purdy's boldly titled monograph, The Cause of Cancer (14). Most attention centered on the solid tumors. By 1933, laboratories in the USA, Japan, Britain, Germany, France, Italy and Argentina had described 28 transplantable tumors in fowl of which 20 were known to be transmissible by cell-free filtrates (7). All but two of the latter were sarcomas of various histologic types (the exceptions were endothelioma and leukosis). A good deal of the early effort was given to description of tumor histology and countering the arguments that filterable fowl tumors were not true neoplasms, or that they were a disease "sui generis", distinct from true tumors of birds (15). It was

generally accepted that cell-free extracts from tumors of a particular histologic type gave rise to tumors of the same type. It was recognized that chicken sarcomas were not contagious, and that the tumor agent played little or no role in metastatic spread. Opinions about histogenesis were controversial, with, in the case of Rous No. 1 sarcoma, various groups favoring fibroblasts, macrophages or mononuclear cells as targets of the virus (13).

Characterization of the physical properties of the infectious agents was necessarily limited by the available techniques and the absence of reproducible quantitative assays. Stability to heating, freezing and dessication were determined in a rough way and estimates of size generally fell between 10 and 100 nm (13). The agents were shown to be sensitive to inactivation with lipid solvents (14) and, in a very early paper, Rous (16) reported that the agent was more resistant to ultraviolet light than were tumor cells. As advanced techniques such as ultracentrifugation developed, they were applied to fowl tumor agents, most notably in Claude's studies of the Rous No. 1 virus (17), concluding that the agent was composed of protein, lipid and nucleoprotein (18,19), though it was recognized that the analyzed preparations probably were not pure (20).

Much of the early work on the antigenic properties of the fowl tumor agents was done with antisera prepared by injection of crude tumor extracts into heterologous species such as rabbits or goats (13,14). Consequently, results were confused by the presence of antibodies to host tissue, giving rise to a controversy involving neutralization of Rous No. 1 virus by antisera directed to normal chicken tissues that was not resolved until 1956, when Rubin (21) demonstrated that such antisera acted primarily against tumor cells, not virus. The most informative work was that of Andrewes (22), who found antibodies that neutralized the virus in sera of birds with slow-growing sarcomata. After a series of studies he concluded that the agents associated with solid tumors were antigenically related, though not necessarily identical (23). The fact that neutralizing antibodies sometimes were present in the blood of apparently "normal" chickens (now easily understood as the result of inapparent infection with avian leukosis viruses (ALV)), led some investigators to question the validity of these results and to propose the existence of an inhibitor other than antibody (7).

After a long period of neglect, interest in avian leukosis was revived in the early 1930s, when new virus strains were isolated in several laboratories, notably by Furth (24), Engelbreth-Holm (25) and Oberling and Guérin (26). Progress in the ensuing decade was reviewed by Engelbreth-Holm (27) and an interpretation, based on later knowledge, of the rather confusing and variable findings during that

period was given by Beard (28). Most viruses were isolated from the two leukemias, erythroblastosis and myeloblastosis. Unfortunately, in some cases the two were mixed in the donor, or produced both diseases upon transmission. The frequent appearance of "neurolymphomatosis", i.e. Marek's disease, in both treated and untreated chickens did nothing to alleviate the confusion, although the distinctive pathology of that disease was recognized (27). Ultimately, a number of virus strains were sorted out that induced predominantly one or the other form of leukemia, and that occasionally gave rise to other tumors, most commonly sarcomas, but also endotheliomas and myelocytomatosis; thus the concept of multipotency of ALVs was established. Of special interest was the report by Furth (29) of the transmission of lymphomatosis, a disease previously believed to be non-transmissible with filtrates (2).

Little information was generated prior to 1940 on the physical, chemical or immunologic properties of ALV, owing largely to the absence of quantitative assays. It was generally assumed, without real evidence, that ALV and avian sarcoma virus (ASV) were similar, and experience with stability to heat, cold and chemical agents supported this supposition, as did size estimates based on filtration (27). The exhaustive review published in 1953 by Harris (30) made clear how little was known of the properties of Rous' virus and the (presumably) related ALVs, 40 years after their discovery. The viruses had not been isolated in pure form, hence their chemical composition remained a matter of conjecture. Although reasonably accurate estimates of size were available, nothing substantial was known of the morphology or structure of the agents. Their immunologic properties were still best described in the work done 20 years earlier by Andrewes (22,23) and nothing was known of the mechanism of virus multiplication. The relationships between the agents isolated from various leukemias and solid tumors were unclear, and little was known of the natural history of infection. No criticism of the early workers is intended; similar statements could be made, for example, with reference to the status of poliomyelitis virus research at that time.

2. AVIAN RETROVIRUS RESEARCH DURING THE PERIOD 1945-1960

Vogt's 1965 review (31) documents the remarkable advances made in little more than a decade following publication of Harris' review. By this time, the chemical composition, physical properties, antigenic make-up and morphology of virus particles were understood in considerable detail. The kinetics of virus multiplication at the cellular level had been studied and clarified, although as yet

little could be said of the molecular events involved. A remarkable series of novel phenomena had been described; defectiveness, helper viruses, subgroup-specific interference, phenotypic mixing, and the host range of Rous sarcoma virus (RSV) was revealed to include many mammalian species. The etiology of lymphoid leukosis, the prevailing pathologic expression of ALV infection, had been described.

The developments coincided with and were fueled by the information explosion in the general field of virology during that interval and were encouraged by a renewal of interest in tumor virology resulting from the discovery of murine leukemia viruses (32) and polyoma virus (33,34), and demonstrations of the oncogenicity of simian virus 40 (35) and human adenoviruses (36). The advances that took place specifically in avian tumor virus research during that interval were based to a considerable extent (not exclusively) on foundations laid in four laboratories whose contributions to different aspects of the problem were fundamental to subsequent progress. In the opinion of this writer, much of the credit should be apportioned to the leadership provided by four individuals; Ben R. Burmester at the Regional Poultry Research Laboratory, Joseph W. Beard at Duke University, W. Ray Bryan at the National Cancer Institute and Harry Rubin at the California Institute of Technology and the University of California.

An early development of great importance took place in 1939, when increasing losses in the poultry industry from "lymphomatosis" caused the U.S. Department of Agriculture to establish the Regional Poultry Research Laboratory in East Lansing, Michigan (37), principally to study and ultimately to control this form of avian leukosis. Initially, emphasis was on genetics with a view to breeding for resistance and susceptibility to lymphomatosis (38). The inbred lines of chickens which were developed by this program were then, and remain today, a basic resource in avian retrovirus research (39). The key contribution of this group was the unequivocal demonstration, in 1946, by Burmester and his associates (40), of the transmission of lymphoid leukosis with cell-free filtrates from a transplantable lymphoid tumor (41). The virus, designated RPL12, became the prototype strain of ALV and was employed in a series of studies to document its physical properties (42) and pathogenicity (43). Additional virus isolations were made from transplantable tumors and directly from field cases of leukosis, and careful studies established the incidence of infection and the fact that both vertical and horizontal transmission of virus infection took place (44).

The group of investigators led by Beard chose to concentrate their efforts on the acute leukemias, erythroblastosis and myeloblastosis. An especially judicious

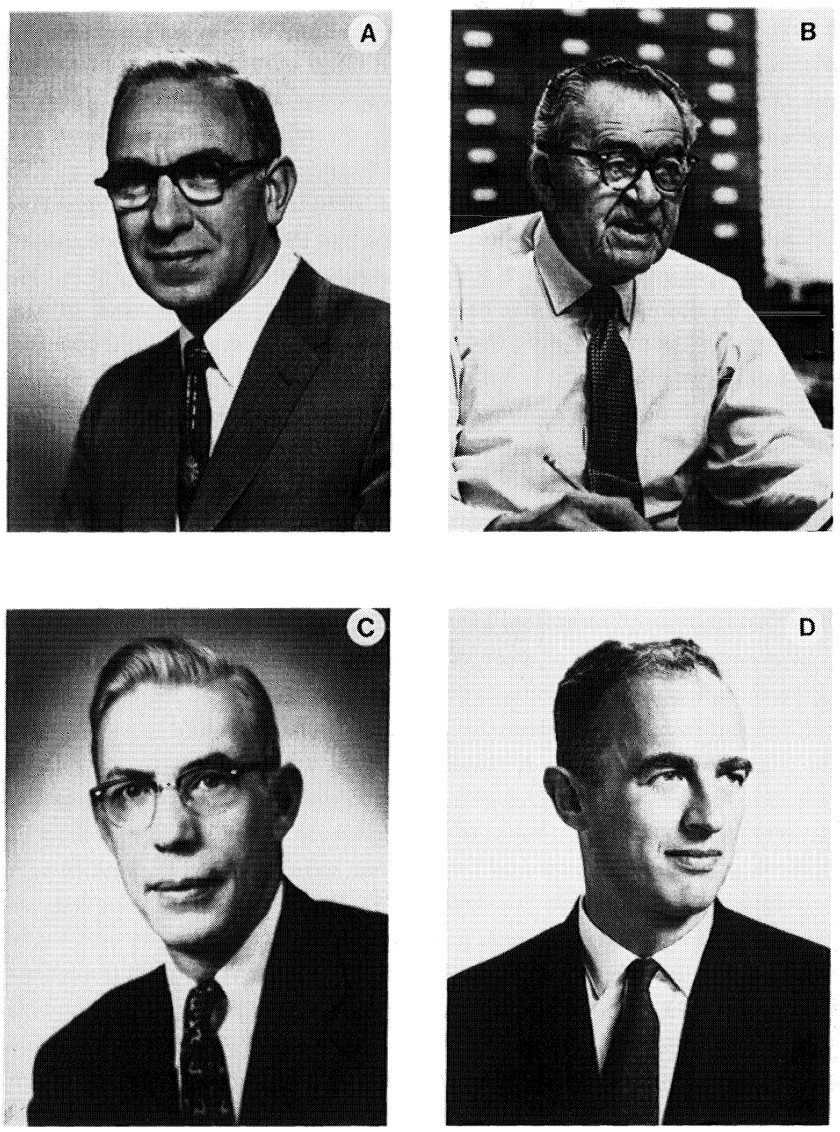

Figure 1. (A) Dr. Ben R. Burmester at the Regional Poultry Research Laboratory, East Lansing, Michigan; (B) Dr. Joseph W. Beard at Duke University, Durham, North Carolina; (C) Dr. W. Ray Brian at the National Cancer Institute, Bethesda, Maryland; (D) Dr. Harry Rubin at the California Institute of Technology and the University of California, Berkeley, California.

choice of an experimental model was made with the selection of the BAI strain A virus for much of their work. This agent, which we now recognize as a combination of defective acute leukemia and helper viruses, produced mainly myeloblastosis in chickens with a short incubation period and release of enormous amounts of virus (up to 10^{12} particles per ml) in infected plasma (45). With such quantities available, nearly pure preparations of virus could be obtained by differential centrifugation, making possible, for the first time, reliable estimates of the physical, chemical and morphologic properties of an avian retrovirus (28,46). Additionally, for the next three decades, avian myeloblastosis virus (AMV), often supplied directly and in large quantities from Dr. Beard's laboratory, was a primary research resource for laboratories throughout the world; not only those involved in retrovirus research per se, but also in many diverse fields of study, since much of the technology of modern molecular biology depends on the use of reverse transcriptase obtained from AMV. Beard's group developed quantitative methods for measurement of several strains of leukemia virus by physical, chemical and biological techniques, and an enormous volume of work was produced on the oncogenic spectrum, pathogenesis and fine structural morphology of the viral leukemias (47). An important contribution was confirmation, in collaborative studies with Burmester and Bryan, that the viruses of avian sarcomas, lymphomas and leukemias were related immunologically (46).

A puzzling and frustrating aspect of the early work with avian sarcomas was the great variation in potency of cell-free preparations. Not infrequently, the property of filterability was lost altogether, and it was found necessary to carry tumors with cell transplants for several passages before extracts regained filterability, and virus preparations were unstable, often losing infectivity in stored preparations (13,14,15,48,49). The absence of reliable, reproducible methods for virus assay and for quantitative evaluation of host response were a serious impediment to research with avian sarcomas. These problems were addressed in a systematic way by Bryan and his associates in studies conducted with RSV starting in the late 1940s. Based on high positive correlations between initiating dose of virus, tumor growth rate and virus yields (50,51,52), a strain of high potency virus was derived, and methods for its purification and stabilization developed (53). Biometrical methods for analysis of host response to RSV and other tumor viruses were developed and reproducible quantitative assays were described (54).

Studies of the interactions of RSV and host at the cellular level were initiated around 1955 by Rubin and continued with a series of students and postdoctoral fellows. At first, quantitative studies of virus replication in cell

culture were carried out with assays done on chicken embryo chorioallantoic membrane (55), a useful though cumbersome technique. A crucial technical advance was based on the observation by Manaker and Groupé (56) that discrete foci of altered cells appeared in chicken cell cultures infected with RSV and that enumeration of these foci could be used to assay virus. Temin and Rubin (57) exploited this finding to develop precise, reproducible focus assays for RSV and RSV-infected cells. The importance of this development is difficult to overestimate, not only because of its impact on avian retrovirus research described here, but also in that it was the prototype in vitro transformation assay from which were derived the multitude of assays currently in use for evaluation of oncogenic potential of viruses, physical and chemical agents, oncogenes, etc. With this tool in hand, detailed information on the kinetics of RSV growth and maturation in cell culture was obtained in Rubin's laboratory and elsewhere (31).

A disturbing feature of the RSV focus assay was the occurrence of embryos whose cells were highly resistant to infection, resulting in occasional, unpredictable failure of titrations. Investigating this phenomenon, Rubin and his associates (58, 59) found that immunity to infection could be transmitted from resistant to susceptible cultures by an agent in culture supernatants, designated Resistance Inducing Factor (RIF). Procedures for the detection and assay of RIF were developed and it was established that the agent was not distinguishable from viruses associated with lymphomatosis (60). The acute leukemia virus, AMV, was also shown to exhibit RIF activity (61). Thus, cell culture methods were made available for the study of viruses that caused the three major forms of avian retrovirus disease; sarcomas, leukemias and lymphomas. This led directly to major discoveries about host range, genetics, mode of replication, immunology and epizootiology of these agents and of retroviruses generally.

One other important event in the development of avian retrovirus research was the establishment in 1964 of the Special Virus Leukemia Program (later the Virus Cancer Program) by the National Cancer Institute (62). Although the objectives of the program were directed principally to research on mammalian, and especially human cancer, substantial support was provided for basic research on avian retroviruses and, more importantly, talented investigators in the fields of biochemistry, molecular biology and cell biology were attracted to the study of tumor viruses in general, resulting in enhancement of the level of research in the field. The individuals who created and maintained the program for many years, Frank J. Rauscher and John B. Moloney, deserve credit for their foresight and energy.

3. CONTEMPORARY RESEARCH WITH AVIAN RETROVIRUSES 1960-1984

It should be understood that although this review is directed to research with avian retroviruses, and so makes only passing reference to other subjects, starting in the 1950s research with avian and mammalian retroviruses advanced concomitantly, often in the hands of the same investigators, so each area depended heavily on the other.

3.1. Etiology of avian viral tumors

By the 1950s the term "Avian Leukosis Complex" had come into general use to include the entire spectrum of virus-induced neoplasms of chickens. This was unfortunate in that it suggested, incorrectly as we now know, that the etiologic agents of the various diseases were closely related. Especially confusing in retrospect was the widespread use of the similar-sounding terms "visceral lymphomatosis" and "neurolymphomatosis" to designate lymphoid leukosis and Marek's disease respectively, even though it had long been suspected that the two were etiologically distinct (27). The problem was highlighted in 1961 by Campbell (63) and Biggs (64), who proposed a classification scheme that separated Marek's disease from the other components of the leukosis complex based on differences in pathology and symptomatology, with the presumption of separate etiology. In 1964, Biggs and Payne (65) confirmed by transmission experiments that the agents of the two diseases were different, and three years later Churchill and Biggs (66) identified the causative agent of Marek's disease as a herpesvirus, biologically distinct from the retroviruses. Subsequent developments with Marek's disease (67, 68) had a significant effect on retrovirus research, not only by removing (or controlling) a source of error, but also by bringing about the means to control Marek's disease through vaccination, which left lymphoid leukosis as economically the most important neoplastic disease of chickens.

The retroviruses associated with avian neoplasms have themselves proved to be diverse. In 1958, Twiehaus isolated a virus from turkeys that subsequently was shown to produce reticuloendotheliosis in chickens, ducks and turkeys (69). A number of similar viruses from turkeys, ducks and chickens have since been characterized and classified separately as the reticuloendotheliosis virus (REV) group. Morphologically REVs resemble type-C retroviruses, but are distinguished from the avian leukosis/sarcoma viruses by different gag, pol and env proteins (see section 3.9). The two groups are immunologically unrelated and share little or no nucleic acid sequence homology (70). Yet another retrovirus was identified by

Biggs in 1972, associated with a lymphoproliferative disease in turkeys. Although not fully characterized, because it has not yet been grown in vitro, the agent has the morphological characteristics of a type-C retrovirus, but seems not to be related to the other avian retrovirus groups (71).

On the basis of biological properties and genome structure, the avian leukosis/sarcoma viruses fall into two groups; the slowly or weakly transforming viruses that mainly cause lymphoid leukosis with a long latent period (ALV), and the rapidly transforming avian sarcoma viruses (ASVs) and acute leukemia viruses. As described in section 3.6 and Chapter 2, most ASVs are replication-defective, producing infectious progeny only in the presence of a helper virus, while the slow-acting ALVs are replication-competent. It later became evident that the acute leukemia viruses also were defective. In 1967, Moscovici (72) developed a focus assay for AMV based on earlier observations that AMV produced visible transformation of chicken hematopoetic cells in culture (73, 74). As with defective strains of RSV, cells from single AMV-transformed foci failed to release infectious progeny unless they were superinfected with a replication-competent helper virus (75), and stocks of AMV were found to contain helper viruses, called myeloblastosis-associated virus (MAV) (76). Thus, an analogy was drawn with defective RSV and its helper, Rous-associated virus (RAV). Similar results were subsequently obtained with other acute leukemia viruses that cause a variety of neoplasms, including erythroblastosis, myelocytomatosis, endotheliomas, carcinomas and sarcomas (77, 78, Chapters 2 and 9). Thus, the avian leukosis/sarcoma viruses are also divided into two groups, replication-competent and defective, each with its characteristic pathogenicity.

3.2. Oncogenic spectrum and host range

Early studies generally agreed that avian retroviruses were multipotent, i.e., they induced a spectrum of disease responses, depending to some extent on variables such as virus dose and genetics of the host animal. For example, AMV predominantly caused myeloblastosis but occasionally also "lymphomatosis", sarcomas, renal tumors, osteopetrosis, and so on. Even RSV in high dilution produced a spectrum of oncogenic responses other than sarcomas (47). The stage of maturation of target cells may also influence responses (79). The revelation that sarcoma and acute leukemia viruses were necessarily mixtures that often included several helper viruses of different serotype, and that the helper viruses themselves were pathogenic (80), cast doubt on the reality of the multipotency concept. However, further studies with cloned virus stocks established that

replication-competent ALVs still cause a variety of neoplasms in addition to lymphoid leukosis, including osteopetrosis, nephroblastoma, erythroblastosis and sarcomas (81, 82, 83). Conclusive evidence of multipotency was harder to obtain with acute leukemia viruses, owing to the presence of helpers; however, it seems clear that they too each produce a characteristic spectrum of responses in the host (77).

The original isolate of Rous No. 1 sarcoma virus was restricted in its host range to close relatives of the donor animal (5) and only after a number of passages did it become more generally transmissible in chickens. The first heterotransplantation of an avian sarcoma was accomplished in 1928 by Fujinami and Suzue (84), who transplanted the Fujinami myxosarcoma in ducks with cell grafts. RSV was passaged in pheasants by Andrewes, who did much of his immunological work with antisera from that species (22, 23), and in 1942 Duran-Reynals transmitted RSV to ducks (85) and subsequently to a number of other avian species (86). The early literature contains numerous reports of failure to transplant avian sarcomas in mammals (13), however, the first successful experiments were not described until 1957 when the Russian investigators, Zilber and Kryukova (87) and Svet-Moldavsky (88), reported infection of rats with RSV. Confirmation came quickly from a number of laboratories, most of which were located in eastern Europe. However, the results were at first greeted with scepticism (89) on account of the failure to duplicate them in a number of laboratories in the USA and Great Britain (unreported, except by word of mouth). The heterogeneity of strains of RSV with different passage histories (90,91) provided an explanation for these discrepancies and, once virus strains were exchanged, confirmation was soon obtained and extended to include many other species, including primates (31, 92). Hanafusa and Hanafusa (93) found that infectivity for mammalian cells was conferred on RSV by the presence of the subgroup D envelope glycoprotein. In addition, at least one subgroup C virus (strain B77) also infected mammals (94). Certain strains of ASV-transformed rat and hamster cells release small amounts of infectious virus (95, 96), however, the majority of such interactions fall into the category termed "virogenic" by Svoboda (97, 98). While virogenic cells synthesize no infectious virus, intact RSV can be rescued by injecting them into chickens (99) or by co-cultivating or fusing them with cultured chicken cells (100). Virogenic cells contain viral group-specific antigens, i.e., gag proteins (101, 102), the oncogene, src, is expressed (103), but pol and env genes are not.

3.3. Epizootiology and pathogenesis of avian leukosis virus infections

The existence of both vertical and horizontal ALV transmission in flocks of chickens was established by Burmester and his colleagues in the early 1950s (44). With the newly developed cell culture methods (the RIF test) for assay of virus and neutralizing antibody, Rubin and his colleagues (58, 59) clarified the role played by immunity and immunological tolerance in the spread of infection. Congenitally infected chickens frequently are immunologically tolerant to the virus, develop viremia that persists for life and shed large amounts of virus in secretions and excretions. The congenitally infected shedders become the principal source of horizontally transmitted virus. Birds that acquire infection more than a few days after hatching usually develop antibodies within a few weeks and are, therefore, seldom persistently viremic, and only occasionally transmit virus congenitally (see Chapter 3).

The central role of the bursa of Fabricius in the pathogenesis of avian lymphoid leukosis was first demonstrated in 1964 by Peterson et al. (104) who showed that bursectomy prevented development of lymphoid leukosis (but not erythroblastosis or osteopetrosis) in chickens infected with RPL12 virus. Replacement of bursal cells in chemically bursectomized chickens restored susceptibility to development of lymphoid leukosis (105). Studies of the histogenesis of the disease revealed the earliest signs of infection in bursal lymphocytes (106) and immunofluorescent staining established that tumor cells and cell lines derived from them are B-cells (107, 108). Tumors arise as separate clones of transformed bursal cells, most of which are destroyed by host immune responses. The occasional surviving malignant clone continues to grow and metastasize, resulting in a B-cell lymphoma that is, in most cases, monoclonal (see Chapters 4, 7 and 9).

3.4. Molecular biological characteristics

The earliest reliable descriptions of retrovirus morphology are those of Claude et al. (109) who, in 1947, disclosed electron micrographs of spherical particles of appropriate size at the thinly spread edges of RSV-infected chicken cells in culture, although resolution by that technique was not adequate to provide structural detail. These results were confirmed by observations on shadowed, purified AMV (45), however, no information on virus substructure was forthcoming until the newly developed thin-sectioning techniques were applied, starting in the mid-1950s (110). The morphologic classification of retroviruses into types A, B and C was proposed by Bernhard (111), and Benedetti and Bernhard (112) obtained the earliest images of type-C retrovirus maturation by budding from the cell

membrane. This finding was in harmony with measurements of the kinetics of virus maturation showing the rapid release of infectious virus from intact cells in culture (55, 57) and the observation that host components from the cell membrane were incorporated in the viral envelope (113). Negatively stained preparations revealed the presence of spikes protruding from the viral surface (114); thus, a reasonably accurate picture of type-C retrovirus fine structure was drawn at a time when knowledge of the chemical make-up was largely absent.

An important advance was the application, in 1960, of density gradient centrifugation to RSV by Crawford (115). The low buoyant density so revealed, and the relatively large size of retrovirus particles, made possible development of rapid and gentle methods of purification (116, 117). With virus purified by these techniques, W. Robinson et al. showed that RNA in mature virions existed principally in the form of a molecule that sediments at 60-70S (118) and Duesberg found that it was dissociated by heating, or treatment with dimethylsulfoxide, into fragments that sedimented at 30-40S (119). A variety of smaller RNA fragments were also present, including 4S molecules with the properties of transfer RNA (tRNA) (120).

When proteins of purified viruses were isolated and analyzed by gel electrophoresis (121) or gel filtration (122), a consistent pattern emerged of seven major structural proteins, two of them containing carbohydrate, and, after a period of some confusion, a convention for nomenclature of the avian and mammalian retroviral proteins was arrived at (123). By analysis of subviral structures purified from partially disrupted virions prepared by a variety of methods from avian and mammalian viruses, it was possible to construct a model of retrovirus structure that identified the location of the viral macromolecules with the morphologic features demonstrated by microscopy. In general, RNA, proteins and phosphoproteins form the internal core structures and group-specific antigens, while the glycoproteins carry subgroup-specific antigens and are the surface spikes embedded in the lipoprotein envelope, which is derived from the host cell membrane (124).

Studies cited earlier (22, 23, 46) supported the idea that the avian leukosis/-sarcoma viruses were closely related antigenically and that cross-neutralization was generally observed between otherwise distinct viruses. It was therefore unexpected when, in 1961, two strains of Rous No. 1 sarcoma virus with different passage histories were shown by Simons and Dougherty (90) to be distinct in neutralization tests. Additional serotypes were discovered by Ishizaki and Vogt (125), and eventually a total of five serotypes were described in the viruses

indigenous to chickens. Two other serotypes were obtained from endogenous retroviruses of pheasants (126). As noted above, the subgroup-specific antigens were localized on the viral envelope and were associated with the viral glycoproteins (127, 128). The close relationship between the several avian oncogenic retroviruses was confirmed in 1964 by Huebner et al. (101), with the discovery of group-specific antigens. These investigators obtained antibodies from rodents with tumors induced by RSV that fixed complement with avian retroviruses regardless of envelope serotype. This became the basis of a diagnostic test (COFAL) for noncytopathogenic ALVs (129), still in use. Group-specific antigens were associated with the internal structural proteins (124) and subgroup-specific antigens were shown to be located in the viral envelope (127). Notwithstanding these distinctions, it is possible, with more discriminating immunologic techniques, to detect group-specific or serotype-common determinants on glycoproteins and subgroup-specific determinants on some internal proteins. It is also evident that avian retroviruses contain host cell antigens (130) localized principally in the envelope (113).

3.5. Interactions of envelope glycoproteins

In addition to being the target of neutralizing antibodies, the envelope glycoproteins were found by Vogt and his collaborators to control interference patterns and host range of avian retroviruses. As noted earlier, infection of chicken cells with "non-transforming" ALVs confers high levels of resistance to challenge with RSV (58, 59). This was shown by Steck and Rubin (131) to result from failure of the challenge virus to penetrate the resistant cell, due to blockage of a cellular receptor by the interfering virus. Cross-interference tests between different strains of viruses revealed that interference occurred only between viruses bearing related envelope glycoproteins (132). At about the same time it was established that genetically determined resistance to avian tumor viruses was directed selectively to the same envelope glycoproteins (133), which thus determine the host range of the viruses. It was therefore understood by the late 1960s that an early essential step in infection with avian retroviruses was the interaction of viral glycoprotein with specific cellular receptors, and that infection could be blocked by the reaction of neutralizing antibody with viral glycoprotein, or by blockage of receptors with interfering virus, and that susceptibility or resistance to infection depended upon the genetically determined presence or absence of the appropriate cell receptor. During the same period, studies of the genetics of retrovirus cell receptors in chickens resulted in a description of a

series of autosomal loci that govern their expression, with the alleles for susceptibility (i.e. presence of receptor) dominant to those for resistance to infection (134).

3.6. Defectiveness and helper viruses

In 1962, Rubin and Vogt (135) found that stocks of Bryan RSV contained a mixture of RSV and a non-transforming ALV which they named Rous-associated virus (RAV). At the same time, Temin (136) showed that RSV-transformed cells of single foci isolated from cell cultures infected at high dilution proliferated readily, but released no infectious RSV. Hanafusa et al. (137) found that the "non-producer" (NP) transformed cells produced infectious RSV only if they were superinfected with RAV or a related ALV, in which case they released both RSV and the second virus. Thus, RSV was "defective" in that it completed its replication cycle only in the presence of a replication-competent "helper" virus. Soon thereafter it was revealed that the "rescued" RSV acquired the antigenic properties and host range of the helper virus (138), thus in that instance the defect lay in the viral gene controlling expression of envelope glycoproteins. Later work identified viruses with defects affecting expression of other regions of the genome (139). The missing function of the defective virus was supplied through complementation by the helper virus resulting in phenotypically mixed progeny.

Although replication-competent strains of RSV were soon discovered (140, 141), they proved to be the exception; all other retroviruses that cause acute transformation, including those associated with the acute leukemias of chickens (see Chapters 2 and 8), are defective (139). The concept of defectiveness was modified in 1965 when Dougherty and DiStefano (142) found that "non-producer" RSV-transformed cells released apparently non-infectious particles with the morphologic properties of RSV, and W. Robinson et al. (143) demonstrated that the particles had the physical and chemical properties of RSV. Not long afterwards, it was shown by Weiss (144) and Vogt (145) that in some cases the particles released by NP cells were, in fact, infectious, but with a more restricted host range (in chickens) than the previously described subgroups. The release of modified infectious particles from NP cells was found to be governed by the presence, in some cells, of a "chick helper factor" (chf) which complemented the defective genome in much the same way as helper virus (146, 147). The factor was then identified with endogenous viral (ev) genes carried as autosomal loci in cells positive for chf (148).

3.7. Endogenous viruses

The first suggestion that viral information might be present in normal cells was provided in 1966, when Dougherty and DiStefano (149,150) observed that some chick embryos that were free of detectable infectious virus contained ALV group-specific antigens, demonstrable by complement fixation or immunodiffusion as well as virus-like particles visible by electron microscopy. Payne and Chubb (151) then found that group-specific antigen expression segregated as a dominant autosomal gene in crosses between two lines of chickens, only one of which expressed the antigen, a finding that strongly supported the concept of integration of viral information in the host cell genome. Subsequently, as discussed earlier, the Hanafusa's (146) and Weiss (147) identified chf, which is an expression of endogenous viral genes controlling the envelope glycoproteins (148). It was then established that chicken cells could harbor intact endogenous viral (ev) genes, which could be rescued by infection with exogenous ALV (152), or induced to replicate with mutagens or carcinogens (153). An endogenous virus, RAV-0, was shown to replicate spontaneously in some strains of chickens (154, 155). Molecular hybridization was later used to show that endogenous viral DNA was present in uninfected cells, integrated within the host cell genome (156). Definitive proof that endogenous viruses were integrated in the germ line and inherited like classical genetic loci was provided by Astrin (157) and Astrin and H. Robinson (158), who used restriction enzymes and Southern's DNA transfer method together with classical breeding methods to identify and map ev loci in chickens. More than 20 ev genes have since been identified and their origin and function studied (159, 160 and Chapter 5).

3.8. The provirus and virus replication

As noted earlier, the general outline and kinetics of avian tumor virus replication were established relatively quickly once cell culture methods for virus cultivation and assay came into use (31); however, the underlying biochemical events remained a matter of controversy for some years. In April of 1964, a conference on avian tumor viruses, sponsored by the National Cancer Institute and chaired by J.W. Beard, was held at Duke University, with 150 participants that included very nearly all of the investigators then active in avian tumor virus research, worldwide. The conference was memorable for many reasons, not the least of which was the closing banquet, which was a spectacular and boisterous southern barbeque. In retrospect, the most important scientific event of the conference was the exposition by Howard Temin of his provirus hypothesis (161),

an idea he had alluded to in several earlier papers. His radical proposal, that RNA tumor virus genomes replicate through DNA intermediates, was based on the stability of virus-induced properties in converted, non-virus-producing cells during passage in culture, and on studies with inhibitors that established a requirement of DNA synthesis and transcription during the virus growth cycle. The idea was not generally accepted for many years, despite the accumulation of supporting evidence from several quarters. Bader (162) described a variety of inhibitors of DNA synthesis or expression that blocked retrovirus multiplication, but did not affect most other RNA viruses, and Temin (163) established that both new DNA synthesis and cell division were needed to integrate the putative provirus. The work of Svoboda and his colleagues on the persistence of the RSV genome in transformed mammalian cells and the rescue from them of intact virus (97, 100) provided strong support for the concept of an integrated DNA provirus, as did the demonstration by Payne and Chubb (151) of the inheritance of endogenous retroviruses through the germ line. Two factors were missing; a direct demonstration of the DNA intermediate, and an enzymatic mechanism for its synthesis. The first attempts to detect viral DNA in retrovirus-infected cells by nucleic acid hybridization were confounded by the presence of virus-like DNA in uninfected cells (161, 164, 165), which only later was recognized to reflect the presence of endogenous viral DNA (156). Scepticism regarding the hypothesis was largely eliminated in 1970 with the simultaneous publication of papers by Temin and Mizutani (166) and Baltimore (167) describing the presence of the missing enzyme, an RNA-directed DNA polymerase ("reverse transcriptase"), in extracellular particles of RSV and murine leukemia virus. Conclusive demonstration of the DNA intermediate was provided by Hill and Hillova (168), who transfected chicken cells with DNA from hamster cells transformed by a temperature-sensitive mutant of RSV and showed that the progeny carried both the serotype and the temperature-sensitive marker of the original virus.

Once the existence of the provirus was established and the enzymes for its synthesis identified, the rapidly maturing methods of molecular biology were applied to delineate the synthesis, integration and expression of the retroviral genome. To summarize: The incoming viral RNA is transcribed, through exquisitely complex mechanisms, into double-stranded DNA, which is transported to the nucleus and there inserted into the host genome, forming the provirus. Proviral DNA is dealt with by the cell as a cellular gene, though with enhanced expression due to the presence of viral promoter sequences located in the terminal redundancies of the proviral genome. It is duplicated with each mitotic division, thus

remains integrated in the DNA of daughter cells. Several classes of viral RNA are transcribed and processed by cellular mechanisms to form progeny viral RNA and viral mRNAs. The latter are translated as polyproteins that are later cleaved to form the mature viral proteins; some are glycosylated by host enzymatic mechanisms, and the products are assembled at the cell membrane and released by budding (169, 170).

3.9. Genome structure and genetics

A graphic illustration of recent progress in retrovirus genetics can be made be comparing the two volumes of Molecular Biology of Tumor Viruses published by the Cold Spring Harbor Laboratory in 1973 and 1982. In the earlier volume (171) retrovirus genetics is discussed in 40 pages, nine of which are devoted to the structure of the virus genome. The size, complexity, and ploidy of viral RNA were disputed, the relationships and organization of the several size classes of RNA were poorly understood and the number of viral genes and their order were unknown. In the second edition (172), viral genome structure occupies 86 pages (not counting 21 pages of references) and most of a 100-page appendix is devoted to genome structure, including the complete base sequences of three retroviruses. A separate chapter on genetics occupies 124 pages. The viral genome is known to be composed of two identical RNA strands (i.e. is diploid), from 4 to 9 kilobases in size in different strains. In the virion, the two strands are joined by complex, base paired structures near the 5' end. Each has the properties of eukaryotic mRNA with a capped 5' end and a 3' polyadenylated "tail" and is translatable in vitro. The binding site for a tRNA primer, needed for initiation of reverse transcription, an initiation site for a second DNA strand, and terminal redundancies, all required for duplication of the genome, have been identified. Non-coding sequences for regulation of gene expression, packaging of virion components and formation of the RNA dimers as well as coding sequences for the structural and non-structural virus proteins have been identified and mapped. All replication-competent retroviruses are known to contain three genes in the same order from 5' to 3': gag, encoding the group-specific, structural core proteins; pol, the polymerase; and env, the envelope glycoproteins. In addition, the sarcoma and acute leukemia viruses have viral onc genes, usually substituted in place of sequences deleted from the gag, pol or env domains (see Chapter 2). The expression of the genome and processing of gene products have been described (169, 170). A detailed exposition of the development of this field is beyond the scope of this paper (139, 173).

3.10. The oncogene theory

The oncogene theory was propounded in 1969 by Huebner and Todaro (174), who noted the widespread existence of genetically transmitted endogenous retroviruses in diverse species of vertebrates. They postulated that endogenous retroviral information (the "virogene"), including a hypothetical portion responsible for transforming a normal cell into a tumor cell (the "oncogene"), was ubiquitous but repressed, and that most or all cancer resulted from partial or complete activation of endogenous viral oncogenes by carcinogens, aging or other factors. As it turned out, an inverted version of their idea may be valid. The oncogenic sequences of retroviruses that cause sarcomas or acute leukemias apparently were derived from non-viral sequences in normal cells (proto-oncogenes) that have become incorporated into retrovirus genomes. However, it has also become evident that cellular oncogenes (c-onc), some of which have no known connection with viral oncogenes (v-onc), play a role, possibly the central role, in the initiation and maintenance of both viral and non-viral neoplasms.

The first oncogene to be identified and characterized was src, the v-onc sequence of RSV. Direct evidence for the presence of specific oncogenic sequences in RSV came with the publication of two papers in 1970. Duesberg and Vogt (175) showed that genomic RNA from non-defective RSV was about 20% larger than the corresponding molecule from "non-transforming" ALV, and suggested that the additional RNA in ASVs encoded the gene responsible for acute malignant transformation. Martin (176) developed a temperature-sensitive mutant from a replication-competent strain of RSV, that initiated and maintained the transformed state in infected cells only at the permissive temperature, but multiplied under both permissive and restrictive conditions, thus demonstrating that the replication and transformation functions were controlled separately. This was soon confirmed when Vogt (177) established that replication-competent ASVs regularly segregate transformation-defective (td) mutants that grow normally but fail to induce transformation. The mutants proved to have deletions of RNA (178), corresponding in extent to the differences previously described between transforming and "non-transforming" viruses (175). The td mutants provided a tool for Stéhelin et al. (179) to make a radioactive probe specific for the transforming gene. Labelled DNA, complementary to wild-type RSV RNA, was prepared by reverse transcription, then absorbed by hybridization with td mutant RNA, leaving free only DNA complementary to the deleted oncogenic sequences. When this probe for the src gene was applied to a variety of cells the unexpected result was hybridization with DNA from uninfected chickens and other birds (180). Even

more surprising was the report by Spector et al. (181) that src sequences were present in the DNA of all vertebrates, and it is now apparent that even insects and fungi can be included in the list (182). At first these results were taken to support the hypothesis of Huebner and Todaro (174) since, as they predicted, viral oncogenes seemed to be present in every individual of every species examined. It soon became evident, however, that the opposite was true, the src sequences in uninfected cells are normal cellular genes. Unlike the viral sequence, v-src, which is a continuous open reading frame, the cellular gene, c-src, is divided into separate domains (exons) by intervening, non-translated sequences (introns) (183). This structure, which is typical of eukaryotic cellular genes, along with genetic evidence that shows no linkage of c-onc genes with proviral DNA, are taken to establish conclusively the non-viral origin of c-onc genes (184).

The gene product of v-src was detected by immunological methods in 1977. Brugge and Erikson (185) found that antisera from rabbits with RSV-induced tumors precipitated a 60,000 dalton protein from RSV-infected cells that was not present in td RSV-infected cells, and shortly thereafter the same method was used to detect an identical protein synthesized by in vitro translation of viral RNA (186). The gene product was found to be phosphorylated, and was designated $pp60^{v\text{-}src}$ to indicate a phosphoprotein of 60,000 daltons encoded by the v-src gene, functionally with tyrosine kinase activity (187). In normal cells the proto-oncogene, c-src, is translated and mRNA transcribed into a protein, $pp60^{c\text{-}src}$ that is nearly identical to the viral protein. Its normal function is yet to be determined. The oncogene of RSV was the first to be identified and characterized because of the unique existence of replication-competent strains of that virus which, together with their td mutants, facilitated the identification of src, while its position, near the 3' end of the viral genome, made it relatively easy to map. All other known acute-transforming retroviruses, from whatever species, are replication-defective by reason of the substitution of an onc sequence for some deleted portion of their genome that is required for replication (possible exceptions are avian osteopetrosis viruses which induce disease with high incidence and short latent period, but have no demonstrated onc gene (77)). Isolation of mutants in the transforming genes of the defective acute-transforming viruses has proved difficult and time consuming (188); in fact, no mutants have been identified in some cases. Consequently, onc genes of acute-transforming viruses other than RSV have been identified and characterized by various immunological and biochemical criteria, and with probes constructed by molecular cloning. Without the example of RSV, their existence might not yet be established. More than 20

viral oncogenes have been found in acute-transforming retroviruses from six species of birds and mammals, and in a number of cases the corresponding cellular oncogenes have been identified as well. The analogy between the several oncogenes and src seems close. Although the protein products differ in size, several, like $pp60^{src}$, are protein kinases associated with cell membranes. Some appear to have hormone activity similar to substances like epidermal growth factor or platelet-derived growth factor. However, the mechanisms whereby oncogenes influence oncogenesis, and their normal cell functions remain to be determined (182,189,190).

A separate set of cellular transforming genes, unrelated to any known v-onc, has been identified in viral and non-viral tumors of birds and mammals, including man, by transfection of mouse fibroblasts with tumor DNA (191), a technique based on the pioneering work of Hill and Hillova with RSV (168). Development of the appropriate probes led to discovery of enhanced expression of the cellular homologs of avian and mammalian retroviral oncogenes in a number of human tumors. In Burkitt's lymphoma, chromosomal translocations place promoters of immunoglobulin genes adjacent to c-myc, the cellular homolog of the onc sequence of avian myelocytomatosis virus, resulting in enhanced expression of the oncogene. Amplification of c-myc is also seen in human promyelocytic leukemia, in a neuroendocrine tumor, and a related gene, N-myc, is enhanced in human neuroblastomas. C-myb, the proto-oncogene of AMV is enhanced in a human adenocarcinoma of the colon (182).

3.11. The role of oncogenes in avian leukosis virus infections

Lymphoid leukosis, the most common naturally-occurring retrovirus-induced neoplasm of birds, is caused by viruses that lack oncogenes. The mechanism of oncogenesis in this disease remained obscure until 1981, until Hayward et al. (192) demonstrated that the ALV integration site in bursal lymphomas was adjacent to the locus of c-myc. Presence of the viral promoter sequence in the long terminal repeat of ALV near c-myc resulted in greatly enhanced expression of that gene, presumably accounting for the transformed phenotype. The mechanism of this augmentation of c-myc expression is not entirely clear, as viral insertion in the opposite transcriptional orientation, or downstream of c-myc, also resulted in increased expression (193). Activation of c-myc in itself may not be sufficient for tumor activation. Using the mouse cell transfection assay, Cooper and Neiman (194) demonstrated a c-onc in chicken bursal lymphomas (B-lym) that is unrelated to myc or any other known viral oncogene. Since lymphoma genesis seems to be a

multi-step process, it has been suggested that activation of these two genes may be involved in different stages of development of the transformed phenotype (192,194,195). This subject is discussed in detail in Chapters 4, 7 and 9.

4. CONCLUDING REMARKS

In writing this article, one could not help being impressed with the fertility of avian retroviruses as an experimental subject. The list of phenomena and concepts first revealed or explored with avian RNA tumor viruses is long; viral etiology of tumors, in vitro transformation, defectiveness, helper viruses, endogenous viruses, the provirus and oncogene concepts, to name only a few, were all based on work done first with the avian system. It is therefore interesting to recall that for much of the period covered in this review, most of the work was done apart from the mainstream of cancer research. Ellerman's findings were largely ignored for more than 20 years, and nearly 60 years passed between the discovery of RSV and the award of a Nobel prize to its discoverer. In his Nobel lecture, Rous remarked on the disbelief that greeted his papers, and in fact, after a few years he dropped the problem for more acceptible areas of research, and did not return to the study of viral oncology until a mammalian virus tumor (the Shope papilloma) was described, many years later (196). Even in recent years, although research with the avian system led the way more often than not in determining the structure and biology of retroviruses, it is probably correct to say that the work of Gross and his successors with mammalian systems was more important in popularizing the idea that research with tumor viruses was important, and might have some bearing on human medicine. Nowadays, all that is changed; retroviruses are known to cause the Acquired Immune Deficiency Syndrome (AIDS), leading articles on retroviruses appear in the New York Times, and oncogenes identified in avian viruses are involved in human cancers. Perhaps we now can declare that Vilhelm Ellerman was mistaken in 1923 when he wrote "Avian leukemias provide no clues for the understanding of leukemogenesis in man" (quoted by Graf and Beug (77)). It is hard to believe he would regret being wrong.

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2

STRUCTURE AND VIRUS-ASSOCIATED ONCOGENES OF AVIAN SARCOMA AND LEUKEMIA VIRUSES

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1. INTRODUCTION

The aim of the review presented here is to give a brief overview of avian retroviruses, the disease they cause and genes and gene products that are thought to be associated with transformation of target cells, in order to provide a common basis of knowledge for readers of this monograph. Retroviruses are of interest first as pathogens in a number of vertebrate species, and second as a major tool of experimental oncologists. The speed and reliability with which these small viruses cause tumors in animals, and analogous changes in tissue culture, suggested early on that an understanding of retroviral functions would provide basic information on the primary mechanisms of cancer causation. This belief is being justified by the results of recent years. A number of genes, originally described in retroviruses

G.F. de Boer (ed), Avian Leukosis.

and subsequently in uninfected vertebrate cells, have been found to be associated with a variety of tumors of non-avian origin. However, in many respects the virus remains the most productive avenue in the investigation of oncogene function.

A subdivision of avian leukemia/sarcoma viruses, reticuloendotheliosis virus (REV) is not discussed here, is based on the spectrum and latency of the neoplasia induced by these viruses in vivo (Table 1). While the members of the exogenous avian leukemia virus (ALV) group, which are commonly called avian leukosis virus, induce neoplasia after a long latency period and do not transform avian fibroblasts in vitro, the avian acute leukemia viruses, also called defective leukemia viruses (DLV) and the avian sarcoma viruses (ASV) group induce disease with fairly short

Table 1. Classification of avian leukemia and sarcoma viruses

Family	Retroviridae		
Subfamily	Oncovirinae		
Induction of/ transformation by			
	Viral transforming (onc) genes	Cellular onc genes	No transformation
	- Sarcoma viruses (ASV) - Acute defective leukemia viruses (DLV)	- Exogenous leukemia (avian leukosis) viruses (ALV)	- Endogenous leukemia viruses (ALV)

latency and transform a variety of avian cell types in vitro. This activity is conferred in each case by a nucleotide sequence that has been incorporated into the viral genome. This sequence, the oncogene sequence, encodes a protein which has been shown to be directly responsible for transformation of cells in vitro and tumor induction in vivo for most of the viruses to be described. It is this sequence, its protein product and the activities of that product in the context of viral transformation and pathogenicity which will be described in this Chapter. The amount of information gathered over the last 10 years about these viruses precludes a comprehensive review of each and the reader is referred to a more detailed compendium for further information (1). The exogenous and endogenous avian leukemia viruses, here both abbreviated ALV, will be dealt with elsewhere in this monograph.

2. DEFECTIVE LEUKEMIA VIRUSES

Upon injection into young chickens viruses of the DLV group rapidly bring about death of the birds from a variety of neoplasms, usually, but not exclusively, of the hematopoietic system (2). The DLVs to be discussed here fall into three groups on the basis of the oncogenes that are contained in their genomes and diseases they induce. These oncogenes are erbB, which is found in viruses which cause erythroid leukemias and sarcomas; myc, which is associated with a variety of neoplasms including myelocytoma, sarcoma and carcinoma; and myb, which is primarily associated with myeloblastic leukemia. The viruses which contain these oncogenes are listed in Table 2 as are the most common diseases associated with these viruses. A recently described DLV, S13, whose oncogene has yet to be defined will be discussed briefly. This virus causes acute erythroblastosis and sarcomas when injected into young birds.

Table 2. Oncogenes and diseases associated with defective leukemia viruses

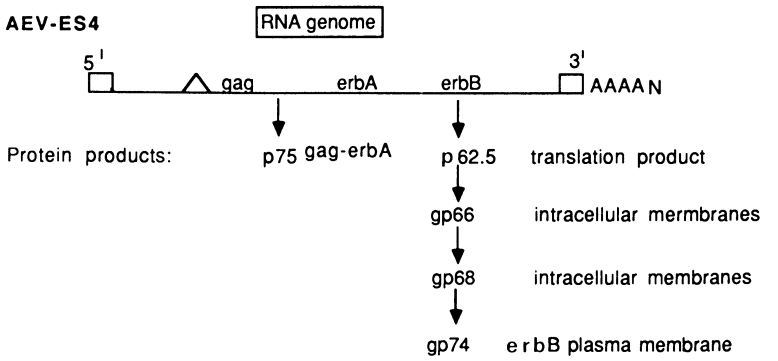
Virus	Oncogene	Disease
AEV-ES4	erb	erythroblastosis, sarcoma
AEV-H	erb	erythroblastosis, sarcoma
MC29	myc	myelocytoma, carcinoma, endothelioma
CMII	myc	myelocytoma
OK10	myc	endothelioma
MH2	myc/mil(mht)	endothelioma
AMV	myb	myeloblastosis
E26	myb	myeloblastosis, erythroblastosis

2.1. The erb oncogene

Two independent isolates of avian erythroblastosis virus containing the erbB sequence, AEV-ES4 and AEV-H, have been reported. Both viruses cause erythroblastosis and fibrosarcoma upon injection into newly hatched chickens and transform avian fibroblasts and erythroblasts in vitro. The best understood of these viruses is AEV-ES4 and, therefore, the protein products of this virus will be considered in more detail.

Both viruses (AEV-ES4 and AEV-H) contain a gene derived from a normal cell sequence termed erbB. AEV-ES4, in addition, contains another cell-derived sequence erbA. For reference, the genome structure of these two viruses can be seen in Fig. 1. The role of erbA and erbB in cell transformation has become more

clear because of two lines of investigation. In the first instance, deletion of erbA sequences from AEV-ES4 generates a virus which can still transform both fibroblasts and erythroblasts, suggesting that erbB alone is sufficient for malignant transformation in vitro (3,4). This thought was strengthened by the isolation of AEV-H which has very similar properties to AEV-ES4 but lacks the erbA sequence (5). However, matters are not so clear-cut since the presence of erbA renders AEV-ES4 more tumorigenic in vivo, implying that erbA plays some role in the development of the malignant phenotype, especially erythroblastosis (3).



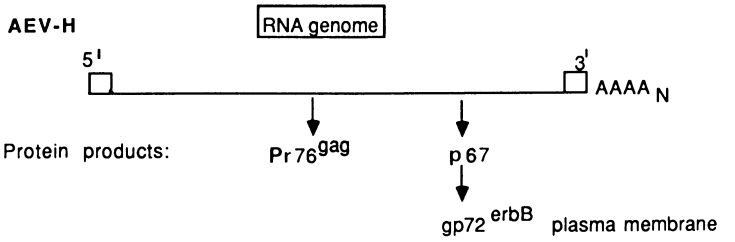


Figure 1. Genome structure and protein products of erb-containing viruses, causing erythroblastosis.
The abbreviations used in the figures are: Pr to indicate that this is a precursor protein to structural proteins found in the virion; gp to indicate the protein is glycosylated; and p for protein. The figures refer to the molecular weights of the proteins in kilodaltons.

Characterization of the erbB gene products of AEV-H and AEV-ES4 has shown them to be membrane glycoproteins (6,7; see Fig. 1). In the case of AEV-ES4 mutants that are temperature-sensitive for transformation have been analyzed. These studies demonstrated that synthesis of the plasma membrane gp74 protein is required for transformation (8). In ts mutants gp68 is not processed into gp74 at the non-permissive temperature and remains localized in internal membrane compartments.

Recent work comparing the protein sequences of erbB with various other proteins revealed extensive homology between epidermal growth factor receptor (EGFR), a 170 dalton glycoprotein, and erbB (9,10). From this data it is clear that erbB is in effect a truncated form of the avian EGFR, having lost portions of the amino and carboxy terminal regions of the protein presumably when it was transduced into the viral genome of AEV (see Fig. 2). It is not known at present what effect truncation has on the function of the protein (i.e. it may render the protein constitutivily active such that the cell receives a constant growth signal).

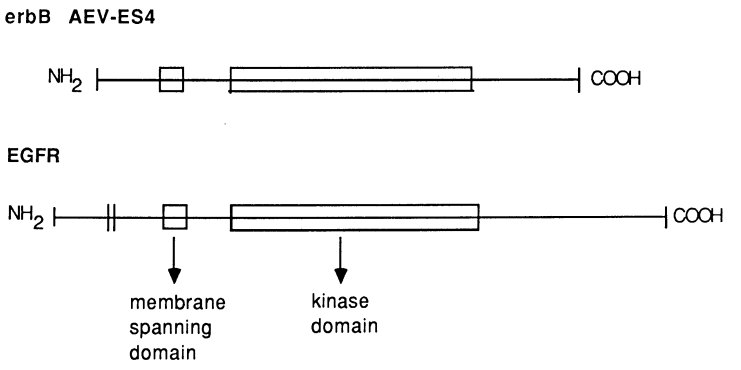


Figure 2. Comparison of erbB with EGFR. Comparison reveals that erbB is truncated at the amino terminus lacking the EGF binding domain. In addition it lacks a portion of the carboxy terminus (74 amino acids) found in EGFR.

However, it has recently been demonstrated that the erbB protein has an associated tyrosine protein kinase activity when assayed under specific conditions in an immune complex assay in which erbB is immunoprecipitated with antisera that recognize the protein (11,14). Indeed, both the intracellular gp68 and membrane, gp74, forms are active; when complexes are incubated with ^{32}P-ATP phosphate is transferred to both forms. The finding of kinase activity associated

with erbB is not unexpected since EGFR also has an associated tyrosine-specific protein kinase. In addition, erbB shows homology with a region of the src oncogene (which will be described below), a member of a family of oncogenes possessing tyrosine kinase activity (15). The importance of this activity with regard to transformation is unclear since mutants of AEV-ES4 (both conditional and non-conditional) express the kinase activity while certain cells infected by the mutants appear to be normal morphologically (14).

2.2. The myb oncogene

There are two isolates of viruses containing the myb gene, avian myeloblastosis virus (AMV) and E26. Interestingly, these viruses have significantly different pathogenic properties. AMV causes only myeloblastic leukemia whereas E26 causes both myeloblastic and erythroblastic leukemia (16,17). Examination of the genome structure of these viruses shows that AMV contains the myb gene while E26 contains two genes, myb and ets. The genome structure and protein products of each can be seen in Fig. 3. The myb gene product of AMV has been identified as a protein with a molecular weight of 45-48,000 dalton ($p48^{myb}$) and is phosphorylated and nuclear in location (18).

The myb gene in E26 is expressed as a larger fusion protein of 135,000 dalton. The remaining information includes a portion of the gag gene and the ets gene (19). Both the AMV myb protein, $p48^{myb}$ and the $p135^{gag\text{-}myb\text{-}ets}$ protein of E26 are nuclear and have been shown to bind DNA (20).

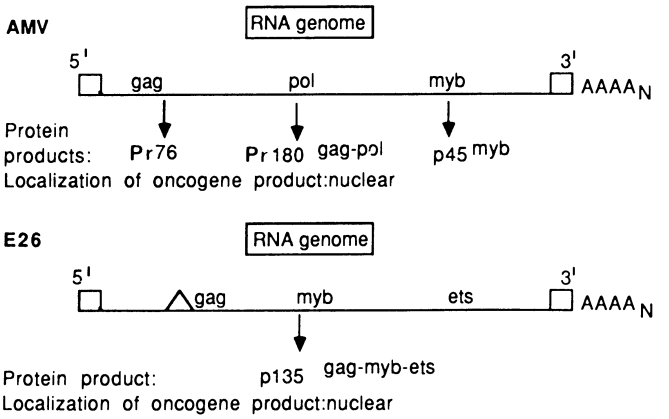


Figure 3. Genome structure and protein products of myb-containing viruses, avian myeloblastosis virus and E26.

Recently, mutants of E26 which are temperature-sensitive for the transformation of myeloblasts have been isolated. When myeloblasts transformed by such mutants were shifted to the non-permissive temperature, they differentiated into resting macrophage-like cells. In contrast, erythroblasts and fibroblasts transformed by these mutants and kept at the non-permissive temperature were indistinguishable from cells transformed by wild-type virus, suggesting that the myb gene of E26 is responsible for myeloblast transformation, whereas ets might cause transformation of erythroblasts and fibroblasts (21). Moreover, the E26 p135 protein of these mutants binds DNA in a thermolabile fashion, implying that interaction with DNA is in some way relevant to transformation (22).

2.3. The myc oncogene

Four avian viruses have been isolated which contain the myc gene (MC29, CMII, MH2, and OK10). These viruses can transform fibroblasts and macrophages in vitro. In vivo, they have been associated with a wide variety of tumor types (see Table 2). As with the myb gene, the myc gene can be expressed as a fusion protein with gag or, on its own, via a spliced subgenomic message (see Fig. 4). Both gag-myc fusion proteins and myc expressed on its own are found in the nucleus of transformed cells. Their function in the nucleus is unknown but the proteins have been shown to have a DNA-binding activity and may be associated with the nuclear matrix (23,24,25). These data have prompted speculation that myc may be involved in the regulation of transcription or DNA replication. Others have also postulated that myc may act as an "immortalizing" function since it can complement the action of other oncogenes such as ras in bringing about the morphological transformation of primary rodent cells (26,27). It should be noted, however, that avian cells can be morphologically transformed by myc-containing viruses but do not become immortal.

Non-conditional mutants of MC29 have been isolated that are essentially non-pathogenic in vivo and have lost the ability to transform macrophage-like cells efficiently (28). They do, however, morphologically transform fibroblasts, but apparently these fibroblasts are not malignantly transformed since no sarcomas are seen in vivo (29). All these mutants were shown to have deletions in the 3' half of myc and, although they encode smaller proteins than wild-type virus that are located in the nucleus, they are no longer pathogenic. No mutants of OK10 or CMII have been reported. However, mutants of MH2 have been isolated. These mutants are still transforming but are no longer able to synthesize the p100 protein (30; see Fig. 4). MH2 is unusual among the other myc-containing viruses in

that it contains an additional cellular sequence termed mil which has been shown to be homologous to the mammalian oncogene raf (31). This sequence has been shown to have homology to the src family of oncogenes (see section 3) and possesses a protein kinase activity, although in this case it is not a tyrosine kinase but is specific for serine and threonine (32). The function of mil and the mechanism by which myc and mil interact with one another is not clear at present; however, it is speculated that v-raf (mil) and v-myc provide two complementary signals for growth to infected cells. These may constitute a "competence" signal from v-myc and a "progression" signal from v-raf.

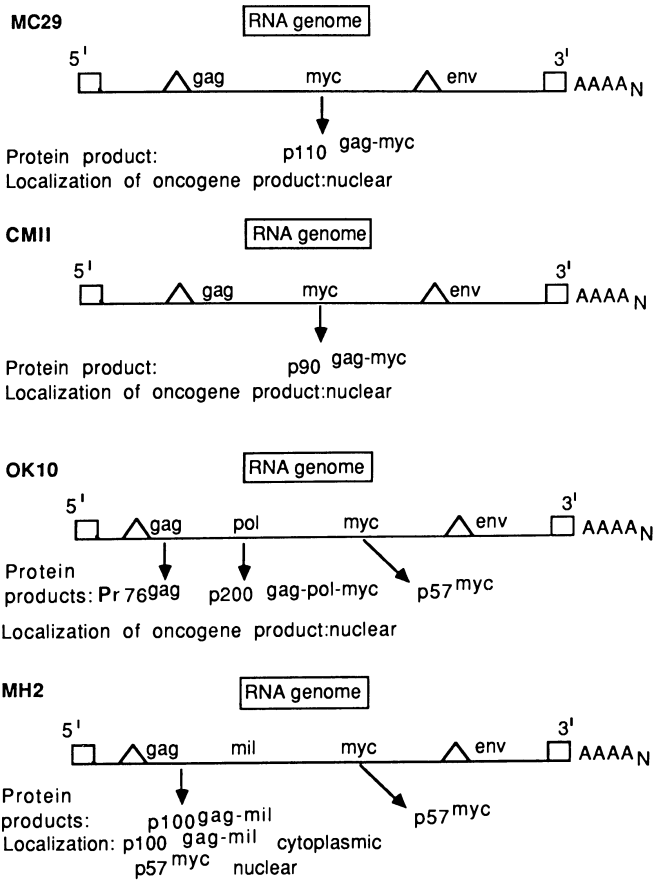


Figure 4. Genome structure and protein products of myc-containing viruses.

A novel myc-containing virus has been isolated recently and examined extensively. When one of the non-conditional deletion mutants of MC29 which could not transform macrophages was passaged in bone marrow cells, a colony of transformed macrophages was obtained. From this colony a virus was isolated which had unusual properties. It could now transform fibroblasts and macrophages in vitro unlike the parental deletion mutant and when tested in vivo it was shown to induce a novel disease spectrum, lymphoid tumors of T- and B-cell origin (33,34). When analyzed by molecular biological techniques, this virus was found to be a recombinant between the MC29 deletion mutant and the helper with which it was passaged, ring-necked pheasant virus (RPV). In addition, it contained a recombinant myc gene made up of sequences derived from v-myc and the cellular homologue c-myc (35). However, it remains to be ascertained which of these changes is responsible for the changes in biological activity.

2.4. S13 erythroblastosis virus

At this point it is worth mentioning briefly the defective leukemia virus, S13, which is capable of transforming erythroblasts and fibroblasts in tissue culture and can cause fibrosarcoma and erythroblastosis on injection into young chicks (36). Although these characteristics are very similar to AEV, this virus has been shown not to contain the erbB gene (37). Its oncogene has not been fully characterized as yet. However, it is known to encode an env-related protein of 155,000 dalton (155 kd); as the normal env gene product is only 95 kd, it is presumed that this protein is an env-oncogene fusion protein. This assumption has recently received more weight with the observation that this protein, and especially its cleavage product, gp70, has an associated tyrosine protein kinase activity (38). Therefore, although the structure and nature of the S13 oncogene remains unclear, it appears as though this virus synthesizes a glycosylated membrane protein with tyrosine kinase activity. This is a property that it shares with the erbB containing erythroblastosis viruses and herewith is demonstrated the sensitivity of erythroblasts to transformation by this class of oncogene.

3. AVIAN SARCOMA VIRUSES

Several ASVs have been isolated which encode transforming proteins which have in common a protein kinase activity for tyrosine residues. Most early work on ASV concentrated solely on Rous sarcoma virus (RSV) which was considered to be the prototype ASV. However, RSV has been found to be the exception rather

than the rule. In RSV the oncogene is situated outside the genes encoding the viral structural proteins and the virus can replicate efficiently in avian fibroblasts. As with the DLVs discussed above, most of the ASVs to be described are replication-defective by virtue of the fact that the oncogene sequence has been inserted into replicative genes. This category of viruses includes Fujinami sarcoma virus FuSV, isolates from the Poultry Research Centre (Edinburgh) PRCII and IV, and more recently recovered, Yamaguchi virus Y73, URI and URII (1,39,40,41,42,43).

Viruses of this class share the ability to induce fibrosarcomas when injected into young birds; they invariably metastasize and kill the bird in a matter of days or weeks. The rapidity with which the virus acts appears to be the result of recruitment, the spread of virus from one cell to another. In vitro, these viruses have been shown to transform avian fibroblasts and more recently were shown to transform erythroid cells from chicken bone marrow (44,45). Because most work has been done on RSV, this virus will be discussed first and most extensively for neoplastic transformation.

3.1. Rous sarcoma virus

As mentioned above, RSV is unique amongst the ASVs discussed here in that it can replicate in, as well as transform, chicken cells in vitro. Fig. 5 outlines the genome structure of RSV and, as can be seen, the cell-derived transforming sequence src is located outside the viral genes required for replication (gag, pol and env). Numerous studies using conditional (temperature-sensitive) and non-conditional (deletion) mutants of RSV have shown that the src gene is required for the initiation and maintenance of the tumorigenic state (for review see (1)). This led to a search for the protein product of the src gene. Using antisera raised in rabbits against tumors induced by RSV, the protein product of the gene was identified as a 60 kd protein ($pp60^{src}$) which was modified by phosphorylation on serine and tyrosine. Soon after its discovery, a function was assigned to the protein in an unusual assay. When $pp60^{src}$ was immunoprecipitated with antiserum directed against the protein and incubated in the presence of ^{32}P-ATP, phosphate was transferred to the precipitating immunoglobulin and in small amounts to src itself.

This protein kinase activity was subsequently found to be tyrosine-specific, a novel substrate for a kinase. It is now generally accepted that $pp60^{src}$ is a tyrosine-specific protein kinase. However, the notion that this activity is solely responsible for neoplastic transformation has not been proven. Several experiments have touched on this subject (46). Parsons described a mutation (a deletion

that removes amino acids 202-255 from pp60$^{v\text{-}src}$) which rendered neoplastic transformation temperature-sensitive, but did not affect protein kinase activity (kinase domain lies about the tyrosine residue at amino acid 416; see diagram of protein structure). In another set of experiments, mutation of the tyrosine at 416 had no effect on kinase activity (as measured by the immune complex assay) but the virus failed to confer tumorigenicity on mouse cells (47).

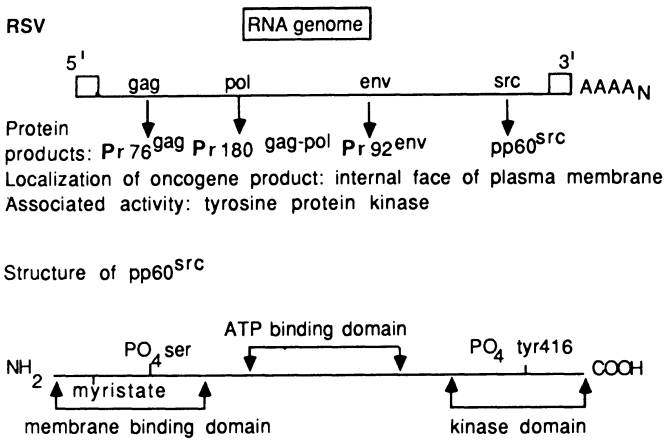


Figure 5. Genome structure and protein products of Rous sarcoma virus. Structure of the oncogene product, pp60^{src}, is included for reference.

The majority of the protein can be localized at the plasma membrane but is not an integral part of the membrane protein. The protein synthesis takes place on free polyribosomes, joins a complex with two cellular proteins (50 kd and 90 kd) in the cytoplasm, and leaves the complex within 5-10 minutes when it reaches the interior surface of the plasma membrane. While in the complex, the tyrosine kinase activity of pp60$^{v\text{-}src}$ is modified and full kinase activity only becomes apparent when the protein is attached to the plasma membrane. The role of the complex is unclear since it is present in normal cells (containing only the 50 kd and 90 kd proteins) and binds at least two other retroviral transforming proteins (v-fps and v-ros products, see below) (48). While the pathway to the plasma membrane is relatively clear, the nature of the attachment of pp60$^{v\text{-}src}$ to the membrane is unclear. It has recently been demonstrated that the protein is modified not only with phosphate but also with myristate (49), which is linked to a

glycine residue present at the amino terminus of pp60$^{v\text{-}src}$ (see Fig. 5). The role that this modification plays in membrane attachment, however, is unclear although it is known that attachment occurs in the amino-terminal position of the molecule. The role of membrane attachment in tumorigenesis is unclear as well. Mutants of RSV which have elongated or shortened amino-terminal halves display reduced affinity for the plasma membrane and induce non-invasive benign tumors in vivo rather than metastatic lethal tumors (50). Information regarding the localization and presumed tyrosine specific protein kinase activity of pp60$^{v\text{-}src}$ has led to a search for cellular proteins that might serve as a substrate for pp60$^{v\text{-}src}$. Several proteins have been identified (by virtue of an increase in level of tyrosine phosphorylation after transformation of cells by ASV), which have been thought at various times to be substrates for pp60$^{v\text{-}src}$ kinase activity. The most readily apparent and abundant potential substrate for pp60$^{v\text{-}src}$ is a protein of 36-39 kd, now known to be located on the inner surface of the plasma membrane. Phosphorylation of the 36-39 kd protein occurs in cells transformed by any of the oncogenes that encode tyrosine-specific protein kinases (src, fps, yes, ros; see below). Phosphorylation of this protein has been provisionally associated with induction of anchorage-independent growth and tumorigenesis by the use of mutations which render v-src partially transformation defective (51). However, it is not the only contributor to neoplastic transformation by tyrosine phosphorylation because the protein is absent from lymphoid cells transformed by v-abl (the oncogene carried by the murine Abelson leukemia virus which encodes a tyrosine-specific protein kinase (52)).

Several other potential substrates for pp60$^{v\text{-}src}$ activity have been identified. Vinculin, a cytoskeletal protein found in adhesion plaques, is phosphorylated on tyrosine in cells transformed by RSV. However, it does not inevitably accompany cellular transformation by oncogenes that specify tyrosine-specific protein kinas es. Three other potential substrates are enzymes in the glycolytic pathway, however, they are not involved in pathway regulation so it is not clear whether phosphorylation of these enzymes could have any physiological consequences (53,54). Thus it is not yet possible to argue that phosphorylation of proteins on tyrosine is essential for neoplastic transformation by pp60$^{v\text{-}src}$ (or any of the other tyrosine kinase encoding oncogenes) or identify cellular proteins whose phosphorylation is responsible for the phenotypic changes observed in the neoplastic cell.

3.2. The fps oncogene

As described above, several replication-defective ASVs have been identified which carry cell-derived transforming sequences, which, like src, encode tyrosine-specific protein kinase activities. One of these, fps, has been found in several virus isolates; the Fujinami sarcoma virus (FuSV) and PRC viruses (PRCIV and PRCII; see Fig. 6). Interestingly, it is derived from the same cellular gene as the v-fes oncogene found in the Gardner-Arnstein and Snyder-Theilen strains of feline sarcoma virus (55). Most work on the protein product of the fps genes has been carried out with FuSV and PRCII; therefore the discussion will be confined to these viruses. As with the DLVs discussed previously, FuSV has been found to be replication-defective and encodes a single protein which is a fusion between gag and fps sequences (see Fig. 6) and has a molecular weight of 130-140 kd ($p140^{gag-fps}$). Nucleotide sequence analysis of molecular clones of v-fps have shown that about 40% of the amino acids at the carboxy terminus of $p140^{gag-gps}$ are identical to a region near the carboxy terminus of $pp60^{v-src}$ around the tyrosine 416 that is phosphorylated (43,56).

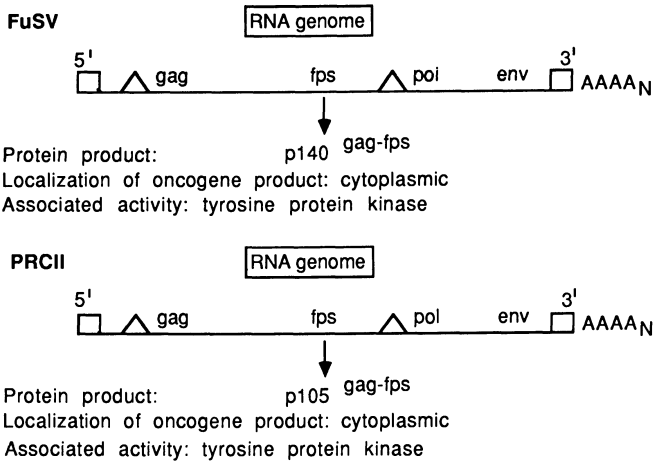


Figure 6. Genome structure and protein products of fps-containing viruses, Fuyinami sarcoma virus and PRC II. PRCII fps contains a deletion of 1 kb and several base changes when compared to FuSV fps.

As predicted, $p140^{gag-fps}$ appears to encode a tyrosine-specific kinase activity (57). However, in immune complexes this activity is predominantly

autocatalytic (in contrast to $pp60^{v\text{-}src}$ where the predominant phosphorylation is on immunoglobulin). The kinase activity resides in the region of homology with $pp60^{v\text{-}src}$ within the 29 kd carboxy terminus of $p140^{gag\text{-}fps}$. As with $pp60^{v\text{-}src}$, $p140^{gag\text{-}fps}$ is modified in vivo by phosphorylation and is associated with the two cellular proteins of 50 and 90 kd for a portion of the time (58).

The gag-fps protein of FuSV appears to be located in the cytoplasm (in contrast to the src protein). Fractionation of rat cells transformed with FuSV suggests that $p140^{gag\text{-}fps}$ is present in heavy cytoplasmic "granules", sensitive to salt concentration. In chicken cells the protein appears in a fraction enriched for plasma membranes in a salt-sensitive form. When cells transformed by FuSV have been examined for phosphotyrosine-containing proteins, a similar set of proteins have been identified when compared to src-transformed cells (59).

The PRC viruses, while containing an fps gene, differ slightly from FuSV. In vivo, FuSV induces tumors relatively efficiently; PRC viruses, however, are only poorly tumorigenic. When the genomes of FuSV and PRCII were compared, it was found that a region of about 1 kb present in FuSV was missing in PRCII on the 5' side of the region homologous to v-src. The effect of this deletion on the phenotype which PRCII induces is unclear since there are also 13 amino acid differences between the products of the shared regions of FuSV-fps and PRCII-fps (60,61). While PRCII behaves differently in vivo, its behaviour in vitro is very much like FuSV. The protein product of PRCII (a gag fusion protein $p105^{gag\text{-}fps}$) has kinase activity similar to that of FuSV and PRCII can fully transform chicken fibroblasts in culture (55).

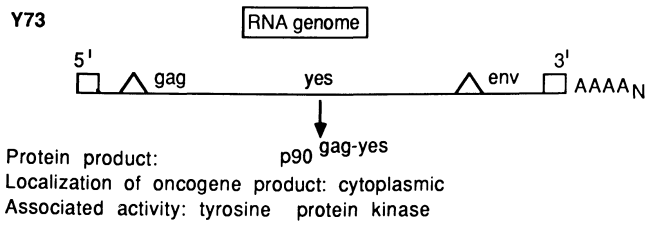


Figure 7. Genome structure and protein products of the yes-containing virus Yamaguchi 73 virus, Y73.

3.3 The yes oncogene

The oncogene yes is contained in the genome of Yamaguchi virus, Y73. Nucleotide sequence analysis of the genome of Y73 and comparison with src has revealed extensive similarity between src and yes (62); 82% of the 436 amino acids from the carboxy terminus of $pp60^{v\text{-}src}$ are identical to amino acids from the homologous area of $p90^{gag\text{-}yes}$, the fusion protein product of Y73 (see Fig. 7). This close resemblance between src and yes is reflected in the immunologic cross-reactivity between the two oncogenes (58), the panel of proteins phosphorylated on tyrosine in Y73- and RSV-infected cells, and in the association of $p90^{gag\text{-}yes}$ with the cellular 50 and 90 kd proteins (51).

3.4. The ros oncogene

Two recent isolates of ASV, URI and URII, were reported by Balduzzi et al (63). URI virus came from primary tumor material supplied by Dr. B.W. Calnek in 1969, and URII came from material provided by Dr. R.E. Luginbuhl in 1963. Tumor material was inoculated into chicks and the virus strains were isolated from resulting tumors by cocultivation of tumor cells with chick embryo fibroblasts. The pathogenesis of URI and URII is novel in the morphology of the tumors induced. With URI, tumors were produced in 4-week-old chickens, 8 days after inoculation into the wing web and two types of tumors were found: soft tumors with mostly mucoid components and tumors containing lymphoid nodules. URII produced a slightly different and more uniform cellular morphology, showing only spindle cells. In vitro, however, the viruses transform avian fibroblasts in a manner similar to the other ASVs (55). The sole protein product of the avian sarcoma virus URII is a fusion protein $p68^{gag\text{-}ros}$ (64). This protein is phosphorylated on both serine and tyrosine residues and has an in vitro kinase activity specific for tyrosine (see Fig. 8). Therefore, like the other ASVs, in URII the transforming gene product is a tyrosine protein kinase that is found associated with the membranes of transformed cells.

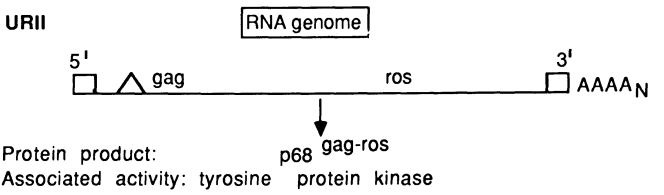


Figure 8. Genome structure and protein product of the ros-containing virus URII.

4. CONCLUDING REMARKS

As stated at the outset of this review, the aim of this Chapter is to provide the reader with a brief overview of the avian sarcoma viruses (ASV) and defective (acute) leukemia viruses (DLV) and their gene products that are thought to be associated with disease. Chapter 7 will deal with the pathogenesis and pathology in much more detail. The other chapters describe non-pathogenic endogenous ALV and exogenous ALV that transform cells mainly by insertional mutagenesis. As will become apparent, this latter group involves the cellular proto-oncogenes erbB and myc.

Hopefully, this Chapter will provide some useful areas of comparison for mechanistic consideration. There are, however, other aspects that are outside the range of this Chapter and indeed this whole monograph. Therefore, in conclusion, a list of recent reviews is included that provide more details for interested readers. The effects of avian retroviral oncogenes on hematopoietic cell differentiation (65), the role of tyrosine kinases in transformation (48), a review of oncogenes from avian retrovirus and other viral model systems (66), and the molecular genetics of cellular oncogenes (67,68), are all areas of intense interest and together with this monograph provide the reader with many hours of interesting and hopefully intellectually stimulating reading.

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3

EPIZOOTIOLOGY OF AVIAN LEUKOSIS VIRUS INFECTIONS

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G.F. de Boer (ed), Avian Leukosis.

1. INTRODUCTION

This Chapter is concerned with the spread and survival of avian leukosis virus (ALV) in populations of domestic fowl, and the various factors which influence infection rate and incidence of disease. The term ALV as used here denotes the common "wild-type" exogenous avian retrovirus which causes mainly lymphoid leukosis (LL). Elsewhere it has also been termed LL virus. The avian retroviruses are remarkable for the strategies they have envolved for their survival. Some, those belonging to the endogenous virus group, are transmitted from generation to generation as viral genes in the genome of the host germ cells, where they may be regarded as forms of parasitic DNA, the eucaryotic equivalents of plasmids in procaryotes. Others comprise the exogenous virus group, being transmitted as infectious virions which invade cells, individuals and populations from their environment. These viruses are transmitted vertically, maintaining the infection from generation to generation, and also horizontally, spreading between contemporaries. The exogenous viruses fall into four subgroups, A, B, C and D, on the basis of viral envelope properties, whereas the endogenous viruses belong to subgroup E. The exogenous ALVs are usually fully expressed, while endogenous viral (ev) genes encode some of the structural proteins. However, some of the ev genes encode infectious virions which behave as exogenous viruses (see Chapter 5). Viruses of subgroups A, B, C and D differ genetically from those of subgroup E in several ways, and the relationship between them is not clear. Temin (1) believes that the endogenous subgroup E viruses represent an evolutionary stage between normal moveable genetic cellular elements termed transposons, and more virulent

genetic entities capable of extracellular existence which we know as the exogenous viruses. Knowledge is being gained that genetic recombination can occur between endogenous and exogenous viruses and that they can interact in the host in other ways (2). The avian retroviruses are remarkably successful parasites: those of subgroup E have little if any detrimental effect on the host, while those of the other subgroups usually cause serious disease in only a small minority of the fowl they infect. For the commonly occurring wild-type exogenous ALV induction of neoplasms is an uncommon accident of infection and forms no essential part of the life cycle of the virus.

Recently, the relationship between exogenous ALV and other avian tumor viruses, more common in the laboratory than in the field, has become clearer. Many of these rarer viruses appear to be the consequence of another type of viral accident, involving genetic recombination between ALV and host cell oncogenes, with the emergence of new oncogenic viruses (3).

The emphasis of this Chapter will be on the epizootiology of exogenous ALV. This knowledge has been acquired mostly since the late 1940s and has become particularly important over the past 10 years as a basis for ALV eradication schemes operated by the poultry industry (see Chapter 13).

2. VIRUS CLASSIFICATION

2.1. Introduction

Viruses of the avian leukosis/sarcoma group are termed avian type C oncoviruses and form a subgroup of the subfamily Oncovirinae of the family Retroviridae (4). They are classified by two main criteria: the predominant type of neoplasm they induce (pathotype) and the viral envelope subgroup. Within these classes, type variation occurs, and numerous strains or isolates exist.

2.2. Pathotypes

Avian leukosis/sarcoma viruses are divided, under the pathotypic criterion, into acutely transforming viruses and slowly transforming viruses (see Chapter 2). The former carry transforming v-onc genes, and the particular gene(s) present determine the target cell transformed and the neoplasm produced: e.g. erythroblastosis, myeloblastosis, myelocytomatosis, sarcomas. Slowly transforming viruses cause mainly LL. They do not possess v-onc genes, but induce neoplasms by the "promoter insertion" mechanism whereby a cellular onc gene, c-myc is activated (see Chapters 4, 7 and 9). Experimentally ALV sometimes also induces

erythroblastosis, apparently again by promoter insertion and activation of another cellular onc gene, c-erbB (5). This finding may clarify the relationship between ALV and the rarer neoplasms that occur sporadically, they probably arise by activation of cellular onc genes. These neoplasms may contain acute transforming viruses which arise by recombination between viral and cellular genetic sequences (3). The endogenous ALVs (see later and Chapter 5) have little or no oncogenicity in domestic fowl.

2.3. Subgroups

Avian leukosis/sarcoma viruses that occur in domestic fowl can also be classified into five subgroups, A,B,C,D and E, on the basis of viral envelope glycoproteins, which control their antigenicity and host range. Viruses of two other subgroups, F and G, have been isolated from pheasants. ALVs of subgroups A,B,C and D are oncogenic and, as mentioned already, are transmitted horizontally and vertically (see sections 4.2 and 4.3) in an infectious form, and are termed, accordingly, exogenous viruses. Subgroup E viruses are non-oncogenic, and are usually transmitted in a non-infectious form as viral genes as part of the host genome; they are termed endogenous as they arise from within the host cell.

Many of the acute transforming viruses are genetically defective and lack the viral envelope gene, env. Although they can still transform cells and produce neoplasms, they cannot form infectious virions. They can replicate, however, if the transformed cell is also infected with a non-defective virus, for example ALV. In this situation, the acute transforming virus acquires viral envelope from ALV (the "helper virus") and thus also the subgroup of the helper virus.

2.4. Types

Viruses within a subgroup show minor variations in antigenicity, with antisera tending to neutralize homologous virus more strongly than heterologous virus (6). Viruses within a subgroup also vary in their ability to produce immunological tolerance to other members of the subgroup (7). Subgroup B viruses appear to be more heterogeneous than subgroup A viruses, but in general information on serotypic variation is meagre.

2.5. Strains

Numerous laboratory isolates, termed strains, of avian leukosis/sarcoma viruses exist, with representatives of most pathotype-subgroup combinations. The rather arcane method of designating strains is described elsewhere (8).

3. INCIDENCE AND PREVALENCE

3.1. Exogenous virus infection

3.1.1. Infection. Infection of commercial flocks by exogenous ALV is almost certain to be present unless the stock is genetically resistant to infection or efforts have been made to eliminate the infection. In the USA, UK, and the Netherlands subgroup A viruses occur frequently and subgroup B viruses more rarely, either as isolates from infected flocks or from cases of LL (9,10,11). In a survey of 19-month-old stock in 1978/79 laying trials De Boer et al. (11) found antibodies to subgroup A and B viruses in 58% and 16% respectively in white layers; corresponding figures in brown layers were 16% and 1%. Subgroup C and D viruses have not been recognized in the field in the USA or UK, but were reported to be quite prevalent, along with subgroup A and B viruses, in Finland by Sandelin and Estola (12). Morgan (13) detected antibody to the Bryan strain of Rous sarcoma virus (RSV) in domestic chickens and wild fowl in Tanganyika and Mozambique. In a survey of 17 species of birds mainly from eastern North America, Rabin and Sladen (14) found specific antibodies only in domestic chickens. Antibodies to subgroup A and B viruses were common among wild fowl and domestic fowl in Kenya and Malaysia (15,16) and there was some evidence for antibody to subgroup D viruses in Kenya.

3.1.2. Clinical disease. Sporadic cases of LL occur in most flocks. De Boer et al. (11) reported LL mortality in the Netherlands as 2.18% of 11220 white layers and 0.57% of 7920 brown layers recorded in random sample tests over the period of 1973 to 1979. Occasionally more heavy losses from LL occur, e.g. 23% in certain commercial breeder flocks (17). Crittenden and Witter (18) investigated flocks with high mortality from LL, including an extreme instance in which birds were dying at 6% a month for the first half of the 10-month laying period, mainly from LL. In the UK, Randall et al. (19) found LL constituted 1.4% of 2615 autopsies from laying flocks, representing about 0.17% of all birds at risk. Almost identical percentage figures were observed in broiler breeders (20).

The other leukoses, erythroblastosis, myeloblastosis and myelocytomatosis, occur sporadically, and are much less common than LL. Rare epizootics have been reported, as of erythroblastosis in 5-week-old birds (21). Haemangiomas and nephroblastomas are the most frequently observed non-leukotic tumors (17,22). They usually occur sporadically and rarely, but epizootics have been recorded. Perek (23) observed an outbreak of histiocytic sarcomas in a flock of adult hens in

which tumors were found in 90% of 400 birds examined over a 4-month-period. Contact transmission of RSV has been observed experimentally (24), suggesting that epizootics of the rarer, non-leukotic tumors might be caused by horizontal spread of virus. Epizootic outbreaks of haemangiosarcomas have recently occurred in laying flocks in Israel (25). Osteopetrosis also occurs sporadically or, more rarely, as epizootics.

3.2. Endogenous virus infection

3.2.1. Infection. Endogenous viral (ev) genes have been identified in all domestic fowl in which they have been sought, with the exception of RPRL line 0 developed for freedom from ev loci. Most of the defined loci have been identified in White Leghorns, but surveys of other breeds indicate these and other ev loci are numerous (see Chapter 5). Frisby et al. (26,27) surveyed other galliform birds for ev genetic sequences. They were demonstrated in red jungle fowl (Gallus gallus) the progenitor of the domestic fowl, and in true pheasants (Phasianus), partridges (Perdix, Alectoris) and grouse (Lagopus), but not in other species of jungle fowl (G. sonnerati, G. lafayettei, and G. varius), several other pheasant genera (Tragopan, Lophura, Crossoptilon, Catreus and Chrysolophus) or in guinea fowl (Numida), quails (Coturnix), pea fowl (Pavo), or turkeys (Meleagris). It was concluded that the ev sequences in the domestic fowl were not related to evolutionary changes within the family Phasianidae but that they had become incorporated following speciation but before domestication.

3.2.2. Clinical disease. Endogenous subgroup E viruses, as typified by RAV-0, have little or no oncogenicity in domestic fowl (28,29) although they may be oncogenic in other species (30). Genetic recombinants between endogenous and exogenous subgroup A or B viruses can be oncogenic in domestic fowl probably as a consequence of the presence of the strongly promoting long terminal repeat (LTR) genetic sequence of the exogenous virus (31). It is probable that such recombinants occur in nature, but their biological significance is unknown.

4. MODES OF NATURAL TRANSMISSION

4.1. Introduction

Three modes of natural transmission of ALV are recognized:

(1). Horizontal transmission, in which virus spreads from bird to bird within a generation either by direct contact of infected birds with non-infected birds, or indirectly, by contact between uninfected birds and fomites. This mode of spread

is of particular importance for the high incidence of the infection within a flock.
(2). Congenital transmission, the form of vertical transmission in which infectious virus is transmitted from hen to offspring (also termed egg transmission). Although usually only a minority of chicks become infected in this way, the route is important for maintaining the infection from generation to generation, and for providing infectious foci for horizontal transmission.
(3) Genetic transmission, a form of vertical transmission in which viral genome, sometimes capable of coding for infectious ALV, but often genetically defective, is transmitted in a Mendelian fashion from parents to offspring. This mode of transmission has only been recognized for the endogenous ALV of subgroup E. Details of these three modes of transmission are given next.

4.2. Horizontal transmission

Most infections by exogenous ALV are acquired after the chick has hatched by exposure to virus shed by infected birds. Natural sources of infection include faeces, saliva and skin. The presence of virus in faeces and saliva was demonstrated by Burmester and Gentry (32,33) by induction of LL (termed "visceral lymphomatosis") in susceptible chickens inoculated at 1 day of age with faeces and oral washings from cases of LL and from normal appearing hens. Judged by the incidences of LL induced, virus was detected more consistently in oral washings than in faecal extracts. Virus could be found as early as 10 days of age, and subsequently, in oral washings from chicks inoculated with ALV at 1 day of age, and at 30 days of age in chicks in contact with them. Oral washings from day-old chicks from an infected dam also caused LL when inoculated into susceptible chicks. These findings were of importance in demonstrating that ALV infection can remain inapparent for long periods, and may indeed never result in overt disease, but that virus may be shed from such carrier birds in saliva and faeces.

As these workers recognized, chicken faeces ("droppings") include both intestinal and urinary excretions. Furthermore, in the hen they may also be contaminated with secretions from the oviduct which often contain large amounts of ALV (see later). Thus ALV in faeces may come from several sources. Some at least is likely to be derived from the alimentary tract as Spencer et al. (34) detected viral antigen in Lieberkühn glands of the gut, and in the proctodeal region of the cloaca, and virus particles were observed among gut epithelial cells. Earlier, Ziegel et al. (35) observed large amounts of virus in pancreatic acinar cells, but whether this virus finds its way into faeces is not clear. Weyl and

Dougherty (36) detected 10^6-10^8 infectious units of ALV per gram of faeces from immunologically tolerant chicks. Similar high titres of virus were observed in meconium from day-old congenitally infected chicks (37).

Recently, ALV has been found in swabs of the skin surface of newly-hatched and adult chickens (38), suggesting that this may be a source of airborne virus. These sources of virus explain the contact transmission that occurs in chicks from a relatively disease-free source when reared in close contact with chicks from an infected flock (39,40). Virus shed by congenitally infected chicks is important in early horizontal transmission leading to a high incidence of LL (41,42). Various routes of infection have been identified, notably, tracheal, nasal, oral, conjunctival and cloacal (36,43) (see also section 8.5).

4.3. Congenital transmission

Transmission of ALV from the dam to her progeny through the egg is important in maintaining the infection from one generation to another. Although suspected for many years, direct evidence for its occurrence was first reported by Cottral et al. (44) in 1949 and described in detail in 1954 (45). In a series of experiments they inoculated 1- to 4-day-old susceptible chicks with unfiltered or filtered suspensions prepared from livers of embryos from clinically normal hens, and induced a significantly high incidence of LL, overall in 29% of chicks compared with 4% in isolated controls. Next, Burmester et al. (46) found that 16 out of 17 dams from two LL-susceptible infected lines (lines 9 and 15) shed ALV to their embryos, as judged by disease transmission, compared with one out of five hens from an LL-resistant infected line (line 6) and none out of two isolated line 15 hens. Burmester and Waters (42) then reported that the incidences of LL in progeny from shedding and non-shedding hens were not significantly different: they observed that transmission of virus to embryos and chicks did not necessarily result in a high incidence of neoplasia, but that such chicks were a source of infection to chicks from other parents not similarly infected and hence lacking maternal antibody. They concluded "The importance of egg transmission lies not in the disease which may or may not occur in chicks hatched from infected eggs, but in the disease which is transmitted by direct or indirect contact from chicks which hatched from infected eggs to chicks which hatched from eggs of hens that have had no experience with the virus". Subsequently evidence was presented that the amount of virus shed by hens decreased as the hens became older (47).

The experiments of the East Lansing group stand as classics in the avian leukosis field, remarkable for their planning and execution, with each transmission

experiment of virus detection taking some 9 months to complete, and for the clarity of the information they provided on the natural transmission of ALV. Soon after, studies on the natural history of ALV infection were transformed by the discovery by Rubin (48) of the RIF test (see Chapter 11) whereby ALV could be detected in a few days by its ability to induce resistance of chick embryo cells to transformation by RSV. Using this method Rubin et al. (49) demonstrated the presence of ALV in chick embryos and yolk of unfertilized eggs following congenital transmission. Most embryo infection was attributable to ALV viremic hens which lacked antibody; congenital transmission by non-viremic, antibody-positive hens was much more erratic. Furthermore, viremic roosters, even when their testicular cells produced ALV in cell cultures, were shown not to infect their progeny. Subsequently, it has been shown that insemination with semen containing virus does not result in infected embryos (50). Although congenital transmission is important in transferring the infection from one generation to the next, Payne and Bumstead (51) have argued on theoretical grounds that congenital transmission alone would be inadequate for maintenance of infection over successive generations. A large amount of horizontal infection leading to new vertically transmitting hens is required. In the field, an average increase of 9% infected embryos to 27% viremic chicks at the age of 7 weeks has been observed (11).

The mechanism whereby exogenous virus is transferred from the dam to the embryo is still unclear. Theoretically, transmission could be trans-ovarial, via the female germ cell, or trans-oviductal, occurring as the fertilized egg passes down the oviduct or subsequently. Although ALV can be detected in the ovum, and could therefore lead to embryo infection, it is believed that infection occurs mainly if not entirely from virus which is excreted from the oviduct into the egg albumen and thence to the embryo. The potential for trans-ovarial infection derives from two lines of evidence. Firstly, Rubin et al. (49) detected large amounts, possibly more than 10^7 infectious units, in the yolk of unfertilized eggs from viremic hens. Spencer et al. (37) also detected virus in the yolk of eggs from viremic, antibody-negative hens, although only rarely eggs from antibody-positive hens. Secondly, DiStefano and Dougherty (52) observed virus replication in the cells of the ovarian follicle which surround the developing ovum. Rubin (53) could not infect embryos by inoculating virus into the yolk sac of freshly-laid eggs, although this route was effective in some instances at 3 and 6 days of incubation. Similarly, Fadly et al. (54) found yolk sac infection of 7-day-old embryos to be effective. However, in a study of statistical associations between different classes of infection of hens and embryo infection, Payne et al. (55) found strong associations between oviduct

infection and embryo infection, but no firm evidence for embryo infection by a non-oviductal route. De Boer et al. (56), on the other hand, believed embryo infection to be more closely related to viremia than with virus in the albumen, suggesting the possibility of a non-oviductal, possibly ovarial, route.

Nevertheless, strong evidence has been adduced for the importance of trans-oviductal infection of embryos. Spencer et al. (57) discovered the presence of virus in the albumen of unincubated eggs, consistent with earlier evidence from DiStefano and Dougherty (52) for abundant virus replication throughout the magnum of the oviduct. Subsequently Spencer et al. (37) observed strong associations between virus in vaginal swabs, egg albumen and embryos, and these associations have been amply confirmed by others (55,56,58,59). It is believed that transfer of virus from the albumen to embryo takes place at an early stage after ovulation, since storage or heat treatment of eggs does not prevent congenital transmission (37,60). The exact mechanism of transfer remains unclear: it does not appear to be highly efficient, since congenital infection frequently fails to occur even when the egg albumen or oviduct contain ALV. For example, in the study of Payne et al. (55) only 25.4% of embryos from eggs with virus in albumen were infected. Spencer et al. (37), in seeking to explain the erratic congenital transmission of virus in antibody-positive hens even though virus was present in albumen, suggested that spermatozoa might mechanically carry virus from the albumen or yolk to the zygote, and that this might be subject to neutralization by antibody in the yolk. An alternative explanation might lie in differences in the amount of virus in the albumen from hens with or without antibodies, as suggested by recent results of Spencer et al. (34). Equally, antibody in the yolk might interfere with passive migration of virus into the ovum. Thermal inactivation of virus in the albumen may also prevent embryo infection.

4.4. Genetic transmission

The term "genetic transmission" applies to a form of vertical transmission of avian retroviruses in which viral genes are transmitted from one generation to the next in a Mendelian fashion along with other host genes (61). This phenomenon represents the ultimate form of parasitism, "infection" of host DNA by parasitic viral DNA. Genetic transmission requires the viral genes to be present in the genome of germ cells. In the fowl this occurs in both sexes, so that either the hen or the cock can pass retroviral genome to their progeny, unlike the situation with congenital transmission of infectious virus, for which only the hen is responsible (see section 4.3). Another important feature of genetic transmission is that it

applies only to avian retrovirus of subgroup E (see section 2.3): genetic transmission of viruses of subgroups A, B, C or D has not been recognized.

The locations of the subgroup E viral genes in the host cell genome are termed "endogenous viral (ev)" loci, and more than 20 loci have been identified (see Chapter 5). Genetic analysis of ev loci has revealed that some contain all the proviral genes necessary for production of infectious retroviral particles but that others are genetically defective and unable to produce virus, although viral antigens, such as group-specific (gs) antigen or envelope antigen, may be produced. When the complete genome is present, infectious virus may be produced, either spontaneously or after certain stimulations, and this exogenous form of the virus will then be transmitted additionally congenitally or horizontally as discussed for exogenous viruses of the other subgroups. The epizootiological implications of endogenous ALV are only just beginning to be studied and appreciated. Although rarely if ever naturally oncogenic themselves, it is evident that they may modify the response of the host to infection by the exogenous, oncogenic viruses (see section 7.4, Chapter 7). They have been shown also to undergo genetic recombination with exogenous viruses: the biological significance of this is not yet known.

5. INFECTION PATTERNS WITHIN FLOCKS

5.1. Exogenous virus infection

Our knowledge of infection patterns within a flock stems largely from the work of Rubin et al. (62). Rubin defined four serological classes of birds: (1) Viremia, no antibody (V^+A^-), (2) No viremia, with antibody (V^-A^+), (3) Viremia, with antibody (V^+A^+), and (4) No viremia, no antibody (V^-A^-). Birds in the V^+A^- class derive from congenitally infected chicks. They are immunologically tolerant to ALV and consequently lack virus-neutralizing antibodies, but have high levels of viremia, as evidenced by testing of blood plasma, and of virus in other tissues. Birds in this class are normally in a minority: 20% of hens in the flock studied by Rubin et al. (62) and 7% and 16% in flocks studied by Payne et al. (55,58). Related to these low proportions are the low proportions of infected embryos or viremic chicks from infected flocks: 5% in the study of Solomon et al. (63) and 6% and 10% in the studies of Payne et al. (55,58). According to Rubin et al. (62), V^+A^- hens transmit virus to most (94%) of their progeny but others have found congenital transmission by such hens to be more variable. In the studies of Payne

et al. (55,58) 24% and 30% of embryos from V^+A^- hens were infected. It should be noted, in comparing the epizootiological findings of Rubin et al. (62) with those of others, that Rubin worked with a flock selectively bred for a high incidence of neoplasms. It is possible that this selection favoured mechanisms causing high congenital transmission rates.

Birds in the V^-A^+ class have virus-neutralizing antibody in their serum and no viremia. They acquire their infection by contact after hatching, commonly from congenitally infected hatch mates. In a susceptible flock they are in the majority: 78% of hens in the flock studied by Rubin et al. (62) and 64% and 56% in the flocks studied by Payne et al. (55,58) which included about 20% genetically resistant birds. V^-A^+ hens transmit virus to their progeny much more erratically than do V^+A^- hens. Rubin et al. (62) observed that 14% (1 in 7) of non-viremic hens (i.e. V^-A^+) was a congenital transmitter of virus, with 6% of embryos from these transmitters being infected. In the study of Payne et al. (58) 24% of V^-A^+ hens transmitted virus to their progeny, with 15% of embryos from transmitting hens being infected.

The third class mentioned, V^+A^+, consists of birds with both viremia and antibodies. This was recognized to be a small class by Rubin et al. (62), two or three birds out of eighty-five, and by Payne et al. (55,58) where they comprised 3% and 5% of hens. Rubin found their viremia levels to be low and it seems that infectious antibody-bound virus in the serum could be responsible for this class.

The fourth class, V^-A^-, consists of non-viremic, antibody-negative birds, and occurs, within a susceptible population, when horizontal spread of infection is still incomplete, and in genetically resistant populations which are insusceptible to infection. These classes apply equally to male and female chickens. However, the infective status of the male apparently has no influence on the rate of infection of his progeny: all congenital transmission of virus is from the female side. The classes are relevant only within the context of a virus subgroup, and status for one subgroup is independent, as far as is known, of status in respect of another subgroup. For example, a bird could be V^+A^- for subgroup A virus but V^-A^+ for subgroup B virus, in a flock with a mixed infection by subgroups A and B.

More recently, a second way of classifying hens has developed from the finding of Spencer and his colleagues (37,57) that some infected hens shed large amounts of virus and gs-antigen into the albumen of their eggs and that this occurrence is correlated with embryo infection. In these and other studies a distinction is made in use of the term "shedding" to denote release of virus into albumen and the environment, and "congenital transmission" to denote infection

of the embryo. Accordingly, hens may be classified as: (a) Shedders and (b) Non-shedders. Shedder hens can be identified by detection of either infectious virus or, more usually, its gs-antigen, in egg albumen or vaginal swabs. This classification is important because, as remarked above, these hens are those most likely to produce infected progeny and their avoidance can greatly reduce congenital transmission and assist in eradication of ALV (see Chapter 13). Thus Spencer et al. (37) observed that 30/33 hens with virus in their vaginal swabs transmitted infection to their progeny, and that overall 37% of their embryos were infected, in contrast to hens with no virus in their vaginal swabs, of which only 3/12 transmitted to 3% of their progeny overall.

In the studies of Payne et al. (55,58) attempts were made to correlate the classification systems of Rubin and Spencer in order to identify more clearly the hen factors associated with shedding and transmitting of virus. Thus the proportions of hens shedding virus or antigen to albumen, or transmitting virus to embryos, and the proportion of infected eggs or embryos resulting, were all significantly increased in hens with virus in vaginal swabs or with viremia (55). These latter traits were partially associated: 89% of 18 viremic hens were vaginal swab test-positive compared with only 31% of 67 non-viremic hens. These studies, in agreement with those of Spencer et al. (37), firmly pointed, as already discussed, to the overwhelming importance of oviduct infection in producing infected embryos; 96% of all infected embryos came from hens which were vaginal swab test-positive or from eggs which had virus or gs-antigen in the albumen.

5.2. Endogenous virus infection

Little is known about the expression of endogenous ALV within a flock. For defective, non-infectious, endogenous viruses, transmission is entirely genetic and congenital viral spread is not to be expected. Some ev genes (see Chapter 5) are not defective, and can be expressed as fully infectious virus. These might therefore be expected to show epizootiological features similar to those of the exogenous viruses. Interesting results of Robinson and Eisenman (64) suggest however that endogenous viruses may differ from exogenous ALV, for they observed that even in susceptible, highly viremic hens, RAV-0, and certain recombinants between endogenous and exogenous viruses, were not shed into egg albumen and were not, by implication, congenitally transmitted. This property appeared to be determined by the presence of the endogenous viral gag gene which encoded a $p27^{0}$ capsid protein of an electrophoretic mobility slightly

different from exogenous viral p27 and which, apparently, restricted the ability of the endogenous virus to replicate in oviduct tissue. However, shedding of endogenous virus has been observed by Crittenden and Smith (see Chapter 5, section 9.3), suggesting an influence of viral strain and host factors.

6. VIRAL FACTORS INFLUENCING INFECTION AND DISEASE

6.1. Subgroup and strain

No influence of the virus subgroup per se on the incidence of LL has been observed, since this is a viral attribute derived from the envelope gene whereas pathogenicity appears to be dependent on the LTR region of the viral genome. Ability of ALV to induce anemia does appear to depend on subgroups, being a property particularly of subgroups B and D (65). Viruses of subgroup B, but not subgroup A, have been reported to be immunosuppressive (66). Of great importance in tumorigenicity, however, is the strain of virus within any particular subgroup. As discussed in Chapter 9, virus isolates or strains vary in the variety and incidence of the different types of tumor they induce under given conditions (67). These differences appear to be a consequence of genetic variation between viruses within the avian retrovirus family and because most viral strains are a mixture of different viral entities. It is important to note, however, that cloned isolates of ALV are shown to be pluripotential in oncogenicity (68). Field isolates of ALV induce mainly LL, but occasionally also erythroblastosis, osteopetrosis and other tumors. Virus strains isolated from tumors other than LL, such as erythroblastosis, tend to induce a higher incidence of that tumor (69), probably because of the generation of variant viruses by genetic recombination (3).

Recently it has been shown that strains of ALV may vary also in the persistence of the viremia they induce, in the antibody response, in shedding rates, effect on body weight, and on antibody response to killed antigens (2).

6.2. Dose

In experimental studies, enlarging the dose of ALV increases the incidence of LL, decreases the age at death, and increases the incidence of erythroblastosis and other tumors (67,70,71) (Fig. 1). However, in the field erythroblastosis and non-leukotic tumours are not common, even though some birds will have been infected with high doses of ALV as a result of congenital infection.

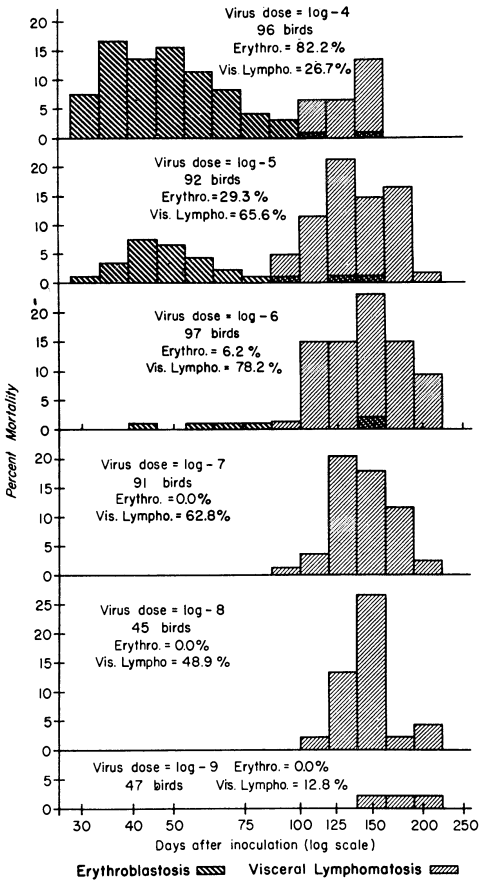

Figure 1. Influence of dose of RPL12 strain of ALV on mortality from erythroblastosis and lymphoid leukosis (visceral lymphomatosis) (from (71), by kind permission).

7. HOST GENETIC FACTORS INFLUENCING INFECTION AND DISEASE

7.1. Introduction

Genetic differences in susceptibility to ALV-induced diseases were first recognized many years ago, although the long-running confusion and controversy about disease relationships within the avian leukosis complex hindered precise study (72). However, even within this limitation, the foundations for our present knowledge were laid by the development of Waters (39,73) of inbred lines of fowl selected for resistance or susceptibility to the disease complex. These and other inbred lines subsequently provided the materials for the genetic analysis of resistance and the recognition of two levels of resistance to LL: (a) resistance to virus infection, and (b) resistance to tumor development (74).

7.2. Genes which influence resistance to virus infection.

7.2.1. Inheritance of resistance. Susceptibility or resistance to infection are encoded by genes which control the presence or absence of virus receptors on the cell membrane. Response to viruses of subgroups A, B, and C, either ALV or ASV, are determined respectively by three autosomal loci, Tv-A (75,76), Tv-B (76,77,78) and Tv-C (79,80), each with fully dominant susceptibility alleles, Tv-A^s, Tv-B^s and Tv-C^s, and recessive resistance alleles tv-A^r, tv-B^r and tv-C^r (Table 1). These gene designations (81) are usually abbreviated further, e.g. A^s, A^r, B^s etcetera. The Tv-A and Tv-C loci are closely linked (82). Responses to subgroup D viruses are strongly influenced by genes at the Tv-B locus (83,84), with cells resistant to subgroup B (B^rB^r) being at least partially resistant to subgroup D viruses. Genetic resistance to subgroup E viruses is controlled in a more complex fashion, the nature of which is still not finally settled. Payne et al. (85) described involvement of two autosomal loci, Tv-E and Inhibitor-E with pairs of alleles, Tv-E^s and tv-E^r, and I-E and i-E at the two loci (Table 1). An epistatic interaction was observed between genes at these loci, the dominant resistance gene, I-E, preventing expression of the E^s susceptibility gene. More recent studies by Robinson et al. (86) indicate that the I-E gene is an ev locus, the viral envelope products of which blocks the virus receptor encoded by the E^s gene. Crittenden and Motta (87) disputed the existence of the Tv-E locus, and believed that multiple allelism at the Tv-B locus explained their observations on genetic resistance to subgroup E virus.

The frequency of resistance genes in commercial flocks varies greatly depending on virus subgroup and type of fowl (Table 2). In the most detailed

survey made to date, by Crittenden and Motta (88), resistance to subgroup A was variable but generally low in the four types of bird surveyed, namely, white egg pure lines, white egg crosses, meat lines and brown egg lines. All types had some resistance to subgroup B, with moderate levels in the white egg types and rather higher levels in the meat and brown egg types. Resistance to subgroup C was low in white egg flocks, and moderate to high in meat and brown egg flocks.

Table 1. Genes controlling cellular susceptibility to the five ALV subgroups.

Virus subgroup	Locus[1]	Alleles	Dominant trait
A	Tv-A	Tv-A^s, tv-A^r	Susceptibility
B and D	Tv-B	Tv-$B^{s1,s2,s3}$, tv-B^r	Susceptibility
C	Tv-C	Tv-C^s, tv-C^r	Susceptibility
E	Tv-E	Tv-E^s, tv-E^r	Susceptibility
	I-E	I-E, i-E	Resistance

[1] The existence of independent Tv-B and Tv-E loci is not settled.

7.2.2. Mechanism and expression of genetic resistance. Resistance to infection is an early event and depends on the lack of specific virus receptors in the cell membrane, preventing viral penetration or uncoating. The biochemical nature of the virus receptors and their distribution amongst different cell types or tissues are poorly understood. Susceptibility and resistance is most easily studied in cultured chick embryo fibroblasts or chorioallantoic membranes of embryos using strains of RSV of different subgroups, where the occurrence of foci or pocks of sarcomatously transformed cells following virus infection, and their number, are a reflection of the presence of virus receptors and of the level of resistance or susceptibility (89). The chorioallantoic membrane assay is useful when large numbers of embryos have to be classified as susceptible or resistant, as in surveys and genetic segregation studies, whereas the chick embryo fibroblast culture assay is valuable when replicate cultures from individual embryos are required. The response phenotypes of cells or embryos identified using RSV are equally applicable to infection by the five subgroups of ALV.

Genetic differences in response to RSV may also be measured by inoculation of chicks by the subcutaneous, intramuscular or intracerebral routes, using tumor

incidence or mortality as a measure of response. Similarly, susceptibility to ALV may be measured by inoculation into chick embryos or newly-hatched chicks, and measurement of the tumor response. The Tv loci described above, of which Tv-A is the most studied, have a strong influence on the incidence of LL and other tumors in birds exposed to ALV (90,91,92,93). Chick assays have the disadvantage that they may only be quantified by quantal methods, and genetically controlled resistance mechanisms not operative in tissue culture or embryos, such as immune reponses, may also influence tumor development (see section 7.3). These other factors which influence tumor development, either by causing regression, as in Rous sarcomas, or by preventing transformation of target cells, as in LL, influence the final outcome of virus infection. They are the "second line of defense" if the "first line of defense", resistance to infection, is absent (93).

Table 2. Proportions of commercial stocks showing genetic resistance[a] to subgroups A, B and C of Rous sarcoma virus.

Virus subgroup	A		B		C	
Type of stock	Flocks with resistance/ total	Mean resistance (%) (range)	Flocks with resistance/ total	Mean resistance (%) (range)	Flocks with resistance/ total	Mean resistance (%) (range)
White egg pure lines	12/15	10.6 (0-89.6)	15/15	28.6 (5.0-72.0)	10/15	2.3 (0-6.4)
White egg crosses	1/10	0.1 (0-1.2)	10/10	36.2 (15.9-88.3)	6/10	1.7 (0-7.8)
Meat	14/17	11.8 (0-88.4)	17/17	61.4 (27.1-89.3)	17/17	21.8 (6.9-48.8)
Brown egg	6/6	7.9 (1.5-12.5)	6/6	89.4 (65.6-100)	6/6	30.0 (2.4-66.7)

[a] Determined by chorioallantoic membrane inoculation. (Data from (88), by kind permission).

The response phenotypes of cells, embryos or chicks from different genetic sources to the five virus subgroups are designated by a convention that indicates chicken cells (C) and the subgroups to which they are resistant (/subgroups). For example, C/ABE cells are resistant to subgroups A, B, E (and D, see section 7.2.1), but susceptible to subgroup C. C/0 cells are susceptible to all five subgroups. The level of resistance, compared with the most susceptible phenotypes, varies, up to 6 $\log_{10}$ units, probably as a consequence of multiple resistance alleles.

7.3. Genes which influence resistance to tumor development

Genes may influence the development of tumors in infected birds. Such genes clearly influence the growth or regression of Rous sarcomas: a dominant gene R-Rs-1 that lies within the major histocompatibility complex (MHC) has been identified as responsible for tumor regression (94,95). The responsible gene was more closely linked to the Ir-GAT locus than to the Ea-B locus in the MHC, suggesting that it is located in the immune response (B-L) region of the complex (96). More recent studies are pointing to a polymorphic family of resistance genes which show viral strain specificity (97,98). While most work on the influence of MHC genes on tumor development has been done with Rous sarcomas, and also with Marek's disease, some lesser influence on LL has also been noted (99). These genes are thought to control immune responses to tumors, to which LL seems to be less susceptible than some other tumors (see Chapter 7). Intrinsic resistance of the target B-cell to neoplastic transformation has been identified as of major importance in one strain of fowl, line 6, susceptible to infection but resistant to LL (100). Some evidence for an influence of the thymus alloantigen locus Th-1 on LL incidence has been reported (101).

7.4. Endogenous viral genes

As discussed in section 3.2.1. and Chapter 5, ev loci are almost ubiquitous in domestic fowl. Depending on their genetic make-up, these loci may be expressed as complete infectious virus, or as viral antigens during embryonic life or later, or they may be unexpressed. Such gene expressions stimulate either tolerance or immunity to viral antigens, and such phenomena may be expected to influence the response of the bird to other related viruses that they may encounter. Studies by Crittenden and his colleagues (2,102,103) have revealed a variety of effects: compared with conventional birds, birds lacking ev gene expression showed, following ALV infection and varying with viral strain, a high incidence of an acute runting syndrome, higher virus-neutralizing antibody responses, lower viremia, and probably lower virus shedding in the cloaca. Mortality from neoplasms was not influenced.

7.5. Other genes

Pani (104) reported that the black plumage colour gene (i^+) was associated with increased resistance to subgroup C RSV. The slow-feathering gene (K) has been associated with increased congenital and horizontal transmission of ALV and, as a result of this, depressed egg production (105). Bacon et al. (106) suggested

that expression of the ev21 gene, which is linked to the K gene, may lead to partial immunological tolerance, and hence greater expression, of exogenous ALV infection.

8. OTHER HOST FACTORS INFLUENCING INFECTION AND DISEASE

8.1. Sex

No effects of sex on infection by ALV appear to have been reported but effects on disease incidence occur. Burmester (107) found that LL (lymphomatosis) was twice as common in females than in males when the overall incidence of the disease was low. Castration abolished the resistance seen in males but this was restored in capons by treatment with testosterone (108,109). Studies on the pathogenesis of LL by Cooper et al. (110) revealed that whereas early neoplastic transformation of bursal follicles occurred equally in males and females, most birds dying from the disease were females. This sex difference is believed to be a consequence of the earlier natural regression of the bursa in males, under the influence of testosterone, accompanied apparently by regression of transformed follicles. In accord with this explanation is the observation that treatment of embryos or chicks with testosterone or testosterone analogues, to cause bursal ablation, markedly reduces the occurrence of LL (111,112). Overall incidences of LL and erythroblastosis and a sex-maternal interaction, suggestive of an influence of sex-linked genes, were observed by Crittenden et al. (93) in diallel crosses exposed to ALV.

8.2. Age

Age at infection markedly influences both the course of infection and disease incidence. As discovered by Rubin (53), infection during embryonic life, either naturally or experimentally, has a most striking effect, leading to immunological tolerance, with high viremia, lack of antibodies, and a high incidence of disease in hatched chicks, in contrast to the immunity and lower disease incidence in chicks infected after hatching (62,113). Burmester et al. (72,114) also clearly demonstrated an influence of age at infection by ALV on neoplastic mortality. With increasing age of inoculation from 1 day to 168 days of age, overall neoplastic mortality from erythroblastosis or LL (visceral lymphomatosis) declined, and early high mortality from erythroblastosis gave way to later mortality from LL (Fig. 2). It should be noted that the high incidence of erythroblastosis seen in these studies, consequent on the use of the RPL12 strain of virus and highly susceptible chicks,

is not observed under natural field situations.

More recently, Maas et al. (115,116) showed that the incidence of LL, and congenital transmission of virus, were inversely proportional to age at infection. In one trial the incidence of LL was 54.3% in birds infected at 1 day of age ranging to 7.4% in birds infected at 8 weeks of age. Congenitally infected embryos were obtained mainly from hens infected at 1 day of age.

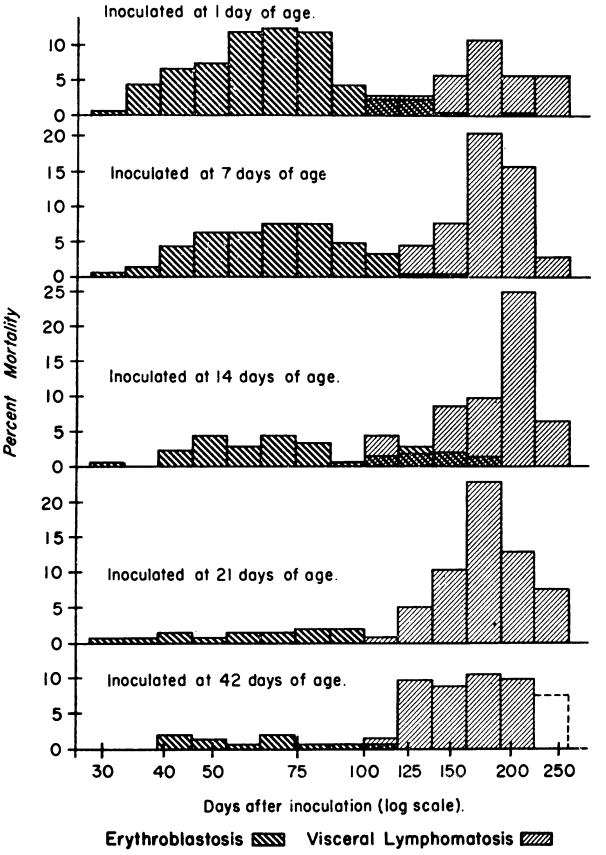

Figure 2. Influence of age at inoculation with RPL12 strain of ALV on mortality from erythroblastosis and lymphoid leukosis (visceral lymphomatosis) (from (71), by kind permission).

8.3. Passive immunity

Immune hens have virus-neutralizing antibodies in their sera which are passed to their progeny via the egg yolk. This maternally acquired antibody persists in the hatched chick for about 3 weeks, and provides a degree of immunity to early ALV infection. Level and persistence of antibody in the chick is related to the titer of antibody in the hen's serum. Rubin et al. (62) found the yolk or chick titers to be 10- to 100-fold less than that in the dam. Witter et al. (117) found that passive antibody prevented the development of permanent viremias following inoculation of 1-day-old chicks with ALV, and delayed the development of actively acquired antibodies. Maternal antibody was found by Fadly (118) to lower rate of viremia and of cloacal shedding following exposure to ALV at hatching.

The influence of passive immunity has also been shown in experiments in which hens were "vaccinated" with large doses of ALV: their day-old progeny were more resistant to ALV by a factor of 3.5 $\log_{10}$ compared with progeny prior to vaccination (119,120,121). Inoculation of day-old chicks with antisera conferred complete resistance to 2.5 $\log_{10}$ LD_{50} of virus.

8.4. Actively acquired immunity

Chickens infected with ALV after hatching normally develop persistent virus-neutralizing antibodies. In the study of Rubin et al. (62), birds naturally infected after hatching first developed antibodies at 9 weeks of age, with a marked increase in the proportion with antibodies between 14 and 18 weeks of age, when 80% were positive, and with 90% positive by 29 weeks of age. A similar slow increase in the proportion of antibody-positive birds was observed in contact-infected birds by Payne et al. (55) (Fig. 3). Development of antibodies is preceded by a transient cell-free viremia. Observations of Weyl and Dougherty (36) indicate that the younger the bird is at infection, the longer the duration of viremia and the greater the delay in antibody production. After inoculation of birds at 4 weeks of age or older with ALV, transient viremia was detectable at 1 week and was followed by antibodies at 3 weeks and later (122). Birds genetically resistant to ALV infection do not develop antibodies (123,124). Virus-neutralizing antibodies serve to restrict the amounts of virus in the bird, which may limit neoplasia, but they are considered to have little direct influence on tumor growth. Cytotoxic lymphocytes directed against viral envelope antigens also occur in birds infected with ALV (125) but their importance in immunity has not been elucidated. It is not known whether antitumor immunity occurs in LL. Antibodies against gs-antigens also occur in ALV-infected birds (126) but these apparently have no influence on

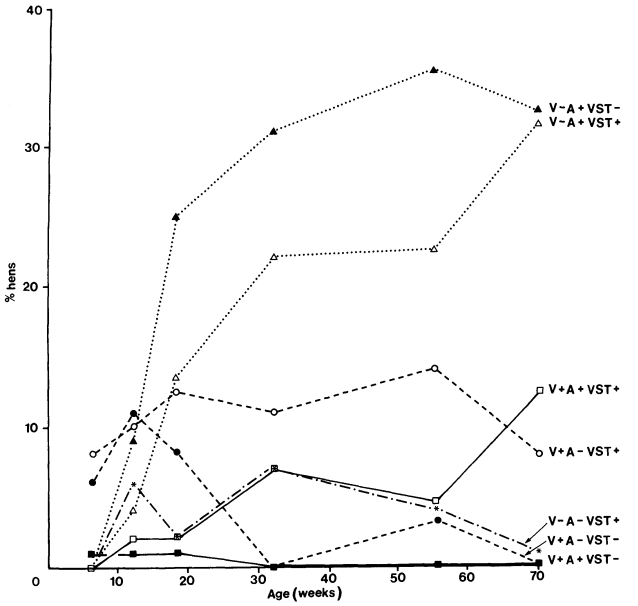

Figure 3. Frequency of different classes of infected hens in a flock naturally infected with ALV of subgroup A. Hen code refers to the presence (+) or absence (-) of ALV viremia (V) in serum, antibodies (A) and virus in vaginal/cloacal swab test (VST) (from (55), by kind permission).

tumor growth. Antibodies against viral reverse transcriptase have also been detected, in virus-infected and virus-free chickens (127).

8.5. Route of infection

The differing features of infections acquired by contact, or by congenital infection, have been discussed in section 4. Routes of horizontal infection which are likely to be significant under natural conditions have been identified by Burmester and Gentry (43) who recorded the following average tumor incidences: tracheal, 83.1%; nasal, 73.0%; cloacal, 57.6%; conjunctival, 47.2%; oral, 45.2%; aerogenic, 39.2%; and oesophageal, 7.7%. In further studies, using the erythroblastosis response to rank the relative efficiency of different routes, Burmester et al. (71) observed, in one series, incidences of: intraperitoneal, 84.1%; tracheal, 23.3% and aerogenic, 6.6%, and in another series incidences of: intramedullary, 46.2%; intravenous, 37.8%; intraperitoneal, 32.6%; intracranial, 26.5%; intramuscular, 25.6%; subcutaneous, 17.3%; and nasal, 1.4%. In these experiments LL

(visceral lymphomatosis) also occurred at relatively high frequencies which were mostly little affected by the route of infection. The authors surmized that variations obtained with different routes were probably mainly due to variations in the amount of virus coming into contact with susceptible cells. Using induction of viremia as a measure of efficacy of different portals of entry of virus, Weyl and Dougherty (36) found rubbing virus onto defeathered skin to be the most effective when compared with oral, nasal and conjunctival inoculation.

8.6. Intercurrent infections

Infectious bursal disease virus (IBDV), the causative agent of Gumboro disease, was shown by Purchase and Cheville (128) to reduce markedly the incidence of LL when administered at 2 or 8 weeks of age, probably as a consequence of its destructive effect on the target cells for leukotic transformation in the bursa of Fabricius (see Chapter 9). Similarly, a moderately virulent strain of IBDV used to vaccinate against Gumboro disease also prevented lymphoid leukosis, but avirulent vaccinal strains did not prevent leukosis (129). Infection by IBDV also increased shedding of ALV, as based on cloacal swab tests (130). Since virulent strains of IBDV are common in commercial poultry, and vaccination is widely used, it is possible that the natural incidence of LL is influenced accordingly.

9. ENVIRONMENTAL FACTORS INFLUENCE INFECTION AND DISEASE

9.1. Management systems

It is believed that the incidence of leukotic disease can be minimized by adherence to principles of good husbandry, including rearing stock in clean surroundings and avoidance of contact with other stock. An experiment conducted by Purchase et al. (131) supported this view: the incidence of "leucosis" in susceptible ALV-free chickens was higher when they were reared in a "dirty" environment compared with those in a "clean" environment and also when they were reared in contact with commercial chickens. Other practices which have been shown to increase contact infection include manual vent-sexing of uninfected chicks when intermingled with infected chicks, rearing on a solid floor compared with a wire-mesh floor, and direct contact with infected chicks (same unit) compared with indirect contact (adjacent units) (54). In this study, spread of ALV infection from infected to uninfected chicks did not occur in the hatcher when chicks were in direct or indirect contact for up to 18 hours. In contrast,

Fairfull et al. (132) observed no difference in the frequency of ALV shedding between birds reared in group cages and those reared in floor pens. Evidence has been presented that ALV can be transferred by the needle during vaccination of day-old chicks against Marek's disease, but only in chicks devoid of maternal antibody (133). The infection can also be transferred to uninfected hens by artificial insemination with infected semen, however, subsequent congenital transmission was not observed (50). Transmission of LL through use of ALV-contaminated live virus vaccines has occurred (134) but is unlikely with modern quality controlled vaccines.

9.2 Diet

A high plane of nutrition has been reported to increase the LL incidence (135,136). Increased leukosis has also been associated with feeding of certain lots of cod liver oil (137). Kakuk and Olson (138) investigated the possibility that the stearine fraction of cod liver oil was responsible, but found that 3% stearine in the diet had no influence whereas 9% stearine increased the incidence, possibly due to inhibition of sexual maturity. March and Biely (139) reported an increased incidence of LL in birds fed high levels of vitamin A, but no evidence of such an effect on other tumors of the avian leukosis complex was observed by Mitrovic et al. (140).

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4

MOLECULAR BASIS OF ONCOGENESIS BY NON-ACUTE AVIAN RETROVIRUSES

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1. INTRODUCTION

A majority of naturally occurring cancers associated with retrovirus infection are caused by non-acute retroviruses. These viruses do not carry oncogenes of their own and induce cancer in their hosts only after a long latency period. They

G.F. de Boer (ed), Avian Leukosis.

are believed to be the progenitor viruses which gave rise to the acute retroviruses through recombination with host oncogenes (1,2). The hosts of these viruses include most vertebrate species. An excellent review of the neoplasms induced by non-acute retroviruses of a variety of organisms is presented by Varmus (3). The focus of this review will be restricted to the non-acute avian retroviruses.

In the avian system, the non-acute or slowly transforming retroviruses can be broadly divided into two groups: avian leukosis virus (ALV) and reticuloendotheliosis virus (REV). The ALV group shares sequence homology and antigenicity with Rous sarcoma virus (RSV). This group includes a.o. RAV-1 (Rous-associated virus-1), RAV-2, RAV-60, MAV (myeloblastosis-associated virus) and RPV (ring-necked pheasant virus). Based on their envelope properties and host ranges, these viruses have been classified into six subgroups, A to F (for review, see ref. 4). The viruses in the REV group are closely related to each other but completely distinct from those in the ALV group (4). Interestingly, REV is antigenically related to certain mammalian viruses (5) and can productively infect several mammalian cell types. REV viruses have been isolated from a number of avian species; for instance, CSV (chicken syncitial virus) was isolated from chicken, REV-A (reticuloendotheliosis-associated virus) from turkey, and SNV (spleen necrosis virus) and DIAV (duck infectious anemia virus) from duck. Both ALV and REV lack the ability to transform fibroblasts in vitro, a property expected for non-acute retroviruses, as they do not carry an oncogene in their genome. However, they can induce a multitude of long-latency neoplastic diseases in chickens and quail, such as B-lymphoma, erythroblastosis, nephroblastoma, angiosarcoma, fibrosarcoma, etc. The incidence of these diseases varies considerably, depending upon the strain of virus, stage of maturation of target cells, and host line involved in the infection (6). The molecular mechanisms underlying the development of these diseases are currently unknown, but recent studies have improved our understanding of key aspects of these mechanisms.

2. STRUCTURE AND REPLICATION OF NON-ACUTE RETROVIRUSES

The genome of retroviruses consists of two identical subunits of a single-stranded RNA molecule (7,8). A genetic map of the non-acute retrovirus is depicted in Figure 1 (for review, see ref. 9). The retroviral genome contains three genes: gag encodes group-specific antigens which are major components of the nucleocapsid, and some of these proteins are involved in packaging the RNA genome into the virion; pol encodes the enzyme reverse transcriptase, involved in

the synthesis of viral DNA and its insertion into the host genome; env specifies the envelope glycoproteins essential for viral attachment to the host cell. These proteins determine the host range and interference patterns of the virus. The RNA genome is bounded by a short repetitive sequence, R. The regions next to R at the 5' and 3' end of the genome are respectively designated U5 (unique 5' sequence) and U3 (unique 3' sequence). In the avian retroviruses these terminal sequences do not contain any protein-coding information, but do contain important transcriptional regulatory signals.

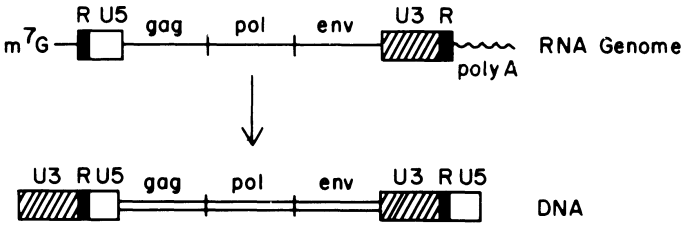

Figure 1. The structure of the RNA genome and the un-integrated viral DNA of a non-acute avian retrovirus. The U3, R and U5 sequences comprise the LTR region present at both termini of the viral DNA. (Arrow denotes the direction of the reverse transcription process; see text for notations).

Upon infection, the RNA genome is first converted into double-stranded DNA by reverse transcriptase (for review, see ref. 10). During this process, the terminal sequences of the RNA genome, namely R, U3 and U5, are duplicated at both ends of the DNA (see Fig. 1). The duplicated sequences are usually referred to as the long terminal repeats (LTR), and these sequences appear to be required for the insertion of viral DNA into the host genome. Only a fraction of the linear DNA is then circularized and inserted at random sites into the host genome. The insertion is aided by a specific cleavage at the LTR-LTR junction by an endonuclease activity associated with the reverse transcriptase II. Cleavage is followed by a recombination event with the host genome. As a result, the inserted viral DNA is always colinear with the un-integrated linear DNA. The insertion sites in the host genome do not appear to be sequence specific and are thought to be randomly distributed throughout the host genome (for review, see ref. 10). The integrated viral DNA, referred to as the provirus, is transcribed by host RNA polymerase II to generate genome-sized RNA. Transcription initiates at the U3/R boundary within the 5' LTR, utilizing the promoter sequence of the 5' LTR (see

below), and terminates at the R/U5 boundary of the 3' LTR. This genomic RNA also serves as the messenger RNA for gag. A fraction of the genomic RNA is further processed to produce the messages for pol and for env (Fig. 2), although direct evidence for the existence of a subgenomic pol message is still lacking. It is important to point out that in ALV, the splice donor site used in the formation of the env message is located 18 nucleotides 3' to the gag initiation codon. As a consequence, the env product shares the first 6 amino acids of the gag gene product (13). In contrast, in REV the gag AUG is located 3' to the splice donor site of the env gene, resulting in env and gag proteins which have distinct amino-terminal sequences.

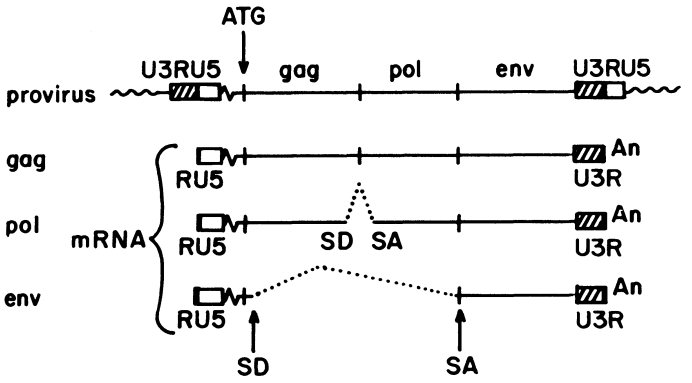

Figure 2. The structure of the ALV provirus and the viral transcripts. The wavy line flanking the provirus represents cellular sequences. ATG: initiation codon used in the translation of all three viral proteins. An: polyadenylation signal sequence, SD: splice donor site. SA: splice acceptor site.

3. THE STRUCTURE OF THE LONG TERMINAL REPEAT (LTR)

In the previous section, we have discussed the involvement of the LTRs in provirus integration, and in initiation and termination of viral RNA transcription. The versatility of the LTR is reflected in its novel structure. Fig. 3 depicts the major features of this element; the LTR has inverted repeat sequences (IR) at both termini, similar to those found in procaryotic transposable elements (2). These inverted repeats are essential to the integrative recombination process.

Genetically engineered viral mutants lacking these sequences fail to integrate into the host genome (81). The LTR also has a "TATA" sequence, located at around 23 bp (base pairs) from the U3/R boundary and an AAUAAA sequence, presumably involved in signaling polyadenylation at a site 10 nucleotides downstream, during maturation of viral RNA. The 5' one-third to two-thirds of the U3 region has recently been shown to contain "enhancer" sequences (14,16). These are sequences which can augment the expression of adjacent cellular or viral genes in a position and orientation-independent manner. It has been postulated that the enhancer elements function as super entry sites for RNA polymerase II, or possibly topoisomerase, rendering the adjacent genes in an "open" chromatin conformation, facilitating the transcription process. It is interesting to note that the strength of the enhancers in the LTRs of exogenous subgroups of ALV is greater than that of endogenous ALV, RAV-0 (17). This observation has led to the suggestion that the enhancers are, at least in part, a determinant of the oncogenicity of non-acute retroviruses.

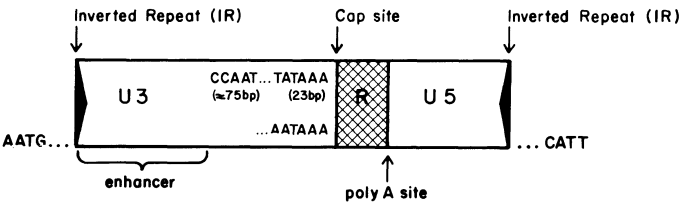

Figure 3. The structure of the LTR. The R region is bounded by the cap site which signals the initiation point of eucaryotic mRNA and the polyA site which signals the end of the mRNA. The "TATA" (TATAAA) and "CAT" (CCAAT) sequences, implicated in promoter function, are located at about 23 bp and 75 bp upstream from the cap site. The enhancer sequences are located further upstream.

4. POSSIBLE SCHEMES FOR HOST ONCOGENE ACTIVATION BY NON-ACUTE RETROVIRUSES

Given the presence of strong transcriptional regulatory elements in the termini of the provirus, and the propensity of the provirus to integrate randomly into the host genome, it is no surprise that non-acute oncogenic avian retroviruses are potent mutagens. It has been hypothesized that when the mutation in the host

genome is in a cellular oncogene, neoplastic transformation occurs. In the following section, we shall consider several possible schemes whereby a host oncogene can be mutated or activated by non-acute retroviruses (see Fig. 4).

(I) Truncation mutation: When an avian retrovirus inserts within the coding sequence of a cellular gene, it causes disruption and potential truncation of the gene. Usually such a mutation event will lead to inactivation of the gene. As will be discussed later, in some cases, the removal of the presumptive regulatory domain of a cellular protein (due to viral insertion) may actually render the protein oncogenic.

(II) Promoter-insertion by 3' LTR: As discussed before, transcription of the viral genes is controlled by the strong enhancer/promoter elements present in the 5' LTR, and these regulatory elements should be capable of promoting high efficiency transcription of downstream or adjacent cellular genes. When transcription is driven by the promoter in the 3' LTR the resulting primary transcripts contain R, U5 sequences at their 5' end, fused to the cellular gene. Gene activation is caused by the provision of an upstream viral promoter, and this mutagenesis mechanism is referred to as promoter-insertion (18,19). The prerequisite for promoter-insertion mutagenesis is that the provirus must be situated upstream from the cellular sequence to be activated, and the LTRs must be oriented in the same transcriptional direction as the cellular sequence.

(III) Promoter-insertion by 5' LTR: Although all the viral transcripts terminate at the U3/R boundary, present evidence suggests that primary viral transcripts usually proceed beyond the polyadenylation site. It is therefore possible that the 5' LTR can initiate a read-through transcript encompassing viral genes and downstream cellular sequences, thereby enhancing the expression of the cellular gene.

(IV) Transcriptional enhancement: If a provirus is inserted downstream from a cellular gene, or upstream, but in the opposite orientation of a cellular gene, the provirus cannot use its LTR promoter to promote the transcription of a cellular gene. Enhancers in the viral LTRs, however, are capable of augmenting the transcription of adjacent cellular genes by using the natural promoters of the cellular genes or by using cryptic promoters.

It should be pointed out that the proviral insertional mutagenesis schemes discussed here are not necessarily mutually exclusive. For instance, it is conceivable that a given proviral insertion can both truncate and enhance the expression of a cellular gene.

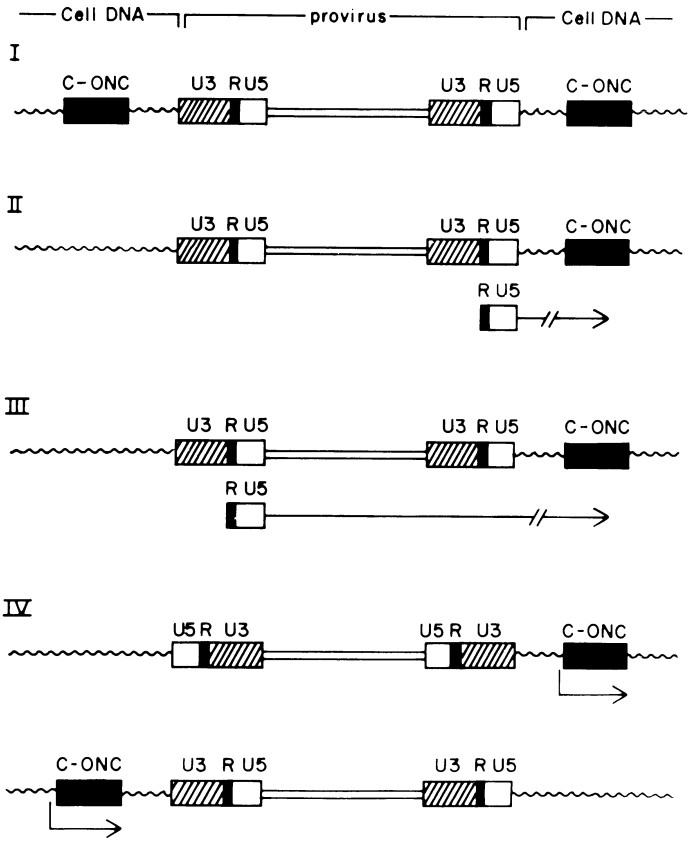

Figure 4. Possible schemes for host oncogene activation by non-acute retroviruses.
(I) Truncation mutation.
(II) Promoter-insertion by 3' LTR.
(III) Promoter insertion by 5' LTR.
(IV) Transcriptional enhancement. Arrows indicate activated c-onc (cellular oncogene) transcripts. The promoters utilized in scheme IV are of cellular origin.

5. B-LYMPHOMA INDUCTION BY ALV: c-myc ACTIVATION

5.1. Development of B-lymphomas

The most prevalent neoplasm associated with ALV strains (e.g. RAV-1 and RAV-2) is a bursal lymphoma (B-lymphomas). This disease is manifested primarily

in the bursa of Fabricius; IgM-producing pre-B-lymphocytes are affected and apparently arrested in this stage of differentiation (20). The first morphological evidence of B-lymphomas is an accumulation of transformed lymphocytes within individual follicles in the bursa, which can only be detected microscopically. Transformed follicles first appear at 4 to 8 weeks post-infection and increase in number until approximately 12 weeks (20,21,22). The number of transformed follicles (usually in the range of 10-100) then decreases. From 10 to 14 weeks of age, macroscopic tumor nodules appear in the bursa in much smaller numbers, usually only one or two tumor modules are observed, indicating that ALV-induced lymphoma formation is a monoclonal event. Eventually, the whole bursa is filled with transformed lymphocytes and often becomes grossly enlarged. Metastasis from bursa to visceral organs such as liver and spleen takes place after 14 to 16 weeks, resulting in massive lymphomatosis and death of the host.

5.2. C-myc activation

To explore the possibility that proviral insertional mutagenesis is the basis for lymphocyte transformation, the insertion sites of the proviruses in RAV-1- and RAV-2-induced tumors were examined. It was found that the proviruses in different tumors are clustered in a specific chromosomal region (18,19,23). Since avian retroviral proviruses are known to integrate randomly under non-selective conditions (see above), this result was tantalizing and suggested that a disturbance of this chromosomal region might be crucial to B-lymphomagenesis. Further studies by Hayward, Astrin and Neel (24) showed that the proto-oncogene c-myc resides in this region and that c-myc expression is elevated 40 to 100-fold in tumors as compared to normal bursal cells. C-myc is the cellular homologue of the oncogene carried by the acute avian leukemia virus MC29. It contains three exons (25,26); the protein coding sequences are contained in the second and third exons. Close inspection of the proviral insertion sites revealed that they are concentrated in a region immediately preceding the second exon (Fig. 5; 26,29). There are also a few insertion sites within the first exon and several are located further upstream (H.L. Robinson, personal communication; not shown in Fig. 5). None of these insertions appear to disrupt the protein coding sequences, but many of them interrupt the contiguity of the first and second exons. The fact that the proviruses integrate at multiple sites in this region suggests that insertion is not dictated by a specific sequence in the host chromosome. DNA sequencing of four proviral-cell junction fragments, molecularly cloned from different tumors, confirms this notion (26,30,31). It thus appears that clustering of the proviruses in

certain regions of the c-myc locus is due to some structural constraint perhaps imposed by successful activation of the oncogene. To examine this issue further, we surgically removed a portion of the bursa from an infected bird in a pre-neoplastic stage (32). The remaining part of the infected bursa was allowed to develop tumors in vivo. Based on an analysis of the DNA, we found that 60 to 70% of the bursal cells were infected by ALV (RAV-1) in the pre-neoplastic stage (4 weeks post-infection) and that the proviruses appeared to be randomly distributed in the chromosomes. By contrast, in the terminal stage of tumor cells, specific proviral insertion near the c-myc locus was invariably detected. This is consistent with the view that initial infection of the bursa by ALV results in proviral insertion into many sites in the host chromosome; those cells that harbor ALV proviruses near the c-myc locus are transformed, outgrow their normal counterparts, and develop into clonal tumors. This data supports the hypothesis that disturbance of the c-myc gene is a critical step in B-lymphomagenesis.

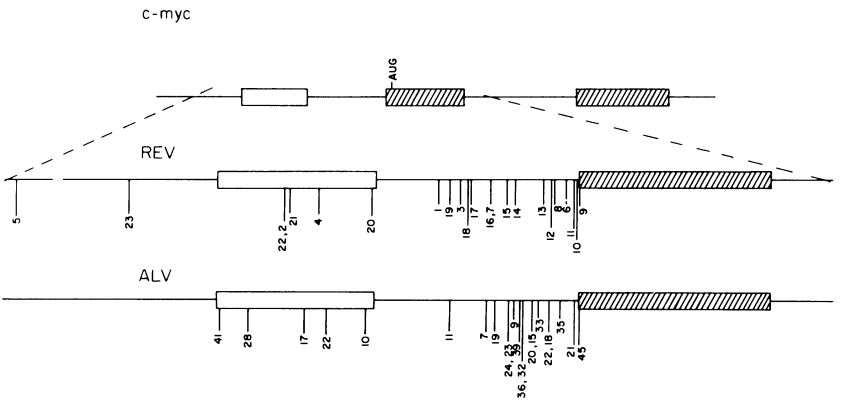

Figure 5. Insertional activation of the chicken c-myc gene. The undisturbed c-myc locus has three exons illustrated in this diagram as boxes. Open box: non-coding c-myc exon (exon 1). Matching boxes: coding c-myc exons (exons 1 and 2, respectively). The numbers in the lower diagrams (exons 1 and 2) represent the insertion sites of individual proviruses in B-lymphoma tumors, induced by the CSV strain of REV, or the RAV-1 and RAV-2 strains of ALV. The REV and ALV diagrams are modified from the data of Swift et al. (52) and Shih et al. (26).

5.3. Activation mechanisms

To understand how ALV provirus activates the c-myc gene, the structure of the proviruses were analyzed by restriction endonuclease digestion. It was shown for the great majority of the proviruses that the provirus is oriented in the same

transcriptional direction as c-myc (24,26,27,28). The transcripts from these tumors contain R, U5, joined to the c-myc coding exons, but no other viral sequences, consistent with a 3' LTR promoter-insertion mechanism. Structural analysis of these proviruses further reveals, curiously, that a significant portion of these proviruses carry deletions at their 5' ends, usually extending into the 5' LTR (18,19,23). In the extreme case, a solo LTR is present in association with the c-myc locus (31). Other viral genes appear to be dispensable after proviral insertion. Why do the deletions occur with such a high frequency? Two hypotheses have been proposed (18,19,23). The first states that deletion of the viral genes or disruption of virus transcription by the 5' LTR would facilitate tumor progression by removing immunogenic surface viral antigens. However, B-lymphoma tumors and cell lines derived from them, which release viruses (presumably due to the presence of a second intact provirus inserted at a distinct chromosomal site) do exist. The second hypothesis suggests that transcription promoted by the 5' LTR may interfere with promotion of the c-myc gene by the downstream 3' LTR, a phenomenon similar to that described as "promoter-occlusion" in procaryotes (33). Using a plasmid with both the 5' and 3' LTRs linked to tester genes in an in vitro transient expression assay, Cullen et al. (34), have shown that the presence of 5' LTR promoted transcription indeed down-regulates 3' LTR promoted transcription.

While most c-myc activations are clearly due to promoter-insertion by the 3' LTR, there are interesting exceptions; at least one RAV-2 provirus has been found to be located downstream from the c-myc gene (28). Several tumors have also been described in which the provirus is inserted upstream from the c-myc coding exons, but in the opposite transcriptional orientation (28, and cited in 26). RNA analysis of these exceptional tumors have revealed that these activated c-myc transcripts are not initiated in the viral LTR, but rather are transcribed from either the natural c-myc promoter or from cryptic promoters located near the proviral insertion sites. Nevertheless, c-myc expression is highly elevated in these tumors, possibly due to an enhancer effect from nearby viral LTRs (i.e., scheme IV, transcription enhancement).

Regardless of the detailed mechanism by which proviruses activate c-myc, all the proviral insertions in the B-lymphomas studied so far are outside the c-myc coding domain. Thus, the c-myc protein expressed in tumor cells is likely to be identical to that in normal cells. This has led to the postulate that inappropriate expression of the normal c-myc protein might trigger B-lymphocyte transformation (24). A direct sequence comparison of the activated and normal c-myc genes

to substantiate this postulate has yet to be performed. However, in one case where a partial sequence analysis of the activated c-myc gene was determined, differences in amino acid sequence between this gene and the normal c-myc counterpart, possibly resulting from point mutations, were detected (31). The possible significance of these mutations to the tumorigenic potential of the c-myc protein remains unclear.

A number of cell lines have been established from B-lymphoma tumors. The proviral insertions in these cell lines are again predominantly upstream from the second c-myc exon with the proviruses oriented in the same transcriptional direction (35,36). The structure of the c-myc transcripts and protein products from these cell lines, however, reveal certain unexpected features. It has recently been shown that in four cases, even though the configuration of the proviruses is such that they can efficiently use the 3' LTR to transcribe the c-myc gene, the transcripts are apparently initiated at the natural promoter of the c-myc gene and include the first exon (36). Transcriptional enhancement thus appears to be the underlying activation mechanism in these cell lines. In several cases, the c-myc protein expressed in these cell lines is of aberrant size (35). Whether these anomalies reflect a typical in situ c-myc activation process, or are the result of changes in these cell lines during their long time in culture needs to be resolved.

Although the correlation between a disturbance in the c-myc gene and the induction of B-lymphomagenesis is now well established, the role of the c-myc gene product in this process remains obscure. Since the unaltered c-myc gene appears to be expressed in a variety of tissues, and since altered c-myc expression is associated with tumors of diverse origin (see below), it has been proposed that the normal c-myc gene product encodes a function not associated with a differentiated phenotype, but rather with some ubiquitous cellular process (37). Several lines of evidence suggest that such a role may be in the process of normal cell proliferation. There is an induction of c-myc mRNA and protein synthesis following growth stimulation in a variety of cell types (37,38,39), and initial studies suggested c-myc expression might be specific to the G_1 phase of the cell cycle (40). Recent reports from two laboratories, however, indicate that the levels of c-myc mRNA and protein synthesis are invariant throughout the cell cycle (41,42). The only other clue regarding the potential role of c-myc is its cellular localization; the gene product appears to be exclusively localized in the nuclear compartment of most cells, and has been implicated to be a DNA-binding protein (43).

In summary, a total of over seventy ALV-induced tumors have been analyzed

by a number of laboratories. These include infections of $15_1 \times 7_5$, 15B and SPAFAS lines, with the RAV-1 and RAV-2 strains of ALV. About 70% of these tumors carry proviruses in the c-myc locus. The majority of the proviruses in these tumors are inserted upstream of the second c-myc exon, are oriented in the same transcriptional direction as c-myc, and appear to use the 3' LTR to direct c-myc transcription. In a few cases, transcriptional enhancement of c-myc expression by an LTR enhancer has also been observed.

5.4. Disease specificity of c-myc

The discovery that c-myc is involved in B-lymphomagenesis presents a paradox concerning the disease specificity of myc sequences, since the acute avian leukemia viruses (e.g., MC29, MH2, OK10) which carry v-myc in their viral genome usually cause myelocytomatosis, carcinomas and fibrosarcomas, rather than B-lymphomas (44). Although c-myc and v-myc are highly homologous, there are differences in their amino acid sequences (24,25). Furthermore in some cases, v-myc is preceded by a portion of the gag protein. These differences, therefore, may account for the different disease potentials observed. Enrietto et al. (45) have isolated an MC29 variant, HB1, which presumably represents a v-myc/c-myc recombinant. Instead of inducing myelocytomatosis and carcinomas as MC29 does, HB1 causes primarily lymphomas of mixed B- and T-cell specificity. This suggests that the disease specificity is inherent in the coding sequences of c-myc and v-myc. On the other hand, Hayward et al. (46) have re-examined the disease spectrum of the MC29 virus and observed that a significant portion of infected birds develop B-lymphomas. These B-lymphomas are macroscopically easily overlooked and may have escaped detection in earlier studies. It is noteworthy, however, that all of these studies have been carried out with different strains of MC29, different lines of chickens, and have used different routes of infection, and different methods of monitoring. As a consequence it is difficult to generalize about the conclusions of these studies.

5.5. A second oncogene

It is generally accepted that oncogenesis is a multi-step process. The activation of c-myc thus represents one of such steps in the development of B-lymphomagenesis. To assess whether other oncogenes are involved in B-lymphoma induction, DNA from B-lymphoma cells was analyzed for its ability to transform NIH 3T3 cells in vitro. A cellular gene, B-lym, was found to be activated (i.e., capable of transforming NIH 3T3 cells) in tumor cells but not in normal control

cells (29,47). B-lym shares limited sequence homology with the transferrin gene, and is distinct from c-myc. The activated B-lym gene is not linked to a provirus, and based on restriction enzyme mapping exhibits no gross structural rearrangements when compared to the normal allele. It is likely that activation of this gene was accomplished by base change(s) during tumor progression. A recent study indeed suggests that c-myc activation occurs quite early, probably before the stage defined by the transformed follicle (48), whereas B-lym activation apparently takes place late and may be involved in tumor nodule development.

6. B-LYMPHOMA INDUCTION BY REV: c-myc ACTIVATION

Despite its completely different origin, REV can induce a spectrum of neoplastic diseases very similar to those induced by ALV (49,50). In line $15_1 \times 7_2$ chickens, the CSV strain of REV induces B-lymphomas at high frequency. When the molecular mechanisms used by CSV and RAV to induce B-lymphomas are compared, a strikingly similar picture emerges. In over 90% of the tumors analyzed, a disturbance in the c-myc gene by CSV proviral insertion can be identified (51,52). The CSV proviruses, without exception, are integrated upstream from the second c-myc exon and are oriented in the same transcriptional direction. The insertion pattern of CSV bears strong resemblance to that of ALV (cf. Fig. 5). Over 70% of the CSV proviruses (indicated as REV in Fig. 5) are clustered in a region 300 bp immediately 5' of the second c-myc exon. There are also a few insertions in the first exon and two 5' to the first exon. Interestingly, the 5' third of the first intron which is devoid of RAV proviruses is also silent for CSV insertion. Most of the CSV proviruses examined also carry deletions. The remarkable similarity of the insertion pattern displayed by the two otherwise very distinct viruses suggests that these insertion sites are probably most effective in activating c-myc, and convincingly establish c-myc as the target oncogene involved in chicken B-lymphomagenesis.

7. ERYTHROBLASTOSIS INDUCTION BY ALV: c-erbB ACTIVATION

7.1. Development of erythroblastosis

As discussed earlier, ALV induces mostly B-lymphomas, but in certain chicken lines, specifically those with the B_5 MHC-haplotype, erythroblastosis incidence is exceptionally high. For instance, in lines 15_1, and $15_1 \times 15I_4$, erythroblastosis incidence after RAV-1 infection reaches 90 to 100%. This chicken

line provides an excellent model system for investigation of the oncogene involved in avian erythroblastosis. ALV-induced erythroblastosis is characterized by a long latency (ca. 10 weeks), followed by an aggressive terminal phase (ca. 1 week) during which an abrupt increase in circulating erythroblasts (sometimes reaching 10,000-fold) is evident (53, M. Raines and H.-J.K., unpublished data). This increase results in the death of the host. At the terminal phase of erythroblastosis, the liver and spleen are enlarged due to the infiltration of leukemic erythroblasts. These symptoms are remarkably similar to the symptoms previously described for erythroblastosis induced by avian erythroblastosis virus (AEV) (44). There are, however, two important differences. First, while ALV-induced erythroblastosis has an average latency of 10 weeks, AEV-induced erythroblastosis usually begins 10 days post-viral infection. Second, ALV-induced erythroblastosis is usually of monoclonal origin, whereas the AEV-induced infection is polyclonal.

7.2. C-erbB activation

In the most extensively studied strain of AEV, AEV_{ES4}, there are two cellular inserts, referred to as v-erbA and v-erbB (54, 56). The fact that ALV and AEV cause symptomatically similar leukemias suggested that ALV might activate the cellular homologue of the oncogene v-erb carried by AEV. Indeed, analysis of 40 leukemic samples obtained from chickens infected with RAV-1 clearly showed that c-erbB (but not c-erbA) is disturbed and activated by RAV-1 insertion (53,57). The region of c-erbB, as defined by homology with v-erbB, spans 24 kb and contains at least twelve exons and eleven introns (58,59) (Fig. 6). The first exon which shares homology with v-erbB is designated VB1. It was found that all the ALV proviruses, without exception, are inserted within a 1.2 kb region 5' to VB1; 70% of the insertions are clustered in a 0.3 kb region (57). Since the size of the intact c-erbB locus, exons and introns included, is probably at least 30 to 40 kb, (see below) the precision of RAV-1 insertion is remarkable. V-erbB has recently been proposed to represent a truncated version of the epidermal growth factor receptor (EGFR; 60, 62). V-erbB contains the transmembrane and protein kinase domains of the EGFR, but lacks the majority of the EGF-binding domain and the carboxy-terminal 34 amino acids of the EGFR, in which the major tyrosine autophosphorylation site resides (Fig. 7). This finding suggests that the VB1 exon, near which all the proviral insertions occur, in its unactivated form does not encode the N-terminus of c-erbB, but instead encodes the middle of this gene. Proviral insertions, therefore, result in the interruption of the coding region of the c-erbB/EGFR locus. The interruption point in this gene with respect to the protein

sequence coincides precisely with the amino termini of the v-erbB protein products (from both AEV_H and AEV_{ES4}). This observation raises the interesting possibility that truncation of the EGFR in this region is critical to the process of oncogenic transformation.

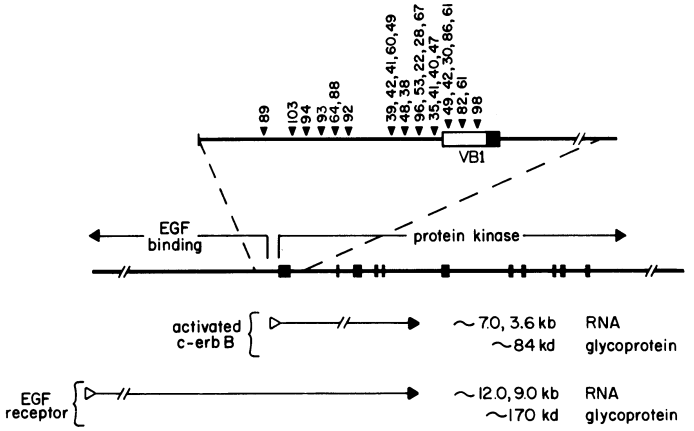

Figure 6. Insertional activation of the chicken c-erbB gene.
The c-erbB locus, represented by a straight line in the center, is crudely divided into two sections: the ligand-binding domain and the protein kinase domain. The solid boxes represent regions that share homology with the v-erbB sequences present in AEV_{ES4} (58). VB1: the first exon with homology to v-erbB. The top line is an enlarged diagram of the region surrounding VB1. The open box corresponds to the intron region which is transduced into the AEV genome. The splice acceptor site of VB1 begins at the boundary between the open and the solid boxes. The numbers above represent the insertion sites of individual RAV-1 proviruses in erythroblastosis samples. The diagrams below the c-erbB locus indicate the initiation sites, the transcriptional direction, and the sizes of the mRNAs of the activated c-erbB and normal c-erbB/EGF-receptor genes. The predicted sizes of the activated and normal c-erbB gene products are also shown.

7.3. Activation mechanism

All the RAV-1 proviruses linked to c-erbB have been found to be oriented in the same transcriptional direction as c-erbB (57). However, in contrast to the c-myc-linked ALV and REV proviruses in B-lymphomas, most of the RAV-1 proviruses involved in c-erbB activation remain completely intact (57). These observations suggest that the activation modes in the two cases may not be identical. When expression of c-erbB in leukemic samples was analyzed, elevated levels of c-erbB transcripts were invariably detected. Two prominent transcripts

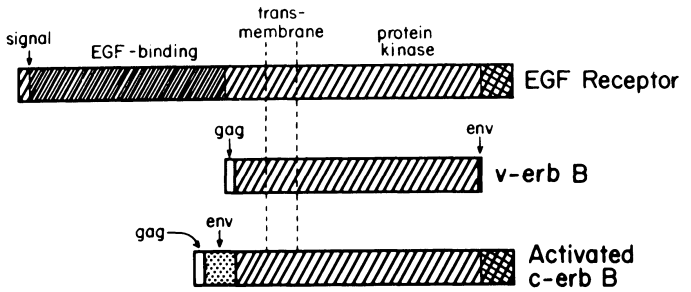

Figure 7. Structural comparison of v-erbB, activated c-erbB, and the human EGF-receptor. The diagrams are based on sequencing data described by Ullrich et al. (61), Yamamoto et al. (65) and Nilsen et al. (67). The gag and erbB splice in v-erbB is predicted from the sequencing data of Debuire et al. (64), and has not been experimentally verified. The c-terminal four amino acids of v-erbB are provided by the viral env gene.

of 7.0 kb and 3.6 kb are evident in many leukemic samples, even though the exact proviral insertion sites in these DNAs are clearly different (63). Recently, the nucleotide sequence of the cDNA clones representing the entire 7.0 and 3.6 kb transcripts have been determined (63; Nilsen, T., Goodwin, R., Maroney, P., Callaghan, T., Raines, M. and H.-J.K., unpublished data). Several extraordinary features of the activation mechanism are apparent: The RAV-1 provirus appears to use the 5' LTR (rather than the 3' LTR) to promote transcription of the c-erbB gene (Fig. 8). This is accomplished first by the synthesis of a read-through message which encompasses the entire viral genome and the c-erbB sequences downstream from the proviral insertion site. The primary transcript is then processed by two novel splicing schemes, so that gag (6 amino acids) and env (53 amino acids) sequences are fused in-frame to erbB. The first splicing (gag to env) is naturally used by the virus to form the env message (see above). The second splicing event (env to erbB) is unprecedented, although the SD (splice donor) site in env contains a consensus sequence that is used in other messages. The SA (splice acceptor) site for the env to c-erbB splice is precisely what has been postulated to be used in the formation of v-erbB (64). Thus, c-erbB and v-erbB have the same precise 5' starting sequences with respect to erbB (cf. Fig. 7).

The gag-env portion of the protein present in the activated c-erbB gene product represents the first 59 amino acids of the total 63 amino acids of the env leader region. The hydrophobic region implicated in signal recognition is present

in this sequence but the signal recognition peptidase site for env is not. It is unclear whether this stretch of sequence functions as a signal peptide in the activated c-erbB gene product. In this respect, it should be mentioned that v-erbB lacks the env signal sequence (although contains the same gag sequence), and is membrane associated. V-erbB in both AEV_{ES4} and AEV_H terminates at the viral env gene (65,66). At the carboxy-terminus of the activated c-erbB gene, however, there is an additional stretch of 34 amino acids (the c-region) not present in v-erbB. Interestingly, this c-region shares homology with the last 32 amino acids of the human EGFR and contains the major tyrosine auto-phosphorylation site of the EGFR (63,67,68). Since both AEV_R and AEV_H lack the c-region, it was originally proposed that truncation of the c-region might be important for oncogenesis to occur. The new finding, that activated c-erbB contains this region, disputes this notion, although the phosphorylation state of the c-erbB protein has not yet been determined. Also noteworthy is that the v-erbB sequences near the c-terminus have been implicated in determining the disease specificity of AEV. It will be interesting to determine whether the presence of the c-region in the activated c-erbB gene renders it exclusively leukemogenic, as a result of its inability to induce sarcomas.

The 7.0 and the 3.6 kb transcripts of the activated c-erbB gene differ primarily in their 3' untranslated regions due to the usage of alternate poly-adenylation sites. Thus, the 3.6 kb transcript carries a 0.9 kb 3' untranslated region, whereas transcription of the 7.0 kb transcript proceeds 3.4 kb further. No significant long open reading frames are present in the 3' untranslated region of either transcript. It is thus likely that the activated c-erbB protein described above is the only one produced by these two messages.

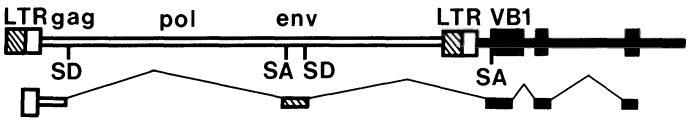

Figure 8. 5' LTR promoter-insertion activation and splicing schemes involved in the generation of the transcript of activated c-erbB. Solid boxes: c-erbB exons (only three are shown). See text for additional notations.

The above cDNA structure demonstrates that the provirus utilizes its 5' LTR to promote the downstream host oncogene by synthesizing a "read-through" message (scheme III), and also interrupts the coding domain of the target oncogene (scheme I). Why does the provirus use such an elaborate mechanism to activate the c-erbB gene, while in c-myc, it can simply use the 3' LTR to accomplish the same goal? One possibility is that the proviral insertion sites in c-erbB are in the middle of the gene and so the gene requires a translational initiation codon to produce a functional protein. It is also possible that the env signal sequence is required for c-erbB activation. Indeed, a higher percentage of the activated c-erbB protein has been found to be associated with the cell membrane than v-erbB (H. Beug, personal communication). The 5' LTR promoter-insertion scheme helps explain why identical transcripts are observed regardless of the proviral insertion sites, since the region between the 3' LTR and VB1 is spliced-out during maturation of the message. It also reveals that transcription of the provirus can read through the polyadenylation signal in the 3' LTR and extend far beyond this site (ca. 25 kb) to transcribe the downstream sequences.

7.4. Activated c-erbB, v-erbB and the EGFR

The finding that the c-erbB and EGFR locus are probably identical is most interesting as it provides a long-sought link between oncogenesis and uncontrolled growth stimulation. The predicted structure of the activated c-erbB and v-erbB genes and the EGFR are schematically diagrammed in Fig. 7. All three proteins contain the transmembrane and protein kinase domains. The major difference resides in the presence of the extracellular ligand-binding domain in the EGFR. It has been demonstrated that prior to EGF binding, protein kinase activity of the EGFR is low; EGF binding triggers tyrosine auto-phosphorylation and "opens" up the active site of protein kinase (see ref. (69) and Chapter 2), which then phosphorylates tyrosine residues of specific targets involved in signal transduction and growth stimulation. Removal of the EGF-binding domain as in the activated c-erbB and v-erbB gene products is thought to render the kinase domain constitutively active, leading to the uncontrolled proliferation of erythroblasts, and hence erythroblastosis. A recent demonstration that v-erbB contains protein kinase activity which is not stimulated by the addition of EGF is consistent with this hypothesis (68). This hypothesis, though attractive, has yet to be vigorously tested. In summary, RAV-1 utilizes a modified promoter-insertion scheme to truncate and activate the c-erbB locus. In addition to a transcriptional promoter also a translational start for the truncated oncogenic c-erbB locus is provided.

8. RPV-INDUCED NEOPLASMS

Ring-necked pheasant virus (RPV) is a member of ALV with subgroup F specificity (70, 72). The genome structure of RPV is very similar to RAV except the sequences in the env region which are entirely different (73, 75). It is generally thought that RPV was derived from ALV through recombination with pheasant DNA sequences. Infection of chickens by RPV produces a variety of neoplasms, of both long and short latency periods (76). Among the long latency (> 8 wks) tumors are B-lymphomas, nephroblastomas and carcinomas. These tumors are of clonal origin and present evidence suggests that they are induced by the proviral insertion-activation scheme as described above (75). Only one B-lymphoma has been examined in detail at the molecular level and in this case the c-myc locus was disturbed and activated by an RPV provirus. Interestingly, c-myc activation by an RPV provirus has also been detected in an omentum carcinoma sample, indicating that the oncogenic potential of the chicken c-myc gene is not confined to lymphoid tissues. In the fibrosarcoma induced by RPV, proviruses apparently integrate near a common cellular site. This cellular site, however, does not seem to be related to v-src or many other viral oncogenes that have been tested, and presumably represents a new oncogene.

An unusual feature of RPV is that it also induces short latency (2 wks) tumors, such as angiosarcomas, which appear to be of polyclonal origin (76). The proviruses in these tumor cells appear to be integrated at multiple sites (75). In this regard, RPV resembles an acute leukemia virus, although no distinct viral oncogene has been identified in the RPV genome and RPV does not transform fibroblasts in vitro. It has been postulated that the env sequence unique to RPV, may play an important role in angiosarcoma induction. Angiosarcomas are generally viewed as a malignant neoplasm, but in some cases they may represent a premalignant or non-malignant proliferation of blood vessel endothelial cells (75). It is possible that the RPV env gene product can specifically interact with endothelial cell surface receptors and induce proliferation of these cells, leading to the appearance of angiosarcomas. A similar type of interaction has been proposed in the stimulation of erythroid cell colony formation by the env gene product of murine Friend viruses in susceptible host cells. In any event, its short latency, mechanism of induction, and polyclonal origin make RPV-induced angiosarcomas an exceptional type of neoplasm induced by non-acute retroviruses.

9. MAV-INDUCED NEOPLASMS

Myeloblastosis-associated virus, MAV, was originally discovered as the helper virus of avian myeloblastosis virus (AMV) (77,78,79). The clonal isolate of MAV is oncogenic in quail and produces long latency B-lymphomas and nephroblastomas (80, H.E. Varmus, C. Moscovici, personal comm.). The genome of MAV, including the LTR, is closely related to RAV. It is therefore perhaps no surprise to find that MAV proviral insertion-activation of the c-myc gene is the underlying basis for MAV-induced quail B-lymphomas. Analysis of 40 MAV-induced quail nephroblastoma samples has yet to yield a common host gene activated by the MAV provirus. In at least one sample, however, cDNA clones carrying the MAV LTR and the quail c-Ha-ras gene have been found, implicating C-Ha-ras in the induction of nephroblastomas. This proposal (D. Westaway, H.E. Varmus and C. Moscovici, personal comm.) is tempered by its current lack of universality.

10. CONCLUDING REMARKS

The molecular characterization of the cancer induction process by non-acute avian retroviruses has not only provided a foundation for our current understanding of how a retrovirus can mutate and activate host genes, but has also led to the discovery of c-myc and c-erbB as the host oncogenes responsible for chicken B-lymphomagenesis and erythroblastosis. The use of the retrovirus as a "tag" to identify host oncogenes has since found wide applicability in a variety of systems and is now a commonly applied method in the search for host oncogenes. It is likely that several new oncogenes will be revealed using this approach in the future. If we look at the few examples given in this review, we find two interesting situations:

First, different avian retroviruses (e.g., RAV, REV, RPV) can induce the same neoplasm (B-lymphoma) by interacting with the same host oncogene (c-myc).

Second, a single virus (ALV) can induce different neoplasms (B-lymphomas and erythroblastosis) by activating different host oncogenes (c-myc and c-erbB). It is thus clear that oncogenic potentials and specificities must reside in the cellular oncogenes activated by the viruses. The oncogenic spectrum of non-acute retroviruses depends not only on the type of cells they can infect but also on the oncogenes with which they effectively can interact.

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5

ENDOGENOUS AVIAN LEUKEMIA VIRUSES

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1. INTRODUCTION

Endogenous retroviruses have been recovered from reptiles, birds and mammals. Virtually all eucaryotic cells harbor DNA sequences that encode structural genes related to species-specific, RNA tumor viruses. Retroviral DNA sequences (proviruses) related to congenitally transmitted avian leukemia viruses (ALVs) occur in the Red jungle fowl, the progenitor of the domestic chicken (1). In this monograph the abbreviation ALV is used for both endogenous and exogenous avian leukemia viruses. During the evolution of Gallus gallus var. domesticus, endogenous viral (ev) sequences were presumably acquired through infection of

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the germ line with a progenitor to contemporary ALVs. Endogenous proviruses have persisted as single-copy genes in virtually all domestic chickens. Individual White Leghorns have been reported to carry as many as 10 ev genes (2). Currently, more than 20 ev genes, identified as distinct restriction fragment length polymorphisms (RFLPs), have been phenotypically associated with various levels of ALV gene expression in inbred and commercial lines of chickens.

In contrast to the replication-defective acutely transforming leukemia viruses (denoted DLV in this monograph), endogenous (subgroup E) and exogenous ALVs (belonging to subgroups A, B, C and D) lack oncogenes. Although endogenous retroviruses penetrate and replicate in cells of susceptible chickens, they, unlike their exogenous ALV counterparts, rarely cause lymphomas or disease (3,4). During the past 20 years, the structure and function of ALV genes have been extensively studied. This has been aided by: the development of phenotypically characterized lines of chickens; sensitive assays for viral gene structure and gene expression; nucleic acid hybridization techniques; and recombinant DNA methodology. An objective of this Chapter is to describe genetic differences between endogenous ALV and exogenous ALV which account for some of their markedly different biological properties. Recent developments in the characterization and biological consequences of ev genes are also presented. Comprehensive discussions of endogenous ALVs are found in earlier reviews (2,5,6,7,8).

2. EVIDENCE THAT NORMAL CHICKENS HARBOR AVIAN RETROVIRAL GENES

The notion that RNA tumor viruses replicate through a DNA intermediate was proposed when it was found that inhibitors of DNA synthesis, e.g., mitomycin C or actinomycin D, blocked Rous sarcoma virus (RSV) multiplication (9). By analogy with the lysogenic interaction of a bacterophage with its host, retroviruses were postulated to replicate through a chromosomally integrated element termed a provirus (10). The occurrence of RNA-directed DNA polymerase in RSV particles supported the role of a DNA intermediate in the life cycle of retroviruses (11,12).

ALVs in infected chicken cells were readily detected in complement fixation avian leukosis (COFAL) tests (13,14). The first evidence that chickens free of infectious ALV harbor endogenous ALV genes was shown when group-specific (gs) antigens were found in organs of "virus-free" chick embryos (15). Evidence that gs antigen expression was genetically controlled, was provided by results of CF tests

on progeny from matings between homozygous, gs^+ and gs^-, Reaseheath lines I and C (16). An autosomal dominant gene was found to control gs antigen expression. Later, it was shown that this locus also determines the coordinate expression of viral envelope, termed chick helper factor (chf) (17). Chf is a proviral-coded glycoprotein which confers unique envelope properties to subgroup E recombinants such as: growth on quail, pheasant and turkey cells, antigenicity and subgroup E interference patterns. Expression of chf in normal cells was shown when defective RSV(-) was complemented by the endogenous envelope (18). Via phenotypic mixing with RSV, ALV-free, chf^+ cells released infectious RSV(0), with a host range distinct from exogenous ALVs of subgroups A, B, C and D (19). Phenotypic mixing is the basis for the chf assay for endogenous envelope glycoproteins. Vogt and Friis (20) initially reported that some normal RPRL line 7 embryos spontaneously released a non-transforming, infectious retrovirus (RAV-0) which also had subgroup E properties. A single dominant gene controlled RAV-0 expression when RAV-0 positive line 7_2 was crossed with a line that did not produce endogenous virus. All of the progeny of the first generation were virus-positive ($V\text{-}E^+$). Segregation of the $V\text{-}E^+$ phenotype in progeny of two additional backcrosses confirmed that the corresponding endogenous retroviral (ev) gene, designated ev2, also segregated in a Mendelian manner (21). Subsequently, other ev genes have been shown to segregate independently. Treatment of both gs^+ and gs^- cells with radiation or carcinogens induced the release of leukemia viruses (ILVs) of endogenous origin (22,23). Confirmation that ev genes were chromosomally integrated came from results of RNA-DNA hybridization experiments. DNA from "leukosis-free" and line 7 embryos contained sequences related to at least 70% of the RAV-0 RNA sequences, whereas DNA from Japanese quail, which lacks RAV-0 hybridized to only 4% of RAV-0 RNA (24).

Although evidence that single genes control proviral gene expression was compelling, the nature of gene regulation was unknown. Viral RNA-cell DNA hybridization experiments which suggested the occurrence of only a few copies of viral DNA per haploid genome led to the notion that one structural gene was prevalent in all chickens and differences in regulatory genes accounted for variations in modes of expression. However, with the advent of site-specific restriction endonucleases and the development of DNA transfer techniques, segregation of restriction fragments among progeny from inbred lines revealed that multiple structural ev loci occurred in chickens and each locus was associated with a specific phenotype of endogenous viral gene expression (25,26).

3. THE ENDOGENOUS ALV GENOME

3.1. Characterization of endogenous viral (ev) genes

The gene order of non-defective ALV consists of three genes: gag, pol and env. The gag gene comprises sequences for five structural polypeptides (p) designated: p19, p10, p27, p12 and p15, where molecular weights are expressed in kilodaltons. Pol encodes the following enzyme activities involved in viral replication: RNA-dependent DNA polymerase, RNA-ase H and DNA endonuclease. The latter activity is required for site-specific cleavage at the LTR-LTR junction of circular proviral DNA prior to integration (27). Env encodes sequences for two envelope glycoproteins (gp), gp85 and gp37. Viral subgroup specificity, i.e., host range, is encoded in gp85 (28) and gp37 is a transmembrane anchor for the glycoprotein complex (29). The structure of integrated proviruses consists of: cell-U3RU5-gag-pol-env-U3RU5-cell DNA sequences. U3 and U5 represent non-coding

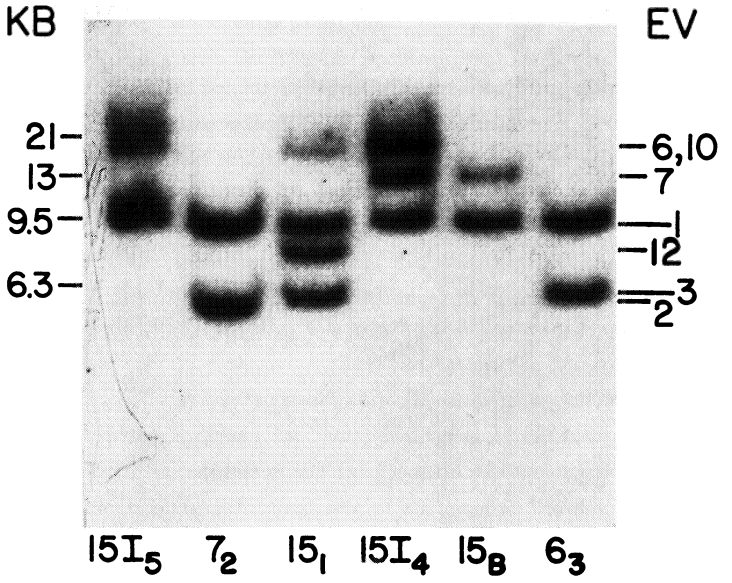

Figure 1. Endogenous viral restriction fragment length polymorphisms generated after Sac-1 endonuclease digestion of red blood cell DNA obtained from inbred lines maintained at the RPRL, East Lansing. ^{32}P-labeled recombinant plasmid containing RAV-2 genomic sequences (pRAV-2) was used as the hybridization probe.

elements that originated from the unique 3' (rightward) and 5' (leftward) ends of virion RNA, respectively. R represents short terminal sequences that are repeated at each end of virion RNA. U3RU5 constitute flanking long terminal repeats (LTRs) generated from the circular replicative intermediate. Different cell-virus junction sequences among ev loci indicate that proviruses integrate non-preferentially (30). Thus, ev genes are situated at loci defined by restriction fragment length polymorphisms (Fig. 1, Table 1).

Table 1. Restriction enzyme fragments and associated phenotypes of endogenous retroviral (ev) genes in inbred and commercial lines of White Leghorn chickens.

ev	Size of the major SacI DNA fragment[a] (kb)	Size of the 3' specific BamH1 DNA fragment[b] (kb)	Phenotype	Line or source[e]	Reference
1	9.5	5.2	gs^-chf^-	Most lines	(25)
2	6	8.2	$V\text{-}E^+$	RPRL 7_2	(25)
3	6.3	7.3	gs^+chf^+	RPRL 6_3	(25)
4	8.7	7.3	gs^-chf^-	SPAFAS	(25)
5	19	13	gs^-chf^-	SPAFAS	(25)
6	21	4.4	gs^-chf^+	RPRL 15_1	(25)
7	13	7.6	$V\text{-}E^+$	RPRL 15B	(25)
8	18	23	gs^-chf^-	K18	(104)
9	23	11	gs^-chf^+	K18	(104)
10	21	14	$V\text{-}E^+$	RPRL 151_4	(104)
11	13	NI	$V\text{-}E^+$	RPRL 151_4	(105)
12	8.1	NI	$V\text{-}E^+$	RPRL 15_1	(106)
14	9.5	15	$V\text{-}E^+$	H & N	(69)
15(C)	4.2	21	None	K28 x K16	(53)
16(D)	5.4	NI	None	K28 x K16	(53)
17	11	NI	gs^-chf^-	RC-P	(33)
18	10.5	25	$V\text{-}E^+$	RI	(33)
19	7.6	9.8 or 18	$V\text{-}E^+(?)$[c]	RW	(33)
20	8.1	9.8 or 18	$V\text{-}E^+(?)$[c]	RW	(33)
21	9.2	20	NI[d]	Hyline FP	(33)

Fragment sizes are approximate and represent a compilation from multiple sources (33). Ev13 is associated with the gs^-chf^- phenotype but restriction fragments have not been characterized (67).
NI = Not Identified. In some cases, comigration with fragments of other ev loci precludes phenotypic definition. [a]Fragments contain viral and adjacent downstream cellular sequences when hybridized with viral genomic probes. [b]Fragments contain viral envelope and adjacent downstream cellular sequences. [c]The presence of five ev loci in Reaseheath line W birds preclude definitive assignment with the $V\text{-}E^+$ phenotype. Definitive association requires further segregation of ev genes. [d] Hyline FP birds also carry ev1, ev3 and ev6. [e]Not exclusive to line or source. K, Kimber; R, Reaseheath; H & N, Heisdorf and Nelson.

Nucleated chicken red blood cells are a convenient source of genomic DNA. SstI (or SacI, an isoschizomer) cleaves near the 5' terminus of endogenous proviruses and produces one hybridizable virus-cell junction fragment for each ev gene. Other endonucleases have been used for further characterization. After endonuclease digestion, electrophoretic separation and transfer to nitrocellulose, ALV-related sequences are detected autoradiographically by hybridization of filters with ^{32}P-labeled probes representative of viral genomic or subgenomic sequences (31,32). Plasmid clones are a convenient source for the preparation of nick-translated probes. The Schmidt-Ruppin strain of Rous sarcoma virus, SR-RSV-A and subgenomic fragments for the group-specific antigen (gag), envelope (env) and sequences unique to the 3' (U3) terminus of viral RNA have been cloned in pBR322 (35). Restriction maps of exogenous and endogenous ALV proviruses are illustrated in Figure 2.

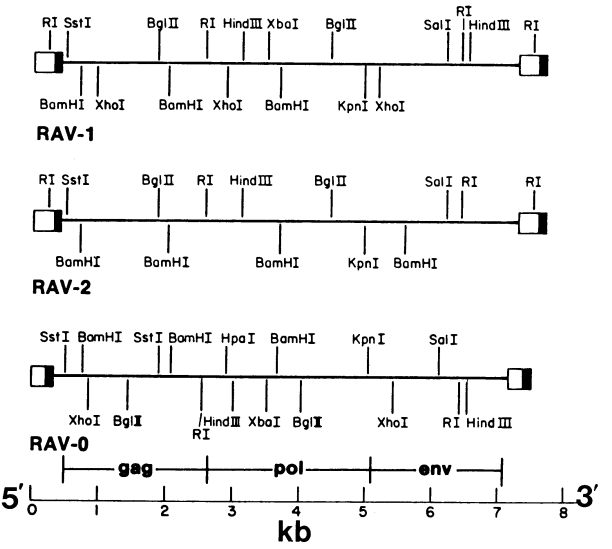

Figure 2. Restriction endonuclease cleavage sites in ALV proviruses, RAV-1, RAV-2 and RAV-0 representative of retroviral subgroups A, B and E, respectively. Although many restriction enzyme sites are shared, polymorphisms occur even within viral subgroups. Flanking boxes represent long terminal repeat sequences generated during viral replication. Open and solid boxes represent sequences unique to the 3' and 5' termini of viral RNA, respectively. (The 21 base repeat (R) sequences are between U3 and U5). Additional nucleotide sequences in U3 tract of exogenous Rous-associated viruses are associated with enhanced viral growth and oncogenicity (8). Adapted from a compilation obtained from multiple sources (36).

3.2. Structural similarities between proviruses and transposable elements

Structurally, retroviruses embrace a symmetry that reflects replicative functions. Both ends of the RNA strands of the diploid genome have short, direct, terminally repeated (R) sequences. Proviral DNA also has longer terminal sequences (LTRs) that represent both ends of viral RNA; the U3 and U5 components each contain tracts of inverted complementary sequences. ALV proviral integration results in the loss of dinucleotides at the termini of each LTR and the duplication of six basepair flanking direct repeats of cell sequences (37,38).

Retroviral proviruses also resemble movable genetic elements found in bacteria, yeast and Drosophila. Transposable elements are inserted at many sites in host DNA; they also have inverted complementary repeats and are flanked by host sequences which are duplicated during integration. Proviruses and moveable elements also begin with thymine-guanine (TG) and end with cytosine-adenine (CA) dinucleotides (38,39,40,41,42).

Transposable elements undergo direct intracellular transposition at the DNA level. However, avian retroviruses proceed through a complete viral replication cycle with subsequent exogenous reinfection and reverse transcription before integration at other chromosomal sites. Direct intracellular transposition at the DNA level has not been shown for avian proviruses.

Although multiple ev loci appear to have originated from rare, independent viral infections or viral translocations, the frequent occurrence of junction fragments that comigrate with ev1 remains an unresolved curiosity. In feline (43) and human (44) DNA, hybridization of endogenous viral-cell fragments implies conservation and amplification of flanking DNA restriction sites. In White Leghorns, the association of six defective ev loci on chromosome 1 suggests tandem gene duplication. However, duplication appears unlikely, because in situ hybridization and genetic analyses show that ev loci are interspersed along the entire length of chromosome 1 (68). Moreover, hybridization with a probe that contained ev1 flanking sequences failed to reveal homologous cell sequences associated with 11 other ev loci (33).

4. EXPRESSION OF EV GENES

Endogenous gs antigens are readily detected in cell extracts and red blood cells in direct enzyme immuno-assays (45). Chick helper factor is frequently determined in a modification of the non-producer (NP) cell activation assay (46,47). Ev gene expression is associated with four basic phenotypes shown in

Table 2. Expression is controlled, in part, by transcriptional and translational control sequences within the LTRs. The 5' LTR is essential for normal splicing and translation of viral RNA transcripts. Absence of gene expression frequently reflects mutations or deletions in the 5' LTR. The 3' LTR of exogenous proviruses plays a crucial role in initiating transcription of adjacent cellular oncogenes.

The relatively low transcriptional level of nondefective ev genes appears not to perturb the host, and in an evolutionary context may explain the persistence of ev genes. Few copies of ev1 mRNA are synthesized and only about 500 copies of infectious RAV-0, encoded by ev2, are expressed (48). However, in susceptible cells, exogenously infected with RAV-0, high titers (10^6) of RAV-0 are released (49). Growth restriction was later shown to be controlled by inhibitory, cis-acting cellular elements structurally linked to ev2. Mechanical shearing of genomic DNA increased significantly (six-fold) the specific infectivity of transfected DNA (50).

At other loci, different levels of transcription are also attributed to modified sequences within or near proviral genes. The low expression of ev1 is attributed to cytidine methylation since 5-azacytidine, a cytosine analogue which cannot be methylated at the 5 position of the pyrimidine ring, induces the release of non-infectious ev1 particles from gs^- cells (51). In contrast, the high copy number of ev3 transcripts relative to ev1 reflects under-methylation of cytidine in critical CG doublets that are found in flanking LTRs. Restriction endonuclease digestion of DNA with ev1 and ev3 by enzymes that discriminate between methylated cytidine residues in the sequence, CCGG, suggests that ev3 is under-methylated. Moreover, autoradiograms indicate that ev3 is preferentially sensitive to DNA-asel digestion, a feature of transcriptionally active genes (51). Nevertheless, a deletion at the junction of the gag-pol genes precludes expression of infectious virus in $ev3^+$ cells (52) and accounts for the smaller size of the genomic transcript (Table 2).

The absence of gene expression with many ev loci reflects mutations or deletions that may confer an adaptive accommodation with the host. Deletions at the 5' end of ev4 and ev5 proviruses which encompass the left LTR account for the lack of detectable transcripts (Table 2) (31,52,103). Provirus ev7 is spontaneously expressed in line 15B (54) and appears to be induced through recombination of constitutively expressed alleles (55). Ev15 and ev16 are vestiges of proviruses that were probably excised by precise homologous recombinations between the 5' and 3' LTRs (53). Ev15 is a solitary LTR that is almost identical with the LTRs of ev1 and ev2 (56). It is not known if ev15 influences transcription of adjacent sequences.

Because retroviruses are potential insertional mutagens, solitary LTRs may reflect reversions to the wild-type. For example, excision of an endogenous murine leukemia virus in DBA/2J mice that carry the dilute-coat-color mutation leads to reversion to the wild-type; only one LTR remains at the site of proviral integration (57). The association of ev21 with the slow-feathering phenotype in three commercial crosses may be the first example of endogenous retroviral-induced insertional mutagenesis in the germ line of chickens (107).

Table 2. Phenotypic expression of representative endogenous viral (ev) genes in normal chicken cells.

			mRNA[a]	
Phenotype	Symbol	ev locus	Size	Copies/Cell
No detectable viral product	gs^-chf^-	1	35S	0.3-0.6
			21S	0.1-0.3
		4,5	ND	-
Expression of subgroup E envelope antigen	gs^-chf^+	9	34S	20-60
			21S	60-150
Coordinate expression of group-specific and envelope antigens	gs^+chf^+	3	31S	50-100
			21S	30-80
			19S	10-30
Spontaneous production of subgroup E virus	$V\text{-}E^+$	2	35S	0.1
			21S	0.05

ND = None detected.
[a] Characterization of viral RNA transcripts from cells of the above phenotypes has been described (31,52)

5. TRANSCRIPTIONAL CONTROL ELEMENTS WITHIN THE LONG TERMINAL REPEAT

During reverse transcription, proviral DNA acquires duplicate copies of sequences found at the 3' (U3) and 5' (U5) termini of genomic RNA. Tandem LTR junction sequences in the circular replicative intermediate provide proviral site-specific attachment sequences for integration into host DNA. U3 sequences

provide putative recognition sites for RNA polymerase II and polyadenylation; transcriptional control signals essential for the initiation and termination of viral mRNA synthesis. U3 of exogenous ALVs contain about 75 more nucleotides than the U3 region of endogenous proviruses (39,58). These enhancer sequences may activate transcription of cellular sequences at sites other than the viral promotor site.

The low copy number of transcripts correlates with the relatively slow growth rate and lack of oncogenicity of endogenous viruses and recombinants containing endogenous U3 sequences (59). In contrast, transformation of thymidine kinase (tk)-deficient mouse cells is enhanced 20- to 40-fold when DNA with RSV U3 sequences adjacent to the herpes simplex virus tk gene is micro-injected into mouse cells. The enhancing sequence of RSV DNA is localized in 143 base pairs (bp) that include 88 bp at the 5' end of the U3 element (60). Transcription promoter activities of the U3 component of LTRs derived from RAV-0, RAV-2, ev1 and ev2 were also compared in envelope complementation assays by measuring induction of foci by plasmid DNA clones with different promoter control cassettes spliced into env-src-LTR sequences; cassettes consisted of flanking regions upstream from the LTR, LTR and 5' leader sequences. As expected, the RAV-2 cassette with enhancer sequences in U3 is most active, whereas RAV-0 and ev2 cassettes are only 7-10% as active. The deficient promoter activity ($<1\%$) of the ev1 cassette is attributed to an additional defect in the leader region (61). In similar reconstruction experiments using Pr-RSV LTR cassettes, enhancement of chloramphenicol acetyltransferase activity is controlled by three independent enhancer domains at the left end of exogenous U3 sequences (62). Osteopetrosis-inducing, myeloblastosis associated viruses contain unique 3' terminal oligonucleotide fragments which are not found in other exogenous ALVs or RAV-0 (63) suggesting that U3 sequences may also be involved in the determination of tissue tropism.

6. CHROMOSOMAL DISTRIBUTION OF EV GENES

Independent segregation of ev2 and ev10 (64), ev3 (65) and ev7 and ev12 (66) has been reported. In contrast to the ev genes cited above, defective proviruses encoded at ev1, ev4, ev5, ev6, ev8 and ev13 are on chromosome 1 (34,67). Although these six inactive ev genes are clustered on chromosome 1 of White Leghorns, ev clusters are not found on chromosome 1 of White Plymouth Rocks (68). Tereba has theorized that multiple ev genes may have originated from an

alteration or duplication of a part of chromosome 1 with subsequent 5' deletions occurring during recombination of defective proviruses that arose from a progenitor viral locus, e.g., ev1 (68). Although hybridizations with probes containing ev1 flanking sequences indicate otherwise, the frequent occurrence of viral-cell junction fragments similar in size to ev1 (33) suggests that there may be preferred proviral integration sites. Ev2 and ev14, which are associated with the V-E$^+$ phenotype, are localized to chromosomes 2 and 3, respectively (69). Ev3 is on a microchromosome (34). Ev7 found in RPRL line 15B is associated with the male (Z) chromosome (66,69) and may provide a convenient marker for the orientation of sex-linked genes.

7. SIGNIFICANCE OF EV GENES AMONG AVIAN SPECIES

In an extensive survey of 68 varieties of chickens, the number of loci per genome ranged from 1 to 10 with an average of about 5 ev loci per bird (2). The apparent ubiquity of ev1 in White Leghorns and the expression of viral genes in embryonic fibroblasts suggested that ev loci may have an important role in ontogeny. However, one normal, ev-negative male was found among 21 siblings in a cross involving RPRL line 7. This ev negative rooster was successfully mated to heterozygous siblings and their progeny formed the foundation stock for the unique ev-negative, RPRL line 0 (70). Other breeds such as Brown Leghorns and Brown Nicks carry similar proviruses, but in contrast to White Leghorns, about 20% of the birds tested lacked ev1 (71).

RAV-0 related sequences also occur in Ring-necked pheasants (71) and Red jungle fowl but not in Sonnerat's, Ceylonese or Green jungle fowl (72). These findings indicate that: (a) ev loci are not essential for normal growth and development, and (b) the RAV-0 genome was introduced into the germ line of chickens following speciation but before domestication (72). Since most initial investigations were done using research laboratory flocks, the rare occurrence of ev-negative chickens is attributed to the multiplicity of ev genes in individual chickens and the limited gene pool inherent in closed laboratory flocks of White Leghorns (2). In this context, Italian partridge-colored chickens appear to be free of endogenous viruses (73).

8. GENETIC DIVERSITY AMONG AVIAN RETROVIRUSES

Characterization of the complete nucleotide sequence (9312 nucleotides) of the Prague strain of Rous sarcoma virus (Pr-RSV-C) has provided a cornerstone for fine-structure comparisons and gene product relationships between endogenous retroviruses and exogenous ALVs (74). Differential and competitive hybridizations of labeled viral RNA with proviral DNA indicate about 90% sequence homology between RAV-0 and RAV-7 (subgroup C) (75). Moreover, sequences essential for reverse transcription, translation and particle formation are conserved among subgroups. Restriction maps of RAV-1 (subgroup A), RAV-2 (subgroup B) and RAV-0 (subgroup E), shown in Figure 2, reveal many common cleavage sites. Nevertheless, spontaneous changes in base sequences frequently occur in the gag, pol and env genes. Direct sequence analysis of RNA in RSV mutants indicated a particularly high mutation rate in pol, gp37 and the 3' end of p19 (76). Substitutions and deletions found in ALV strains are frequently reflected in amino acid substitutions and altered electrophoretic mobilities of structural polypeptides (77). Variant forms of p27 and p19 occur in recombinants between Pr-RSV-C and RAV-0 (78), evl particles (79) and 15B-E viruses (54). Moreover, RAV-0 $p27^{o}$ has 23 more amino acids than p27 of exogenous ALV (80).

RNA-aseT_1 resistant oligonucleotide fingerprints and sequence analyses of ALVs and RAV-60 recombinants revealed variant regions that govern host range, growth rate and oncogenicity. The 5' terminal halves of RAV-0 and the Prague strain of RSV-B are, except for single nucleotide substitutions, almost identical but major differences in oligonucleotide composition are found clustered in the viral subgroup (S) coding region near the middle of gp85 (81). Amino acid sequences deduced from the alignment of host range (hr)-specific nucleotide sequences of Pr-RSV-C, Pr-RSV-B and RAV-0 indicate that two variable regions occur within gp85 (28). Host range 1 sequence homology between subgroups B and E is consistent with a common recognition of cell surface receptors. On the other hand, in other deduced comparisons, amino acid sequences at the gp37 carboxy termini of evl, RAV-0 and Pr-RSV-A were predicted to be remarkably similar (29,58). The latter observation has led to the suggestion that subgroup A and subgroup E envelope proteins are more closely related to one another than to subgroup C envelope (29). Significantly, oligonucleotides present at the 3' end of exogenous ALVs were missing in fingerprints of endogenous viruses (59).

In genetic experiments designed to test the role of env gene products on tumor induction, subgroup E recombinants (RAV-60s) were generated by infection

of ev3$^+$, ev6$^+$ or ev9$^+$ cells with ALV of subgroups A and B. Induction of neoplasms in chickens infected with RAV-60 recombinants, containing endogenous viral envelope and exogenous U3 sequences, led to the conclusion that the high rate of tumor induction is not controlled by the env gene but rather by sequences involving the U3 terminus (82,83). Confirmation of the critical role of exogenous U3 sequences in controlling growth rate and tumor induction was shown in later experiments using another subgroup E recombinant, NTRE-7, which is apparently identical in oligonucleotide composition with RAV-0 except for about 300 nucleotides derived from the 3' end of its oncogenic RSV-B parent (84). NTRE-7 grows to high titer and is oncogenic (83).

9. BIOLOGICAL INFLUENCES OF ENDOGENOUS PROVIRUSES

9.1 Interaction of ev genes with exogenous ALVs

Cellular resistance to ALV infection operates at two levels. Genetic cellular resistance to exogenous ALVs of subgroups A, B and C is specifically controlled by autosomal recessive tumor virus (tv) genes: tv-a, tv-b and tv-c (85). Genes for receptors are distinct from dominant, epistatic inhibitor (I^e) genes which express chf antigens (86,87). Viral envelope glycoproteins block subgroup specific receptors and thus interfere with penetration of ALVs of the same subgroup. Cells with the V-E$^+$ phenotype or those with defective endogenous proviruses which express chf such as ev3, ev6 and ev9 have a markedly reduced susceptibility to subgroup E infection and a slower rate of viral penetration than chf$^-$ cells. Moreover, horizontal spread of endogenous virus infection is also reduced in chf$^+$ chickens (87). However, in subgroup E susceptible, line K28 chickens, RAV-60 recombinants induce lymphomas, sarcomas, osteopetrosis, anemia and wasting. Wasting is associated with internal bleeding and blood dyscrasias (83).

Immunologic tolerance to ALV infection modulated by ev gene expression was shown in a series of experiments comparing antibody responses to RAV-1 infection in hatch-mates with and without ev2 or ev3. A significantly higher percentage of infected ev2$^-$ chickens produced RAV-1 neutralizing antisera with higher titers than ev2$^+$ chickens. Ev3$^-$ chickens also developed a higher incidence of antibodies than ev3$^+$ chickens. However, infected ev3$^-$ chickens also developed a high incidence of a lethal non-neoplastic syndrome (NNS) characterized by atrophy of lymphoid organs, hepatitis and a debilitated immune response to particulate antigens (88). Additional experiments were conducted to study the interaction of different ALV strains in the presence and absence of ev genes. Groups of day-old

semi-congenic chickens with and without ev2 and ev3 in a line 15B background and an unrelated line that lacks ev genes (line 0) were infected with two laboratory strains (RAV-1 and RAV-2) and two field strains of ALV (RPL40 and RPL42). As expected, all four virus strains induced a higher incidence of neutralizing antibodies and a lower incidence of viremia in $ev2^-$ and $ev3^-$ semi-congenic chickens than in $ev2^+$ and $ev3^+$ hatch-mates. A high incidence of NNS was seen only in RAV-1-infected, $ev2^-$ and $ev3^-$ chickens; infection of hatch-mates with RAV-2, RPL40 and RPL42 induced little or no NNS. RAV-1-infected, RPRL line 0 chickens developed an even higher incidence of antibody production and a lower incidence of viremia and cloacal shedding of group-specific (gs) antigens than $ev2^-$ and $ev3^-$ semi-congenic lines, but unexpectedly NNS was not observed (89). Mortality from neoplasms, however, was not influenced by ev gene expression.

Tissue tropism and induction of a specific disease is determined by interactions involving exogenous viral promoter sequences (LTRs), endogenous chf status and host genes. In view of the hyperimmune response raised against subgroup-common envelope antigens, and sequence similarities between RAV-1 and RAV-2 genomes, one would expect to also find NNS in RAV-2 infected chf^- semi-congenic chickens. Absence of NNS in RAV-2, RPL40 and RPL42 infected chickens suggests that targeting may be determined by envelope subgroup variable regions. On the other hand, the influence of host genes other than ev loci in modulating ALV-induced disease was also shown in the unexpected absence of NNS in RAV-1 infected, hyperimmune, RPRL line 0 chickens (89).

Partial tolerance or non-inducibility of humoral antibody reactivity to subgroup-common envelope determinants in RAV-1-infected chickens constitutively expressing ev3 has been reported using assays based on immunoprecipitation of radiolabeled envelope glycoproteins derived from ALV subgroups A, B, C and E (90). Thus, tolerance in birds with the chf^+ phenotype may provide a survival advantage in the prevention of NNS. In this context, it has been noted that many lines of chickens express defective proviruses of the chf^+ phenotype (87,91). On the other hand, chickens that express ev genes may become tolerant to subclinical levels of infection and thus transmit exogenous ALV congenitally (7).

Since Sonnerat's jungle fowl do not carry ev genes and are susceptible to infection with subgroup E viruses, experiments were conducted to determine the leukemogenic potential of RAV-0, RAV-60 (the NY202 isolate) and RAV-1. In Sonnerat's jungle fowl, all three strains produced lymphomas and wasting accompanied by bursal and thymic atrophy (92). The broad anti-glycoprotein reactivity elicited with RAV-1 infection of Sonnerat's jungle fowl indicates that in

chf$^-$ birds, wasting and NNS are associated with a hyperactive humoral immune response to subgroup-common envelope antigens. In contrast, RAV-1-infected Red jungle fowl, which carry ev genes, produced only subgroup A specific antibodies (92). Earlier, immunoprecipitation assays also showed that only subgroup A specific antibodies were induced in chf$^+$ chickens after infection with the Prague strain of RSV-A (93). Turkeys, which lack ev genes, also had a high incidence of inflammatory and lymphoproliferative but non-neoplastic lesions in visceral organs after RAV-1 inoculation (94).

The modulating influence of ev gene expression on the incidence and titers of neutralizing antibodies is specific for ALV-related retroviruses. The frequency of antibody induction in chickens infected with chick syncytical virus (CSV), a reticuloendotheliosis virus (REV) unrelated to ALV, bore no relationship to the presence or absence of ev genes. Moreover, NNS was not observed in CSV-infected, chf$^-$ chickens (88).

9.2. Negative influence of ALV infection on commercially important traits

Flocks subclinically infected with exogenous ALV consistently produce fewer and smaller eggs with thinner shells than flocks selected for high egg production, which were free of ALV infection (95). Moreover, ALV infection was associated with higher general mortality and lower hatchability as compared with strains selected for high egg production (96). This negative effect of ALV infection was confirmed in subsequent studies of commercial meat-type chickens. Broiler-age body weight of ALV-positive females was also lower than in non-shedder hens (97,98). In random-bred populations, gs antigen-positive hens matured later, produced fewer and smaller eggs and grew less rapidly than those which produced gs antigen-negative eggs (99).

Do endogenous viruses impair productivity traits? In one study on the incidence of endogenous virus in unselected random-bred chickens and related strains selected for high egg production, a reduced incidence of subgroup E virus was found in selected strains as compared with unselected, random-bred control stocks (100), suggesting that endogenous viruses may compromise productivity traits. Ev genes were not characterized in these lines, therefore association of specific loci with altered productivity traits was not established (see also Chapter 12).

9.3. Structure of $p27^o$ and restricted congenital transmission of RAV-0

In reproductive tissues of line K28 hens, an absolute, tissue-specific restriction on replication of RAV-0 appears to operate. Structurally, $p27^o$ from RAV-0 is about 2,000 daltons larger than p27 found in RSV (80). Therefore, different electrophoretic mobilities of p27 were used to characterize various RAV-60 recombinants which contained p27 from either an exogenous or endogenous parent. In viremic K28 hens, congenital transmission was associated only with viruses which inherited p27 from the exogenous parent, whereas recombinants which inherited $p27^o$ from either RAV-0 or ev3 were not transmitted (101). Restricted replication of RAV-0 in the oviduct was ascribed to impaired processing of viral capsid $p27^o$ by host factors. Nevertheless, endogenous proviruses are stably integrated in germinal cells and genetic survival is ensured. RAV-0 was detected in embryos and meconia of $ev2^+$ semi-congenic line 15B dams (102). On the other hand, in the evolution of contemporary ALVs, selection for survival through congenital transmission may have required structural modifications of capsid p27 (101). Viral strain and host factors appear to be crucial because p27 was detected in albumens and progeny after exogenous infection of hens with line 15B (ev7) virus. Enzyme immunoassays also indicated that infectious viruses encoded at loci, ev10, ev11, and ev12 are also shed in albumens of semi-congenic hens (Crittenden and Smith, unpublished data).

9.4. Evolution of ev genes and possible origin of exogenous ALV

The structural similarity of proviruses and procaryotic transposable elements suggests an evolutionary relationship between retroviruses and moveable genetic elements (40). Although ev-negative chickens appear normal, most chickens harbor multiple ev genes. Estimates based on oligonucleotide maps suggest that less than 2% of endogenous proviral sequences have diverged between a distantly related pair, i.e., ev1 and ev7 (59). The family of subgroup E proviruses could have multiplied through: (a) rare, independent germ line infections, (b) gene duplications involving both flanking and proviral sequences, (c) reverse transcription of viral transcripts accompanied by re-integration at other sites in germ line DNA, and/or (d) direct intracellular transpositions (2).

Viral repression mediated by host factors, inefficient transcription and extensive interference to penetration by chf have contributed to ev gene accommodation in the germ line. In the evolution of field strains of ALV, selective pressure for variants with modified envelope host range determinants operated to overcome cell surface barriers to further propagation. Insertions or recombinations

generated virulent strains with enhanced transcriptional promoters. Acquisition of rapid growth led to more diverse recombinants with the advantage of an expanded host range. However, retroviruses are potential insertional mutagens (57,103) and germ line infection with pathogenic variants would be lethal (2,59). Selection for defective proviruses which express chf, e.g., ev3, ev6 and ev9, may have led to the protection of the germ line from potentially harmful viral insertions (87). Consequently, only relatively innocuous subgroup E viruses are germ line transmitted. The influence of ev genes as they relate to physiologic traits in commercial chickens remains to be seen.

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6

IMMUNOLOGY OF AVIAN LEUKOSIS VIRUS INFECTIONS

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1. INTRODUCTION

This Chapter will concentrate on three interactions of avian leukosis virus (ALV) with the immune system of the avian host. The first, neoplastic transformation of lymphoid cells, will be treated briefly and is the subject of more detailed discussion in Chapters 4 and 9 of this monograph. The second, immunization, will be treated more thoroughly since immune recognition is a major host defense mechanism. The third, immunosuppression, will be discussed at some length because it is an area of considerable current interest. The interactions of ALVs with the host immune system are almost always harmful. Infection with ALV generally gives rise to either neoplasia or immunosuppression.

G.F. de Boer (ed), Avian Leukosis.

2. NEOPLASTIC TRANSFORMATION OF LYMPHOID CELLS

An obvious interaction of ALV with cells of the lymphoid system is that of malignant transformation. The clonal outgrowth of malignant B-cells after transformation by ALV has been the subject of intense investigation and a discussion of this subject is beyond the scope of the current review. The reader is referred to two excellent reviews of this subject (1,2) and to Chapter 4 of this monograph.

The evidence that ALV induces transformation of lymphoid cells of the immune system is compelling. The involvement of the bursa of Fabricius in lymphoid leukosis was proven by observing that surgical removal of the bursa from chicks infected at hatching resulted in a significant reduction in the incidence of disease (3,4), and by reconstitution experiments wherein the induction of lymphoid leukosis in bursectomized chickens required that target chickens be repopulated with bursa cells (5). Thymectomy, however, had no effect on the appearance of lymphoid leukosis (3). Further proof of the lymphoid origin of the tumor cells was obtained by showing that cells within the tumors contained surface IgM but no IgG or IgA (6). Finally, histological examination of the lymphoid organs during the development of lymphoid leukosis revealed that changes were observed in individual lymphoid follicles within 4 to 6 weeks post-infection (7,8), while the lymphomas appear in the chicken with latent periods of 4 to 6 months (9). Recent results suggest that the susceptibility of a chicken strain to ALV infection can be pre-determined by examining the pre-neoplastic hyperplasia present in the bursa (10).

Little evidence is available that ALV causes transformation of cells of the immune system other than the B-cell. It should be noted that several acute leukemia viruses cause the direct transformation of bone marrow cells which have roles in the lymphoid system. For example, MC29, CMII, OK10 and MH2 transform macrophages or macrophage stem cells, while AMV and E26 transform stem cells of the granulocyte series (11,12). Direct transformation of lymphoid cells by exogenous ALV or acute leukemia viruses may have a profound effect on the function of the immune system, but this area of study has received little attention. It is likely that the animal suffers the normal consequences of a neoplastic disorder such as a large tumor burden, compromised organ function such as would be observed in a liver filled with lymphoid cells or bone marrow teeming with myeloblasts, and non-specific metabolic disorders associated with neoplasms such as anorexia, toxicity from necrotic tissue, and mechnical and locomoter dysfunctions.

3. IMMUNE RESPONSE TO ALV INFECTION

Most ALV infections are acquired as horizontal infections, and the immune system of the chicken serves to protect the host in a conventional manner. In the majority of cases in which chickens are exposed to ALV as immunocompetent adults, the animal develops a transient viremia, neutralizing antibodies develop and infectious virus is eliminated from the serum (13,14,15). However, one mode of persistence has been reported to involve retroviral particle association with white blood cells (16). Congenital transmission of ALV to embryos by excretion of virus from infected cells of the oviduct causes the highest incidence of lymphoma development and virus shedding (17,18). Congenital transmission particularly occurs in a viremic, immunologically tolerant hen, but may also occur in the presence of relatively high concentrations of neutralizing antibodies (15). It is interesting to note that congenital transmission of some endogenous avian leukemia viruses (ALV), related to RAV-0, is restricted by its own gag gene products (19). However, congenital transmission of endogenous ALV seems to be a regular event in other strains of chickens (see Chapters 5 and 13).

The immunocompetent chicken predominantly makes virus neutralizing antibodies which may persist at high levels throughout life (5, 20). These antibodies are responsible for neutralization of the virus, and are subgroup- and partially, group-specific (21). Antibodies are only rarely made against the internal group-specific antigens (22), presumably because most chickens express p27 antigen of endogenous avian retrovirus and therefore are tolerant to the p27 polypeptide (23). Some field strains of ALV appear to be less immunogenic than laboratory-passaged viruses (24).

As a result of subgroup-common antigenic determinants in glycoproteins, the expression of endogenous viral (ev) genes may influence the birds' ability to mount an immune response to exogenous ALV. Chicken strains which lack the ev2 and ev3 loci appear to have a higher incidence of antibody and less viral shedding when inoculated as day-old hatchlings than chickens which were positive for these ev genes (24). Chickens lacking these ev genes seem to be more susceptible to a wasting syndrome (non-neoplastic syndrome) than their ev-positive counterparts. However, the absence of ev genes does not seem to be the only factor determining mortality from non-neoplastic syndrome. After RAV-1 inoculation, the wasting syndrome was not observed in line 0 chickens which do not carry ev genes (24,25). The non-neoplastic syndrome of endogenous ALV-free chickens associated with RAV-1 infection appears to be different from an obesity and wasting syndrome

observed after RAV-7 infection (26). The latter disease appears to involve lymphoid infiltration of the thyroid glands and the stunting and obesity may result from a hypothyroid state (27).

The appearance of neutralizing antibody may accompany the waning of acute manifestations of ALV infection. For example, recovery from an ALV-induced pancytopenia is accompanied by the appearance of neutralizing antibodies and the disappearance of infectious virus from the serum of infected chickens (28). The presence of endogenous ALV may also influence the appearance of neutralizing antibodies. After infection as one-day-old chicks, those which lack endogenous ALV appear to make neutralizing antibodies sooner, and to clear virus from the plasma sooner, than chickens which express endogenous ALV (25). The reader is referred to an excellent review of related aspects of natural and experimental infections (29).

Passive administration of neutralizing antibody may prevent or delay the appearance of the acute effects of certain avian retroviruses. For example, pancytopenia can be prevented if neutralizing antibodies are given within 3 days after MAV-2(0) infection (30). Likewise, prompt intravenous administration of neutralizing antibodies to embryos infected with MAV-2(0) will prevent the development of osteopetrosis in the hatched chick (31). However, it is necessary to administer antibodies within one to 3 days after infection, otherwise little effect is noted (30,31). The role of neutralizing antibodies in recovery from MAV-2(0)-induced pancytopenia is further illustrated by examining the effect of bursectomy on the induction of disease symptoms. The infection of bursectomized chickens leads to a high incidence of progressive and irreversible anemia (30). Administration of neutralizing antibodies to infected, bursectomized chickens delays the onset of anemia and osteopetrosis (31).

It seems apparent that avian retrovirus-induced tumors may differ in their sensitivity to neutralizing antibodies. ALV-induced lymphomas which arise via clonal development of B-cell tumors, appear indifferent to the presence of neutralizing antibodies (3,4,7,15). Nephroblastomas may be found in the presence or absence of antibodies (32,33). Non-clonal proliferative growths, such as osteopetrosis, appear more sensitive to the presence of neutralizing antibody (30,31). In general, neutralizing antibodies may prevent infection, but they are less effective in clearance of an established infection. It is interesting to note that passive administration of neutralizing antibody to RAV-1-infected chicks may lead to the elimination of infectious virus and viral group-specific proteins from the cells of the spleen, liver and thymus, but not from the bursa (34). It would be interesting

to establish in this system whether changes are observed in the nature of the integrated provirus.

Infection of an avian embryo with one ALV may not prevent appearance of neutralizing antibody against a second ALV of the same subgroup administered after hatch (35).

4. IMMUNOSUPPRESSION

Immunosuppression has been defined as "a state of temporary or permanent dysfunction of the immune response resulting from insult to the immune system and leading to increased susceptibility to disease" (36). The presence and extent of immunosuppression may be assessed by examining the following criteria: Morphometric changes in central and/or peripheral lymphoid tissues; changes in concentrations or ratios of immunoglobulin classes within serum and secretions, and changes in complement levels; changes in functional activity of the immune response; demonstration that the suspected immunosuppressive agent will interfere with vaccination and/or exacerbate the course of disease produced by another pathogen (36). Immunosuppression has been associated with retroviral infection for many years (for a review of the early literature, see reference 37). Immunosuppression associated with ALV was reviewed in 1979 (38), and immunosuppression associated with reticuloendotheliosis virus infection recently (39). The reader is referred to these excellent works for further information. Each of the five criteria established by Dohms and Saif (36) will be discussed in relation to current information about ALV.

4.1. Morphometric changes in central lymphoid tissues

The major structural alteration associated with immunosuppression observed in the lymphoid organs of ALV-infected chickens is an apparent involution of the bursa and spleen (40). Specifically, the mass of the bursa is frequently ten-fold smaller in MAV-2(0)-infected chickens than the mass of the bursa of age-matched uninfected chicks. Subsequent investigation of the thymus revealed a similar size reduction (41). The changes observed in the bursa appear to be an arrest of development, rather than destruction of cells already in the organ (42). Specifically, when 10-day-old embryos were infected with MAV-2(0) and bursa morphology was examined at weekly intervals following infection, it was apparent that the infected bursa failed to develop beyond a state of differentiation and size observed in the 20-day-old uninfected embryo (42,43). Histologically, the follicle

of the infected chick bursa was smaller than that of the uninfected chick, contained fewer lymphocytes than the normal, and had a sparse cortex. Far fewer follicles were present in the infected bursa, and the stroma was much more prominent. Similar changes were noted in the thymus (42). The spleen of the MAV-2(0)-infected chick was initially larger, but quickly regressed to a size similar to that of the bursa and thymus of the normal animal (42). Similar morphologic changes have recently been noted in the lymphoid organs of chicks infected with RAV-7 (Adcock, Heidrich and Smith, unpublished; Bolin, Cheville and Smith, unpublished). Infection with RAV-1 of chicks lacking the ev3 locus leads to a severe atrophy of lymphoid organs (23,44). Aside from the above reports, there has been little systematic study of the morphometric aspects of lymphoid organ involvement in ALV infections.

Avian leukosis viruses vary in the degree to which they influence the weight of lymphoid organs. For example, MAV-2(N), an avian myeloblastosis-associated virus of subgroup B which induces a high incidence of nephroblastomas, causes involution of the thymus, but involution is observed only when infected chicks are over 3 months of age (32).

4.2 Changes in concentration or ratios of immunoglobulin classes within serum, secretions, and changes in complement levels.

Infection of chickens with ALV results in changes in the levels of plasma proteins (40,45). In MAV-2(0)-infected chicks, the level of gammaglobulins is elevated (40). A two- to five-fold increase in IgG appears to account for the majority of this elevation in immunoglobulins. A significant proportion comprises probably IgG with specific activity since the increase of immunoglobulins coincides with higher titers of neutralizing antibodies (20,46). However, the impact of this alteration remains obscure. In most cases, a convincing argument may be made that non-specific factors such as stress, anorexia, or tumor burden play an important role. Alterations in secretory (IgA-like) antibody and in complement levels have not been extensively studied.

4.3. Changes in functional activity of the immune response

The response of avian lymphoid cells to mitogens has been employed as a measure of lymphoid cell function. Infection of chickens with an ALV of subgroup A results in a decreased blastogenesis when suboptimal doses of phytohemagglutinin (PHA) are employed (47). ALV of subgroup B causes a marked lack of responsiveness to lectins, while viruses of subgroup A are not as immuno-

suppressive (48). The mitogen responsiveness of lymphoid cells from MAV-2(0)-infected chickens has been studied in some detail. Lymphoid cells from chicks developing osteopetrosis or anemia after MAV-2(0) infection did not respond to a wide range of PHA (41) or Concanavalin A (Con A) concentrations (49). Cell mixing experiments with MAV-2(0) and uninfected spleen cells showed that normal cells restore mitogen responsiveness to infected cell populations (49). These results suggest that MAV-2(0) immunosuppression is not due to the presence of a suppressor T-cell, as has been amply documented for reticuloendotheliosis virus (50,51,52). The normal cells responsible for restoring mitogen responsiveness to MAV-2(0)-infected cells appear to be adherent, macrophage-like cells from the uninfected spleen or peripheral blood (49). Although this result suggests that the macrophage population of the MAV-2(0)-infected chick is impaired, the number of macrophage-like cells present in the infected chick is normal, and these adherent cells possess most of the functional attributes of normal macrophages (49). The nature of the difference between uninfected and MAV-2(0)-infected lymphoid populations remains to be elucidated.

The ability of ALV-infected chicks to raise antibody may depend on the nature of the infecting virus. Subgroup A viruses appear to have little effect on the ability of the host to produce antibody (6,13), while ALV of subgroup B may cause a severe depression in antibody synthesis (41,43). Specifically, subgroup B infected chickens have a diminished capacity to form hemolytic plaques in a direct (Jerne) assay for IgM production (41) and in an indirect test for IgG production (43). In addition, when MAV-2(0) is given to 10-day-old embryos, hatched chicks have significantly depressed ability to form antibodies against sheep red blood cells (SRBC), Brucella abortus and human gammaglobulins (43). Likewise, MAV-2(0)-infected chickens have a depressed delayed hypersensitivity reaction against human gammaglobulin, and decreased mitogenic responsiveness against Con A, PHA, and pokeweed mitogens. Chickens infected within 48 hours after hatch show few signs of immunosuppression (43).

Recent work with a newly isolated lymphoproliferative disease virus of turkeys (another member of the family of avian retroviruses) shows that this virus is strongly immunosuppressive. Mitogen responsiveness to PHA and Con A are suppressed, as is the body weight of the infected turkeys (53).

4.4. Immunosuppressive effects to other pathogens or antigens

This is perhaps the least studied aspect of ALV-induced immunosuppression. Most reference to the susceptibility of ALV-infected chickens to secondary

pathogens is anecdotal, and little current literature on the subject exists. In our laboratory, we note that chickens with long-standing proliferative diseases such as osteopetrosis or nephroblastoma should not be wing-banded since the skin wound is often complicated by suppurative lesions. A more definitive study has established that dual infection with infectious bursal disease virus (IBDV) and ALV causes a higher rate of ALV shedding than when birds are not exposed to IBDV (54). The area of dual infection remains to be thoroughly investigated and should provide important insights.

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7

AVIAN SARCOMAS: IMMUNE RESPONSIVENESS AND PATHOLOGY

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1. INTRODUCTION

The finding by Peyton Rous (1) in 1911 of a viral origin for a spontaneously occurring sarcoma in chickens has led, directly and indirectly, to some of the most exciting research imaginable. Since his discovery, numerous investigations have shown, using a variety of cell culture and animal systems, that neoplastic transformation may be virus-induced. In recent years, it has even become apparent that certain viruses play an important etiologic role in human malignancies (2). The agent which Rous described became known as Rous sarcoma virus (RSV). This agent, together with other similar isolates that can cause fibrosarcomas in chickens, are collectively referred to as avian sarcoma viruses (ASV).

G.F. de Boer (ed), Avian Leukosis.

They closely resemble, both structurally as well as genetically, another group of viruses that are the major subject of this volume, i.e. avian leukosis virus (ALV). Together with the (acute) defective leukemia viruses (DLVs) these viruses comprise the avian leukosis/sarcoma virus group.

ALV infections induce lymphomas in chickens, many months after viral inoculation, but do not result in the development of sarcomas. Furthermore, ALV is unable to transform chicken embryonic fibroblast (CEF) cells in tissue culture. In contrast, ASV is able to mediate the rapid transformation of cells in tissue culture and is able to induce sarcoma development in chickens within days or weeks after injection into wing webs or elsewhere. In many cases, these viruses are unable to cause tumor formation by themselves or indeed to replicate independently. They may, however, accomplish both of these tasks in the presence of ALV which can act as "helper virus". Viruses which require the presence of other complementary viruses for replication and/or tumorigenic function are termed "defective" (3).

Members of the avian leukosis/sarcoma virus group have been described as C-type particles, on the basis of morphological considerations. They usually measure about 120 to 140 nm in diameter and contain a ribonucleoprotein inner core surrounded by an outer envelope (4). Negative staining reveals that spike-like protrusions, about 6 nm long, extend outward from the viral surface. These viruses are all members of the subfamily Oncovirinae of the family Retroviridae. That is to say, they all possess the enzyme RNA-dependent DNA polymerase or reverse transcriptase, and that this enzyme is responsible for initiating viral replication by making a DNA copy of parental viral RNA after infection has been initiated. This proviral DNA, as it is called, can be found in the cytoplasm of infected cells in the form of circular double-stranded molecules. It subsequently migrates to the nucleus where it integrates at random into genomic DNA. Such integrated proviral DNA can then transcribe various viral m-RNA species which can be translated into viral proteins. It can also give rise to full-length viral RNA which can be packaged along with newly synthesized viral proteins, including reverse transcriptase, into progeny virions.

The discovery of RSV and similar oncogenic agents has led to the subsequent identification and purification of reverse transcriptase as the enzyme that specifically mediates initiation of the viral replicative cycle. The use of this enzyme to make c-DNA copies of virtually any segment of isolated RNA has resulted in a revolution in molecular biology over the past 15 years. In addition, research over the past decade has successfully identified a single ASV gene, the

src gene, as one which is responsible for the ability of this class of virus to rapidly induce tumors in animal hosts and to transform cells in tissue culture (5). The src gene is today considered the prototype for a series of so-called onc genes that were initially described as being of retroviral origin (3). We now know that these onc genes are also present in somewhat different form within the normal DNA of every vertebrate species, that has been examined, and can even be identified in some lower species as well. A number of studies indicate that chromosomal translocations of these genes may be crucial events in the cancer induction process (6). Current thinking dictates that the onc genes are of cellular rather than viral origin. Research into their normal physiological function and mechanism of action under both normal circumstances and during malignancy constitutes one of today's most exciting areas in cell and cancer biology.

The purpose of this Chapter is not, however, to review recent developments in either the use of reverse transcriptase in molecular biology or the study of onc genes. Rather we wish now to consider the avian sarcoma model as one for which the presence of defined antigens on the tumor cell surface has been relatively well established. When applicable the current knowledge of the immune response to ALV infection and transformation will comparatively be discussed. We will focus on the surface antigenic composition of ASV-transformed cells and/or ASV-induced tumor cells. We will further deal with the interactions between neoplastic and lymphoid tissue in avian sarcoma-bearing birds which result in the development of anti-tumor immune responsiveness.

2. CELLULAR TRANSFORMATION BY AVIAN SARCOMA VIRUSES

As mentioned above, ASV is able to rapidly induce sarcomas in chickens and to transform CEF cells in tissue culture. These properties are shared with the DLVs and relate directly to the fact that these agents contain an onc gene within their genomic structure (v-onc). The integration of this gene, followed by its transcription and translation, is one of the important contributory factors that result in transformation and/or oncogenesis. In contrast, ALV is able to induce neoplasia in chickens, usually B-cell lymphomas, only after relatively long latent periods. This is because the ALVs lack their own onc genes, and must rely on activation of a cellular onc gene (c-onc) in order for tumorigenesis to occur. In most instances, this takes place as a consequence of the integration of the ALV genome at a site close to the cellular onc gene, known as c-myc. This latter gene can be shown to be activated in almost all ALV-induced chicken B-cell lympho-

mas, in the sense that vastly elevated levels of specific c-myc RNA can be detected in tumor tissue (7,8). The mechanism whereby the ALV genome serves to activate endogenous c-myc sequences has been termed "promotor insertion" (9). Furthermore, it appears that the entire ALV genome is not required in order for c-myc activation to take place. Rather, it apparently suffices for a proviral genomic segment, termed the large terminal repeat (LTR), to integrate somewhere near the c-myc site. The LTR is so termed because it is found at both ends of the proviral DNA genome (10). This is due to the fact that the viral RNA-dependent DNA polymerase copies the same portion of viral RNA twice, both at the initiation and termination of reverse transcription. During induction of chicken B-cell lymphomas, elements of viral genomic material may frequently be lost from infected cells; however, the LTR must remain present in proximity to the c-myc gene in order for oncogenesis to take place. During the period following infection the virus will have replicated in many cells as a consequence of having integrated at sites consistent with production of progeny virus, but not necessarily with activation of the c-myc gene. The large number of virus particles thus produced makes it likely that subsequent infections will result in the type of LTR integration which predisposes to oncogenesis (see also Chapter 4).

As pointed out above, ASVs carry their own transforming gene, termed v-src. Integration takes place at random and is followed by expression of the v-src gene, leading to rapid cellular transformation. The product of the src gene is a 60,000 dalton polypeptide ($pp60^{src}$) that can serve as a protein kinase, with the ability to phosphorylate certain cytoskeletal elements such as vinculin (11). Avian sarcomas are further distinguished from ALV-induced tumors in that they are usually polyclonal. Tumor cells which are induced by ASV are themselves producers of de novo virus in most instances. Thus, progeny virions will infect and transform other previously uninfected cells to become part of a growing tumor mass. Since proviral integration occurs at random, this means that every ASV-transformed cell has the potential to result from an independent integration and transformation event. In practice, of course, tumor cells which have already been established can replicate as well as produce progeny ASV. Thus, tumor enlargement results both from the mitosis of previously transformed cells as well as from virus spread. For this reason, avian sarcomas as well as other tumors of this type, such as the Moloney sarcoma of mice, have been termed "recruitment tumors" (12).

The fact that integration of proviral DNA occurs as a random event can be documented by showing an absence of specific integration bands in Southern blots of electrophoresed agarose gels. In this type of experiment, tumor cell DNA is

digested by restriction endonucleases into smaller fragments, electrophoresed onto agarose and transferred to nitrocellulose paper which is then hybridized with ^{32}P-labelled c-DNA probes that are specific for viral genes. In the case of DNA that is derived from avian sarcomas, no specific integration bands can be detected (3). However, when DNA from ALV-induced B-cell lymphomas is studied in this way, it can be shown that specific integration sites are indeed present (13). Furthermore, the same bands are present both in primary ALV-induced malignant tissue as well as in secondary tumors that have resulted from metastasis of primary tumor cells (13). A comparison of multiple B-cell lymphomas, that have arisen in different hosts, has revealed that the sites for viral integration may vary from animal to animal. However, within a single host, there is always a constancy of integration sites when one compares primary with distal tumors (13). Thus, these ALV-induced B-cell lymphomas are of monoclonal origin, in the sense that every tumor cell examined within a single host has resulted from a common transforming event. In contrast, ASV-induced sarcomas are very heterogeneous, with tumor cells arising both by a multitude of independent transforming events as well as by mitosis of previously transformed cells. In several strains of inbred mice, however, ASV was shown to establish clonal transplantable tumors. This has been accomplished by injecting newborn animals either with high doses of ASV or with avian sarcoma cells that are themselves producers of progeny virus. Transplantable tumors established in this way were demonstrated to be monoclonal, by the criteria explained above for mapping viral integration sites.

3. VIRAL-ASSOCIATED ANTIGENS

Analysis of ASV proteins was first performed by the group of Duesberg which showed by polyacrylamide gel electrophoresis that radiolabelled virus particles contained at least eight distinct proteins (14). Subsequent work revealed that two major glycoproteins (gp) were present and that these were located at the viral envelope (15,16). Since ASV is a virus which egresses from cells by budding at the cell membrane, it therefore seems logical that viral envelope antigenic structures might serve as important immunogenic entities, with regard to potential elicitation of anti-tumor immune responses. In fact, the major viral glycoproteins, termed gp85 and gp37, do indeed appear to possess this function, as will be discussed below. Under conditions of viral infection, it appears that many different viral proteins may also be expressed, albeit transiently in some cases, at the cell surface. Thus, these proteins may be able to serve as both immunogens

and as antigens that are able to interact at the cell surface, under certain conditions, with both anti-viral antibody and with sensitized lymphocytes.

In the case of ALV-induced B-cell lymphomas, it has already been mentioned that LTR sequences must be integrated in proximity to the cellular c-myc gene in order for oncogenesis to occur. This does not mean that integration of the complete viral genome did not also take place at that same site as an early event in the initiation of neoplastic transformation. The fact is, however, that much of the viral genome has been eliminated from that site by the time frank malignancy occurs and that one of the only features common to all such B-cell lymphomas is the presence of integrated LTR (9). This finding is of considerable importance in that it has led to the understanding of the clonal origin of this tumor. In addition, however, it is of considerable importance with regard to the outcome of the relationship between tumor and host. The absence of a complete viral genome in the case of B-cell lymphoma means that few if any virus-specified proteins can be made in such cells. This, in turn, results in the development and replication of tumor cells which express few, if any, viral antigens. As a consequence, chicken B-cell lymphomas are much less reactive against anti-viral antibody and sensitized lymphocytes than are avian sarcoma cells which elaborate virus-specified proteins very efficiently. The result of this lack of viral antigen expression on the part of the B-cell lymphoma is that this tumor is usually successful in escaping immune surveillance and that metastases are common, with death as a usual outcome of tumor growth. In contrast, avian sarcomas almost always regress in the case of immunocompetent hosts. This regression is mediated principally by both humoral and cell-mediated immune responsiveness against viral proteins that are expressed at the tumor cell surface (17). There is little evidence for the existence of non-virion, virus-induced antigens of any significance in the case of both avian sarcomas and ALV-induced lymphomas. Such antigens had been proposed to exist on the basis of early experiments by several investigators (18,19). Indeed, one group went so far as to characterize one such antigen which they called tumor-specific surface antigen (TSSA) (20). However, it now appears that if such antigens do exist that their role in anti-tumor immunity is of limited significance at best (see section 5.1).

4. ASV-INDUCED PATHOGENESIS AND IMMUNOSUPPRESSION

ASV classically induces a fibrosarcoma, regardless of the site of the body at which the inoculum is administered (21,22). This is largely because of the

recruitment nature of the tumor. In spite of the fact that the initial target cell for viral replication may vary according to the site of inoculation, the release by such cells of progeny virus commonly leads to the involvement and transformation of connective tissue cells shortly thereafter. These latter cells become the predominant tumor cell type in most instances. Large ASV-induced neoplasms frequently become necrotic. This can create difficulties with regard to attempts to grow tumor cells in tissue culture. Distal ASV-induced tumors may frequently appear on the basis of either virus spread or by actual metastasis of previously transformed cells or clumps of cells. It is difficult or impossible to distinguish between these possibilities because the tumor cells are themselves producers of progeny virus in each case. Necrosis in avian sarcomas results from a rapid expansion of the neoplasm and a corresponding inability of the vasculature to furnish blood supply to tumor tissue. In the case of wing web tumors, this frequently results in bleeding and bacterial infection. When several animals are maintained together in the same cage, cannibalism may also occur.

Avian sarcomas are also frequently heavily infiltrated by lymphocytes (23). These are mostly T-cells. In the case of chickens that have been neonatally thymectomized prior to viral inoculation, few if any lymphocytes can be detected in tumor tissue. These lymphocytes are thought to play an important role in the anti-tumor immune response that generally develops in ASV-injected animals and that often succeeds in mediating complete tumor regression. ALV-induced B-cell lymphomas, by contrast, rarely display such excessive T-cell infiltration.

Both ASV-induced sarcomas and ALV-induced B-cells lymphomas may result, in part, from the fact that the virus particles involved in each case can be immunosuppressive. The induction of B-cell lymphomas commonly involves a viremic phase, during which time ALV can be isolated from the serum of infected hosts. This period seems to correspond with a time at which responsiveness on the part of chicken lymphocytes to both T- and B-cell mitogens is depressed (24). This effect is transient and disappears once viral dissemination has taken place. However, immunosuppression can commonly recur in animals suffering from established ALV-induced malignancies. Both ALV and ASV can also act to suppress lymphocyte responsiveness to antigenic and mitogenic stimuli (25). This is a feature that these viruses share with many other types of viral agents and has been described in the literature for each of avian (26), feline (27), murine (28), and human retroviruses (29). Indeed, retroviruses seem to be more efficient than other viruses in being able to mediate such lymphocyte inhibitory effects. The scenario through which such abrogation of responsiveness occurs seem to be through the

inhibition of production by T-helper cells of T-cell growth factor (TCGF), also referred to as interleukin-2 (IL-2) (30, 31). This factor serves as a second signal for all types of T-cell proliferation, following initial activation by antigens or mitogens. In the presence of virus particles, it can be shown that production of TCGF is reduced to 20% or less (30). This effect was obtained in several instances through the use of ultraviolet light (UV)-inactivated viruses, indicating that active infection of the cells concerned is not always necessary to attain the inhibitory effect. Rather, these viruses may be structured in such a way as to play an infection-independent role in immune regulation. The addition of exogenous TCGF to cultures of lymphocytes that had been co-incubated with both viruses and mitogens restored responsiveness to near-control levels (32). This indicates both the ability of these viruses to interfere with TCGF biogenesis as well as the fact that receptors for TCGF apparently continue to be expressed in the presence of the viruses concerned. In some cases, certain of these viruses may even be able to complex with TCGF and to inactivate it directly (33).

It is not coincidental that immunosuppression has been reported to occur in the case of pre-leukemic chickens, mice and cats that have been infected by any of the retroviruses that are specific for these different species (34). The immunosuppression doubtless results in each case from a series of complex interactions between the viruses themselves and T- and B-lymphocytes as well as cells of the reticulo-endothelial system.

5. DEVELOPMENT OF ANTI-TUMOR IMMUNITY IN TUMOR-BEARING HOSTS

5.1. Humoral immunity

It was recognized during the 1940s and 1950s that chickens that had been injected with RSV were able to develop immunity of a sort against both the virus and against the tumor which it induced (21,35). The outcome of the host-tumor relationship was found to result from a combination of factors including strain of virus used, dose of the viral inoculum, and strain and age of the host (36). The use of young animals and/or high doses of virus was more likely to lead to irreversible tumor growth than the use of older fully immunocompetent chickens. In addition, early workers in this field observed that lymphocytic infiltration of fibrosarcoma tissue tended to be a good prognostic indicator of ultimate tumor regression (37).

In several studies, it was shown that the immunization of chickens with either ALV or with sub-tumorigenic doses of ASV could protect against the subsequent development of sarcoma caused by tumorigenic doses of the same virus subgroup

(38). Furthermore, the sera of birds that became tumor-resistant contained high levels of virus neutralizing antibodies (39). Further analysis revealed that immunization with ASV of several subgroups could also induce resistance against tumors caused by ASV of other subgroups, although to a lesser extent than that induced by the homologous subgroup (40). Tumor immunity was always correlated with the presence of high levels of virus neutralizing antibody, but was not achieved either by injection of killed RSV or by passively immunizing recipient chickens with virus-neutralizing antibody (41). These findings suggested that resistance to tumor growth might be mediated by cellular rather than humoral immunity.

In other studies, it was shown by immuno-electron microscopy that antisera of chickens immunized with ASV of any subgroup could label both cell surfaces and viral envelopes in target cultures transformed by virus of the same subgroup (42). The use of target cells transformed by viruses of other subgroups resulted in labelling of the cell surface only. On this basis, it was postulated that a non-virion structure called tumor-specific surface antigen (TSSA) might exist, and that this antigen might play an important role in anti-tumor immunity. Subsequent work revealed, on the basis of immunoprecipitation by antisera from chickens that displayed resistance to virus-induced tumorigenesis, that this TSSA was a protein of 100,000 molecular weight, and was distinct from the major glycoproteins, gp37 and gp85. Furthermore, this so-called TSSA could not be detected in cells that had been infected but not transformed by ALV. In addition, TSSA was not subgroup-specific and could be precipitated from cells that had been transformed by a variety of viral subgroups (20). Experiments by other workers, however, failed to confirm the existence of any virus-induced non-virion antigen of significance in this model (43). No independent confirmation of the existence of TSSA has been obtained to this day. Indeed, the consensus of opinion is that the only antibodies of significance that are induced in virus-injected hosts are those that are specific for the various viral proteins (44). Such antibodies can be detected in reasonably high titer in almost all animals that have been injected with ASV. These antibodies, if present following immunization with virus, may be able to play a protective role by neutralizing ASV that is subsequently injected into wing webs or elsewhere. It seems certain, however, that they are relatively unimportant with regard to the mediation of tumor resistance in the great majority of cases. Furthermore, they are of no apparent significance with regard to the ultimate regression of tumors that have been induced by ASV. The best evidence for this statement is the fact that three separate studies have shown that neither surgical nor hormonal bursectomy have any apparent effect on the growth of ASV-induced tumors in

chickens (45,46,47). This was true in terms of duration of the latency period until tumors became palpable, tumor growth rate, tumor incidence, and incidence of tumor formation at sites distal to that of viral inoculation. On this basis, it was concluded that anti-viral antibodies do not play any significant protective role, nor do blocking antibodies play an important function with regard to the survival and growth of tumors that continue to grow progressively. Similar findings have been reported in the case of neonatally bursectomized quails (48).

5.2. Cell-mediated immunity

Neonatal thymectomy, on the other hand, resulted in dramatic impairment of the ability of both chickens and quails to withstand ASV-induced tumor growth (47,48,49). This led to the conclusion that cell-mediated immune responsiveness is functionally responsible for tumor resistance and regression in those cases in which tumors fail to enlarge. In one study, it was shown that chickens that had been thymectomized during the first 3 days of life prior to injection of ASV, developed tumors twice as often as sham-operated controls (49). Furthermore, by 30 days after viral inoculation 86% of thymectomized birds had died in comparison with 23% of controls. Distal tumor formation was also much more common in the thymectomized group. In addition to the foregoing, a number of laboratories have conducted in vitro studies of cell-mediated immunity. In one group of experiments, Kurth and Bauer (18) employed a cytotoxic effector cell microassay, in which spleen cells from immunized chickens were assessed against ASV-transformed CEF targets. They showed that spleen cells sensitized to virus of one subgroup were not cytotoxic against uninfected target cells or against target cells infected by a non-transforming virus of another subgroup. They were cytotoxic, however, for target cells infected by any member of the immunizing subgroup and for targets that had been transformed by a member of any subgroup (50). These findings supported the notion that reactivity must have been directed, in part at least, against a non-virion, virus-induced antigen, which they hypothesized to be the same TSSA described above.

Most workers would argue, however, that functional immunity in this system is exclusively directed against viral glycoprotein. First, it had been shown by Rubin and colleagues (23,37) that immunization with infectious ALV rendered chickens resistant to tumor development upon challenge with ASV. More recently, other workers have shown that specific cytotoxicity reactions involving sensitized chicken lymphocytes and tumor cell targets can be blocked by including purified UV-inactivated virus particles in the reaction mixture (51,52).

Among assays employed to assess cellular immune status in ASV-injected hosts is a lymphocyte blastogenesis procedure which is probably the most reproducible and convenient of all the tests available for this purpose. In an early study, it was shown that peripheral blood lymphocytes from ASV-immunized chickens could be stimulated to incorporate tritiated thymidine in the presence of culture fluids from ASV-infected cells. This reaction was found to be most efficient when sensitized lymphocytes were derived from animals that had been inoculated with the same subgroup of virus employed as antigen (53). However, cross-reactions were also reported to varying degrees. Subsequent analysis showed that the major antigenic determinants responsible for elicitation of this immune response were the internal viral proteins p15 and p27 (54). The usefulness of this test was demonstrated by showing that cell-mediated immunity, as measured by this technique, generally appeared within 2 weeks following inoculation of virus. In the case of tumors which ultimately regressed, animals rapidly lost the ability to continue to mount such immune reactivity (55). Cytotoxicity tests have also been used effectively in this regard. Animals that have been injected with tumorigenic doses of ASV usually mount effective anti-tumor responsiveness within 10 to 14 days and continue to display such immunity for up to 2 months following tumor rejection (51,52). Cytotoxicity is considerably more efficient when cultured tumor cells rather than transformed embryonic cells are used as targets (56). Animals that have been neonatally thymectomized, and whose tumors grow progressively to kill their hosts, are unable to express cell-mediated anti-tumor immune reactions by any of the methods employed (47). Cell-mediated cytotoxicity reactions in the ASV model are apparently governed by classical histocompatibility considerations (57,58).

Further evidence that viral envelope glycoproteins play a crucial role as antigens in the tumor rejection process has come from indirect means. First, early studies by Rubin and his colleagues had shown that those tumor cells which could be cultured from regressing avian sarcomas were poor producers of progeny virus (23,37). Subsequent analysis by Wainberg's group revealed that the tumor cells in question could, in fact, produce some virus but that the particles concerned were often gp-defective, a fact which accounts for their lack of infectivity and transforming ability (59). In fact, it turns out that such producers of defective virus particles are present from the earliest stages of tumor growth, and are apparently selected out by a cell-mediated immune response that is most effective against tumor cells that are efficient producers of progeny virus and which express high viral gp levels at their surface (60). Such cells are difficult to

detect at early stages of tumor growth because they are present as a very small minority. However, they become predominant during regression, when they remain present simply because they do not express viral gp to a significant extent. Tumor cells derived from the regression phase are largely resistant to cytotoxic effector cells and are poor producers of antigen that are reactive with sensitized lymphocytes in blastogenesis assays. Finally, such tumor cell variants can be shown to be present at the earliest stages of tumor growth by clonal outgrowth assays in which cells from progressively-growing tumors are plated in tissue culture at limiting dilution. A percentage of the outgrowths can be shown to possess the "regressor" phenotype. These experiments provide further evidence for the very heterogeneous nature of avian sarcomas, a finding which was predictable on the basis of the recruitment-like nature of the tumor.

One important question which then arises is why such regressor tumor cells, which express viral envelope antigens very inefficiently, do not grow out to kill their hosts? Theoretically, such cells should possess an important growth advantage in vivo, by virtue of their ability to escape immune surveillance. The answer is, in part, that these "regressor" sarcoma cells, while resistant to specific cell-mediated cytotoxicity, are sensitive to the lytic activity of natural killer (NK) cells (52). In fact, they are more susceptible to NK activity than are cells derived from progressively-growing tumors. The addition of autologous virus to these various reaction mixtures is inhibitory to specific cell-mediated cytotoxicity but not to NK-mediated lysis. Finally, chickens which bear tumors induced by ASV usually retain high levels of NK activity throughout the course of tumor growth and regression (52).

6. THE IMMUNOGENICITY OF RETROVIRAL ENVELOPE GLYCOPROTEIN

As discussed above, a considerable body of evidence now indicates that the viral envelope glycoprotein (gp) is a major antigen on cells transformed by ASV. For this reason, factors influencing the immunogenicity of gp should serve as important determinants of tumor immunity in ASV-infected chickens. As for any protein antigen, these factors would include: (a) the major histocompatibility complex (MHC) haplotype of the host because T-cells recognize antigen in association with MHC-encoded class I or class II restriction elements and (b) the degree of non-selfness of the antigen because, under appropriate conditions, self antigens are tolerogenic. A direct analysis of the influence of the MHC on T-cell recognition of avian retroviral gp has to date not been carried out. Nevertheless,

a number of studies have documented a strong influence of the MHC in determining patterns of regression or progression of ASV-induced primary wing web sarcomas (61,62,63) (the subject of the MHC influence on sarcoma growth has recently been reviewed (64)). Under equivalent conditions of sarcoma virus infection, certain inbred chicken lines, which differ only at the B locus, exhibited uniform patterns of either regression or progression, depending on the B locus haplotype (63). Although unproven, it is reasonable to infer that the MHC influence on the growth of primary ASV-induced sarcomas is at least in part mediated via MHC-determined differences in gp-immunogenicity.

With respect to the degree of non-selfness of viral antigen, the oncogenic ASVs exhibit marked antigenic homology with "normal" chicken cell components, the endogenous avian leukemia viruses (ALVs). The genomes of endogenous ALV (designated ev loci) are ubiquitous in the germ line DNA of domestic Leghorn chickens (although a small ev-negative breeding flock has recently been derived, see Chapter 5). As analyzed (65,66) with chick embryo cells in culture, a subset of ev loci specifies the synthesis of complete, replication-competent virus particles (i.e. ev2 which encodes the prototypic endogenous virus RAV-0), whereas other loci are either silent (ev1) or else are defective in the sense that they encode the expression of one or more viral proteins in the absence of complete virion formation (i.e. ev3, ev6, or ev9, each of which determines the chicken helper factor (chf) phenotype defined by gp-expression in fibroblasts; chf was originally defined by its capacity to complement the defective Bryan high titer strain of RSV (38,67)). Although marked differences exist between individual chicken strains, certain inbred lines are homogeneous in terms of their ev loci. These inbred lines have proven to be quite useful because they enable mapping of biological effects of endogenous virus expression to individual loci.

All envelope glycoprotein species encoded by ev loci exhibit subgroup E specificity (i.e. the glycoproteins possess subgroup E-specific antigenic determinants, as recognized by virus neutralizing antibody). As the gp species of the exogenous avian retroviruses are subgroups A-D specific, subgroup E specificity represents a general marker for endogenous ALV genetic information. In addition, all gp species of subgroups A-E possess common antigenic determinants (these do not mediate virus neutralization and were originally detected by antibody recognition of solubilized gp present in lysates of chf^+ chicken cells or in preparations of disrupted virions (17,67,68)). In terms of self-nonself discrimination, it are the subgroup-common gp-determinants of the exogenous ALVs that represent self in chf^+ chickens. Given that expression of several ev genes the

possibility arises that one consequence of expression is the establishment of a state of immunological tolerance to endogenous ALV-gp, including the antigenic determinants shared with exogenous ALV-gp. In the remainder of this section, we will discuss evidence which is suggestive, however not conclusive, that such tolerance exists and has ramifications for tumor immunity and immune response to virus infection.

6.1. Endogenous viral glycoprotein as tolerogen

The phenomenon of tolerance is of course an exceedingly complex one, with two vastly different mechanisms, functional deletion or active suppression, implicated in diverse tolerance states and with different rules of induction operative for different antigens. Although the demonstration of autoreactive B-cells served to weaken an early framework of tolerance induction in which clonal deletion was postulated to be the invariable consequence of antigen encounter by immature lymphocytes (69,70), in modified form this concept retains validity. Nossal and his colleagues (71) have shown that during the pre-B to B-cell transition, the interaction of even very low concentrations of antigen with membrane-bound immunoglobulin induces a state of clonal anergy, i.e. functional deletion without cell killing. B-cells that encounter antigen at later stages of maturation than the pre-B stage are also susceptible to the negative signaling required for induction of the anergic stage, although increasing antigen concentrations are required with increasing B-cell maturity.

The above observations on subgroup-common gp-determinants are relevant to a consideration of the possibility of tolerance to endogenous viral gp. Recent work has shown that those ev loci, 3, 6, or 9, which determine the chf phenotype in fibroblasts, also encode the expression of endogenous gp in lymphoid cells (72). To date, the most detailed characterization of ev expression in lymphoid cells has been carried out for the ev6 locus, which is present in the inbred $15I_5$ line.

Biochemical methods, involving immune precipitation with anti-envelope sera and lysates of (^{3}H)glucosamine-labelled cells, have established that the ev6-encoded gp is constitutively synthesized by both bursal cells and thymocytes (73); in the case of bursal cells, direct evidence for the surface expression of the ev6-encoded gp was derived from the labelling of this component under conditions of lactoperoxidase-catalyzed iodination of viable cells. Mitogen activation of resting peripheral blood lymphocytes served to increase the surface gp-expression on both T- and B-cells to levels detectable by immunofluorescence, as scored by cytofluorimetry or (for late maturational stages) by direct microscopic examination.

The maximal level of surface expression is detected with plasma cells (74).

As analyzed by immunofluorescence microscopy with both fixed tissue sections and viable cell suspensions, all plasma cells in spleen and Harderian gland were found to be positive for ev6 expression (73) (the Harderian gland is a lympho-epithelial organ in the orbit of the eye whose lymphoid element comprises only plasma cells and these are of the IgG-, IgM- and IgA-producing classes). By contrast, since ev6 expression in bursal cells or resting B-cells is below the limits of detection of standard immunofluorescence methods, direct proof is lacking that all such B-cells express the ev6-encoded gp (i.e. the biochemical methods used to demonstrate gp-biosynthesis by mass cultures of bursal cells do not distinguish between high levels of expression by a subpopulation of cells or a lower level of expression by the total cell pool). Nevertheless, the observation that the level of ev6 expression in plasma cells represents an amplification of levels found at earlier B-cell maturational stages (73), coupled with the observation that all plasma cells are positive, provides strong support for the assumption that ev6 expression is a property of the entire B-cell pool. The same conclusion appears valid for the T-cell pool, since most, if not all, of the T-cell blasts in mitogen-activated lymphoid cell cultures are positive for ev6 expression as assayed by cytofluorimetry (74).

The same methods of analysis applied to $ev3^+$ or $ev9^+$ chickens indicated an analogous pattern of endogenous viral gp-expression in the sense that the levels of expression in lymphoblasts exceeded the levels in resting or immature lymphocytes (72); however, plasma cells from only the $ev6^+$ chickens express higher levels of endogenous viral gp than do the lymphoblasts, an effect that may reflect the absence of a 5' LTR on ev6. The observation that the great majority of T- and B-blasts in mitogen-activated cultures prepared from spleen or peripheral blood of $ev3^+$ or $ev9^+$ chickens were positive for endogenous gp-expression by cytofluorimetric analysis again argues that expression is a property of most, if not all, lymphoid cells (72).

On the basis of the observation referred to above, that immature lymphocytes are functionally tolerized by prolonged contact with antigen (71), the endogenous gp-expression on the surface of lymphocytes at all maturational stages provides a conceptual framework for postulating a state of tolerance to this glycoprotein. Were endogenous viral gp expressed by only non-lymphoid cells (i.e. chf production by fibroblasts) or were expression clearly confined to only a subpopulation of lymphocytes, tolerance would be more difficult to rationalize, as considerable precedence exists for auto-immune responses to non-lymphoid-

associated self antigens (the anti-thyroglobulin response in thyroiditis) or to self antigens expressed by only a subset of lymphocytes (the anti-IgG response in rheumatoid arthritis). Even if chf expression by fibroblasts did suffice to tolerize T-cells, which require a lower antigen dose than do B-cells for tolerance induction, the surface expression of endogenous viral gp by the total pool of B-cells would seemingly ensure B-cell tolerance as well.

To date, the few experimental analyses bearing directly on the question of tolerance to endogenous viral glycoprotein have involved assays for serum antibody reactivity to gp-specific antigenic determinants. A general finding has been that ev6$^+$ or ev3$^+$ chickens, whether uninfected or infected with ASV, do not exhibit detectable reactivity for the subgroup-common gp-determinants under assay conditions in which such reactivity is readily scored with infected chf$^-$ chickens (75,76). In certain states of self tolerance, in which autoreactive T-cells but not B-cells are functionally deleted, the introduction of a cross-reactive antigen triggers autoantibody production to the shared determinants. Since ASV infection of chf$^+$ chickens serves to introduce an exogenous gp-species cross-reactive with the endogenous gp, the absence of detectable subgroup-common gp-reactivity in sera of infected chf$^+$ chickens is consistent with the premise of B-cell tolerance to the endogenous gp. Nevertheless, the results do not rigorously establish B-cell tolerance, as the absence of reactivity may simply reflect the complexing of antibody to serum- or tissue-associated endogenous viral glycoprotein.

More compelling evidence for tolerance derives from the observed differences in neutralizing antibody titers inducible to exogenous retrovirus in chf$^-$ versus chf$^+$ chickens. As tested with avian leukosis/sarcoma viruses of subgroups A, B, or C, neutralizing antibody titers in sera of chf$^-$ chickens are 2 to 3 log units higher than those in chf$^+$ chickens (77; unpublished). The differences in neutralizing titers are not explicable in terms of antibody complexing to endogenous viral gp, but are explicable in terms of B-cell tolerance to the shared antigenic gp-determinants: any conformational overlap between subgroup-common and group-specific determinants (i.e. sharing or close aposition of contact residues) would seemingly restrict in chf$^+$ chickens the repertoire of subgroup-specific antibody responses available in chf$^-$ chickens. An additional possibility to explain the low neutralizing titers inducible in chf$^+$ chickens follows from the assumption of T-cell tolerance in these chickens: a reduced antibody response to the subgroup-specific (hapten-like) determinants on exogenous ALV-gp would reflect the absence of T-cell priming to the subgroup-common (carrier-like) determinants.

6.2. Expresssion of endogenous viral glycoprotein as predisposing to ASV-induced sarcoma growth.

Implicit in the premise that tolerance to endogenous glycoprotein impairs the expression of immunity to ASV-induced sarcoma is the assumption that non-responsiveness to subgroup-common gp-determinants would favor sarcoma growth. Evidence supportive of this assumption derives from earlier studies in which the growth of sarcomas induced by infection with ASV of one subgroup was compared between normal chf$^-$ chickens and hatch-mates that had been congenitally infected with ALV of a different subgroup (78,79). Whereas sarcoma regression was the general rule for the ASV-infected normal chickens, sarcoma progression was the rule for the congenitally ALV-infected chickens. As congenital ALV infection has been interpreted as yielding a state of immunological tolerance to the ALV structural antigens (80,81), the use of a different virus subgroup for the ASV challenge emphasizes the central role of the immunogenicity of subgroup-common gp-determinants in resistance to sarcoma growth.

With respect to purely host genetic elements that may influence the expression of immunity to ASV-induced sarcoma, comparatively little work has been done on the possible influence of the ev loci. One study (82) that has addressed this question was carried out on the premise that the ev6 locus would influence the pattern of sarcoma growth by virtue of the tolerogenicity of the ev6-encoded viral gp, as discussed above. To examine this possibility, sarcoma growth was compared in $15I_5 \times 7_2$ chickens infected with either of two ASV strains, which although otherwise closely related, differed in the antigenicity of their envelope glycoproteins (82). The env gene of one virus encoded gp of subgroup B, which possesses subgroup-common ALV-gp-determinants, whereas the env gene of the other encoded a subgroup G viral gp, which lacks these subgroup-common determinants (subgroup G is defined by the gp of the golden pheasant endogenous virus, the env gene of which has no nucleic acid homology with the env genes of avian leukosis/sarcoma viruses, comprising subgroups A-E). As expected on the basis of tolerance to the gp-determinants, the sera of the subgroup G virus-infected chickens exhibited much higher levels of virus neutralizing antibody than the sera of the subgroup B virus-infected chickens (82). Both groups of infected chickens developed sarcomas at the virus inoculation site in the wing web, however, whereas a majority of the subgroup B virus-infected chickens developed sarcomas at sites distal to the wing web, only a small minority of subgroup G virus-infected chickens developed distal sarcomas. Insofar as distal sarcoma formation reflects an increased potential for sarcoma growth, the results with

$15I_5 \times 7_2$ chickens were interpretable on the basis that tolerance to subgroup-common determinants constitutes an immunological deficit that favors sarcoma growth. As the relative importance of ev-determined versus MHC-determined effects on sarcoma growth has yet to be even addressed, the generality of the concept that tolerance to endogenous viral gp enhances the potential for ASV-induced sarcoma growth remains problematic. Nevertheless the usefulness of the avian system for evaluating this concept relates to the availability of inbred chicken lines positive or negative for particular ev loci and the existence of ASV strains positive or negative for gp-species cross-reactive with endogenous envelope glycoprotein.

7. CHARACTERISTICS OF TUMOR CELL GROWTH IN VIVO AND IN VITRO

Immunostimulants such as BCG have often been reported to be able to mediate the regression of certain types of experimental tumors and to retard the growth of others (83). It was therefore astonishing to discover that injection of BCG into chicken wing webs, at various times prior to inoculation of virus at the same site, had a dramatically stimulatory effect on tumor growth (84). In fact, continuous tumor growth, followed by death of the host, was the rule rather than the exception under these circumstances. Subsequent analysis revealed that the reason for this finding is that BCG induces a massive macrophage and granulocyte infiltration at the site at which it is injected. These macrophages can then serve as effective targets for ASV infection and are able to replicate progeny virus very efficiently and to mediate its spread to surrounding tissue. In fact, tumors which are established by this BCG-enhancement procedure can be shown to contain a much higher percentage of macrophage-like cells than do neoplasms induced by ASV alone. The infected macrophages produce progeny virus in culture and express viral antigens at their surface. Inoculation of such cells into chickens leads to tumor development, indicating that they can produce ASV both in vivo and in vitro. They are present most predominantly during the earliest stages of tumor growth and can survive in tissue culture over long periods. The injection of BCG into other sites, besides that used for viral inoculation, had no effect on tumor growth but was stimulatory to the development of anti-tumor immunity. These findings show that macrophages can constitute an important reservoir for viral replication and spread during the earliest stages of tumor growth (85).

As indicated above, avian sarcomas are heterogeneous with respect to viral integration site, virus production and expression of virus-specific antigens.

Differences are especially pronounced when one compares tumor cells derived from the progressively growing as compared to "regressor" phase of the tumor. When cells from these various stages are established in tissue culture, one finds that those which derive from progressively growing sarcomas are rapidly growing, produce large quantities of the enzyme plasminogen activator, and are very efficient producers of progeny virus. In contrast, tumor cells that are obtained from regressors grow much slower and have doubling times which are approximately twice as long as cells of the progressor phenotype (48 hr vs 24 hr). The regressor cells also have elevated levels of hexose transport activity and are greatly enlarged, properties which are characteristic of a senescent cell population (86). These data, of course, provide further clues as to the basis of the inability of tumor cells from regressing neoplasms to continue to grow efficiently in vivo. Of further interest is the fact that levels of $pp60^{src}$ kinase activity are reduced by approximately 75% in regressing as opposed to progressively growing tumor cells. This difference is not reflected at the level of gene expression. Both types of tumor cells produce similar levels of the $pp60^{src}$ gene product on a per mg protein basis. The half-life of both this product and its associated kinase activity, are, however, greatly reduced in the case of tumor cells which are derived from regressing neoplasms (87). These data thus underlie the fact that tumor cells of the "regressor" phenotype differ from their progressively growing counterparts, as measured by each of immunological, molecular biological and biochemical criteria.

This field has moved a long way since the earliest studies on viral inocula and host immune responsiveness were completed (88,89). It is clear that the study of avian retrovirus-induced neoplasia has led to important developments in the understanding not only of the tumors described here but of oncogenic viruses, onc genes and neoplasia in general. Those of us who continue to study these models believe that they will continue to provide important new information and insights in the years to come.

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8

VIRUS-CELL INTERACTIONS OF AVIAN SARCOMA AND DEFECTIVE LEUKEMIA VIRUSES

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1. INTRODUCTION

Avian retroviruses are able to induce malignant diseases, such as sarcoma, carcinoma and leukemia as well as non-malignant diseases, e.g. anemia or auto-immune diseases. In the field of cancerology, avian retroviruses have contributed greatly to elucidate some of the oncogenic processes. One main breakthrough was reached when it was shown that retroviruses induce malignant diseases through the action of cell-derived nucleotide sequences (referred to as oncogenes) either transduced in the genomic RNA, or activated by viral promoters (1,2).

Most of the recent findings have been obtained thanks to the combined efforts of cellular and molecular biologists. The latter have provided new viral agents by engineering site-specific mutations resulting in a total or partial deletion of the viral oncogenes, by constructing molecular recombinants between avian and mammalian retroviruses, and by cloning some oncogenes into bacterial vectors. The cellular biologists have devised new efficient in vitro assays by adding to cell cultures, derived from various embryonic and adult tissues, specific growth factors essential for the proliferation and differentiation of these cells. Such technical advances have revealed new cell-virus interactions that are valuable in understanding the involvement of these oncogenes in the transformation process. Furthermore, it has become possible to separate the malignant process into three different components: (a) immortalisation, (b) tumorigenicity, and (c) interference with the differentiation program of the target cells.

In the last five years, several comprehensive reviews have discussed the relationship between the retroviral oncogenes and the neoplastic process (3,4,5,6, 7). Therefore, the scope of this review is purposely limited to the most recent and significant findings which shed light on the mechanisms involved in the pathogenesis of avian retroviruses. To avoid a lengthy catalogue, we are going to focus on the interactions of avian sarcoma and acute defective leukemia viruses with target cells for infection and transformation, and on the subsequent alterations induced in the phenotypic characters of these cells.

2. AVIAN SARCOMA VIRUSES

All isolates share the characteristic of having transduced a chicken cellular gene which encodes a protein with kinase activity that phosphorylates tyrosine residues. Numerous genetic and biochemical studies suggest that the kinase activity associated with the transforming protein is central to the molecular processes whereby these viral gene products induce cellular transformation. These transforming proteins have a cytoplasmic distribution with a predominant localization in the plasma membrane and the cytoskeleton. It is now postulated that these proteins phosphorylate a number of substrates within the cell, some of which are proteins regulating the process of cell growth, metabolism and architecture (1). The phenotypic changes resulting from the oncogenic expression have been analysed by using virus-transformed chicken fibroblasts. Among these changes, morphological modifications, cytoskeletal alterations, increase in hexose transport, anchorage-independent growth, synthesis of proteases and loss of

extracellular fibronectin have been instrumental in comparing the oncogenic and transforming potentials of these sarcoma viruses (8,9).

The more extensively studied virus is Rous sarcoma virus (RSV) comprising several non-defective and defective strains which all contain the src gene (10). The other avian sarcoma viruses (ASVs) do not carry the src gene, and are all defective: Fujinami sarcoma virus (FuSV), PRCII, and UR1 share the same oncogene, fps; Esh sarcoma virus and Y73 also share a common oncogene yes, while UR2, the most recently isolated ASV, contains the ros oncogene. The genome of these defective sarcoma viruses encodes unique transforming proteins, precipitable by antisera against viral structural proteins encoded by the gag gene. These proteins are therefore chimeric proteins coded in part by this replicative gene and in part by the oncogene (11,12,13,14,15).

3. PATHOGENIC EFFECTS OF AVIAN SARCOMA VIRUSES IN VIVO

The inoculation of these viruses in the wing web of newborn or adult chickens results in the rapid formation of a sarcoma. Upon viral inoculation, tumor formation appears at the site of injection as well as at other distant sites. It has been reported that direct injection of cloned subgenomic src-DNA fragments into young chickens induces sarcomas within 3-4 weeks after inoculation, thus providing evidence that the src gene alone is responsible for tumor induction without the collaboration of other viral genes (16). Differences in env gene expression may determine distal sarcoma formation. Thus, chickens injected with a subgroup B virus exhibited fibrosarcomas at sites distal to the primary wing web sarcomas, whereas only a small percentage of chickens injected by a subgroup G virus exhibited such a phenomenon. This difference is explained by a significantly higher titer of virus neutralizing antibody detected in the subgroup G virus-infected chickens, whereas the subgroup B-infected chickens exhibited lower levels of neutralizing antibody because of the tolerizing effect of endogenous viral gene expression (17, see also Chapter 7).

The fact that two viruses share the same specific oncogene does not imply that they have the same pattern of tumorigenicity. Thus, FuSV and PRCII both encode gag-fps transforming proteins. However, PRCII has a lower oncogenic potential than FuSV in vivo and induces only a partially transforming phenotype in cells infected in vitro (18,19). This difference may be the consequence of a large internal deletion within the fps gene of PRCII in its 5' half. This deletion does not decrease the tyrosine kinase activity of the transforming protein, but seems to

alter its cellular localization. In fact, this protein was found to be present in cytoplasmic patches and is not associated with the plasma membrane of the transformed cells, as is the case with the FuSV transforming protein. Therefore, in cells infected with PRCII, some proteins involved in oncogenic transformation might not have been phosphorylated by the PRCII transforming protein. Such defects may be correlated with the lower oncogenic potential of PRCII. Furthermore, the in vivo tumorigenicity of size-variant viruses encoding proteins with alterations in the amino-terminal membrane proteins is greatly reduced. Chicken embryo fibroblasts transformed by these viruses display a partial transformation phenotype, because of the restricted interaction of the transforming proteins with specific areas of the plasma membrane (20).

Early and recent observations concerning the pathogenicity of RSV in chick embryos in ovo have pointed out that transformation and tumorigenicity are not a direct consequence of kinase activity expression (21,22). For instance, RSV is non-tumorigenic and non-teratogenic when inoculated into 4-day chicken embryos, in spite of its ability to replicate in the limb buds and to synthesize the src-specific protein kinase. However, cells from the infected buds are capable of expressing the transforming phenotype a few hours after being seeded in culture. It seems, therefore, that in vivo developmental cellular factors closely control the appearance of the malignant phenotype. Experiments involving embryos at different stages of development, different viruses and different routes of infection should provide new and challenging informations on the regulatory mechanisms exerted by the embryonic host cells on the transforming program encoded by the oncogene proteins.

4. TRANSFORMING EFFECTS OF AVIAN SARCOMA VIRUSES IN VITRO

The first in vitro studies have been restricted to the transformation of chick embryo fibroblasts. During the last 10 years, a better knowledge of the transforming effects of avian retroviruses has been acquired after studying the response of different cellular types to the viral infection.

4.1. Avian sarcoma viruses in chicken embryo neuroretinal cells

Investigations on the response of neuroretinal cells to RSV infection has allowed the distinction between proliferation and transformation in the tumorigenic process. Chicken embryo neuroretinal cells, maintained in vitro, are non-proliferating cells. Infection of these cells with RSV results in morphological

transformation and sustained proliferation (22). The effect on the growth properties of neuroretinal cells characterizes the mitogenic property of the virus. Two mutants of RSV, PA101 and PA102, have lost the ability of causing morphological transformation, but have retained the ability of inducing proliferation (23). The mitogenic property of these mutants is temperature-sensitive and they both encode $pp60^{src}$ protein with a low kinase activity. Recent experiments using these mutants suggest that the expression of the mitogenic function may not be sufficient for cell tumorigenicity (24).

4.2. Avian sarcoma viruses in differentiated cells

The concept of self-proliferation or self-renewal induced by the src gene has been further supported by the interactions of the src-containing viruses with cells that are close to the terminal stages of differentiation such as myotubes (25), chondroblasts (26), pigmented retina epithelium (27) and neural retina (28). In all these cases it appeared that src gene expression prevents the synthesis of the specific differentiation products while inducing a continuous cell proliferation. However, once cells transformed by temperature-sensitive mutants are incubated at the non-permissive temperature, they resume their differentiation program and lose the capacity to proliferate. In one case, it was found that src gene expression did not impair differentiating functions (29). Thus, infection of chicken skin epidermal cells with RSV leads to the proliferation of these cells which retain certain differentiation markers. In fact, these cells have a typical epithelial cell type morphology and retain the capacity to produce major species of keratins (30).

4.3. Avian sarcoma viruses in hemopoietic cells

An increase in self-renewal of hemopoietic stem cells and committed progenitor cells has been observed after infection of mouse long-term marrow cultures with a molecular recombinant of an amphitropic leukemia virus and RSV (31). The expression of the $pp60^{src}$ kinase activity resulted in an altered balance of the relative numbers of stem cells, granulocyte-macrophage progenitors (which both increase), and mature granulocytes (which decrease). In this case too, the src gene induced the self-renewal capacity of these cells at the expense of cell differentiation. However, src expression did not result in a neoplastic transformation of these infected cells. In fact, injection of these cells into lethally irradiated recipients did not lead to the development of leukemia, but instead resulted in a complete hematopoietic regeneration (32). It is noteworthy that these cells were able to proliferate in vitro under culture conditions adverse to the proliferation of

normal stem cells. Conversely, the same molecular recombinant retrovirus was shown to induce colonies after infection of freshly explanted mouse bone marrow cells (33). Cells derived from these colonies could be established as continuous cell lines with unrestricted self-renewal in vitro and tumorigenicity in vivo. In addition, it was postulated that they were progenitor cells of the T cell lineage.

The ability of src-containing viruses to transform macrophages has been the subject of studies leading to conflicting results. Some investigators have shown that the src gene, although expressed in avian macrophages, failed to elicit a transformed cell phenotype (34). Conversely, we have reported that infection of macrophages from yolk sac, bone marrow and peripheral blood infected with viruses from B and C subgroups, acquired a partially transformed phenotype as ascertained by morphological changes and an increased rate of sugar uptake (morphology and rate of sugar uptake are two commonly measured criteria of transformation). These macrophages did not proliferate and retained their phagocytic ability (35).

Finally, it was recently shown that RSV is able to induce erythroid colonies in infected chicken bone marrow cells in the absence of growth factors (36).

5. DEFECTIVE AVIAN LEUKEMIA VIRUSES

Defective (acute) leukemia viruses (DLVs) induce malignancies in a short period of time following inoculation into birds. All of them are defective because of total or partial deletions of their replicative genes. Therefore, viral progeny is produced only in the presence of helper viruses that supply replicative functions. They are divided into three groups according to the three types of cell-derived oncogenes (37):

1) The myc-containing viruses: MC29, CMII, OK10 and MH2. The latter virus contains an additional cell-derived sequence v-mil, unrelated to v-myc (38).

2) The myb-containing viruses: Avian myeloblastosis virus (AMV) and E26 virus. The latter virus contains an additional cell-derived oncogene, v-ets which is unrelated to known retroviral oncogenes (39).

3) The erb-containing viruses: Avian erythroblastosis virus (AEV), ES4 strain, and AEV-H. AEV ES4 contains two cellular transduced genes v-erbA and v-erbB (40), while AEV-H carries only the v-erbB gene (41).

5.1. Myc-containing viruses: MC29 and MH2

Among the four viral isolates, MC29, CMII, OK10 and MH2, MC29 and MH2 are the most thoroughly studied. No further observations concerning the pathogenesis of CMII and OK10 have been reported since our earlier report (7). MC29 displays a wide oncogenic spectrum comprising renal and hepatic tumors such as endotheliomas or histiocytic sarcomas, together with myelocytic leukemia. Our own experience has indicated that this leukemia is induced only when embryonated eggs are injected with MC29 through the chorioallantoic vein. Hatched chickens injected with this virus by different routes always induced a stem cell leukemia accompanied by solid tumors. Attempts to maintain in vitro leukemic cells of the granulocytic type have always failed. These results suggest that MC29 is able to transform a broad range of hemopoietic cells which remain to be defined. In vitro, MC29 induces transformation of macrophages and immature bone marrow cells resulting in non-adherent cells displaying phenotypic characteristics of macrophages with a finite life in culture. According to several reports, these cells are non-tumorigenic. When MC29 infects cultures of chicken embryo cells, it induces two distinct phenotypes of transformed foci, namely an epithelioid-like focus encircled by a thin lamina of fibroblasts (42), and a second focus resembling the ones induced in chicken embryo cells infected with RSV.

The isolation of partially transformation-defective mutants has allowed the analysis of the multi-oncogenic potential of v-myc. Deletions within the v-myc sequences correlate with a reduced oncogenic potential of these isolates, which lose the ability to transform macrophages in vitro, while retaining that to transform fibroblasts (43). The finding that c-myc, the cellular homolog of v-myc, is involved in the induction of ALV-induced lymphomas (44) was somewhat surprising since MC29 was not reported as a lymphoma-inducing virus. This last question was recently re-examined and, in contrast to earlier reports, MC29 was found to induce bursal lymphomas appearing much earlier (1-2 months) than those induced by ALV (4-12 months) (45). These lymphomas were detected only in tissues expressing high levels of MC29 v-myc RNA. Furthermore, lymphoid tumors were also obtained upon inoculation of chickens with an MC29 variant, HB1 (46). This virus is a recovered avian myelocytomatosis virus which has a myc gene containing c-myc sequences acquired by recombination with the cellular gene and some v-myc sequences. It has also sustained some changes within the LTR region and the gag gene. The lymphoid tumors consist of B- and T-cells, as determined by antigenic markers, and cause the death of inoculated animals within 2-4 months following inoculation.

MH2, in addition to the v-myc sequence, contains another cell-derived sequence defined as v-mil. These two oncogenes are expressed in transformed cells (macrophages and fibroblasts) via two distinct mRNAs which encode two proteins: a fusion protein, containing gag and mil determinants, $p100^{gag\text{-}mil}$ which is localized in the cytoplasm, and $p57^{myc}$ localized in the nucleus. It is speculated that $p100^{gag\text{-}mil}$, which shares homology with a number of other onc proteins (47), (such as those coded for by v-src, v-fps, and v-erbB), might act either in synergy with or complement the action of the v-myc protein. This may explain the differences in transformation-specificity exhibited by MH2 and MC29. MH2 has been shown to induce a higher incidence of liver and kidney carcinomas in inoculated chickens and is much more oncogenic than MC29 when injected into young immunologically competent quail. Moreover, MH2 is capable of transforming quail and chicken macrophages with a higher efficiency than MC29 (48). In addition, MH2 induces proliferation and transformation of neuroretinal cells. Recent experiments have shown that deletion mutants of MH2 (lacking the mil gene) as well as other myc-containing viruses are not able to induce proliferation and transformation of neuroretinal cells in contrast to wild-type MH2. These results indicate that the expression of v-mil is essential for the proliferation and transformation of neuroretinal cells (49).

5.2. Myb-containing viruses: AMV and E26 virus

AMV causes a rapid leukemia in chickens and transforms specific hemopoietic cells in culture. The oncogene of AMV, v-myb, is a truncated version of c-myb. V-myb encodes the transforming protein $p45^{v\text{-}myb}$, located in the nucleus (50,51). Experiments aimed at the characterization of AMV target cells in chicken bone marrow by density, velocity sedimentation, adherence, and phagocytic activity, indicate that the target cells for AMV are recruited among cells which are located just beyond the stage of the myelomonocytic progenitors, i.e. CFC (colony-forming cells) (52), and are already committed towards the macrophage lineage (53).

In addition, in vitro studies from this laboratory established that functionally differentiated macrophages obtained from bone marrow, secondary yolk sac cultures or peripheral blood can serve as target cells for transformation (52). Our findings were challenged by other investigators who suggested that the cultures still contained some adherent immature cells (54). This problem was later carefully re-examined by Durban and Boettinger (55), who were able to demonstrate that the number of contaminating immature cells in the macrophage

culture is less than 1 in 5,000 and therefore does not account for the high level of transformation. Moreover, no correlation was found between the granulocyte-lineage cells and the number of AMV-transformed cells, observed in the yolk sac cultures infected with AMV. They reconfirmed that the target cells for this virus are recruited among the mononuclear-phagocytic lineage. In view of these observations, the leukemia induced by this virus should be referred to as monocytic leukemia.

The AMV-transformed cells can be maintained in culture for long periods of time (56). They were obtained either from colonies of transformed bone marrow or yolk sac cells, from foci of macrophage cultures or from the peripheral blood of moribund leukemic chickens. Regardless of their origin, they are morphologically the same and have the same functional and surface properties (52,57,58). Receptors for the Fc portion of immunoglobulins are expressed on the surface of these cells, whereas receptors for the C3 component of complement are not, both receptors being present on normal avian macrophages. However, immune phagocytosis mediated by Fc receptors did not occur, indicating that these receptors are not functional. AMV-transformed cells can engulf latex particles (phagocytosis mediated by non-specific receptors). Acid phosphatase and adenosine triphosphatase are also found in the cytoplasm and on the membrane of the transformed cells. An additional marker has been detected by using a monoclonal antibody which specifically recognizes cells of the myeloid lineage (59). Finally, when treated with a tumor promoter (PMA), AMV-transformed cells adhere to the surface of the culture flask and differentiate into macrophages (60). Differentiation into macrophages was also obtained with a temperature-sensitive isolate of AMV when transformed cells were shifted at the non-permissive temperature (61).

In conclusion, studies on the transforming activity of AMV have shown that all stages of macrophage differentiation, from the committed progenitor to the mature macrophage, may serve as target cells. The same was true for other related myeloid viruses (MC29, MH2, OK10, CMII). It seems therefore that target cells for these viruses must express some or all of the differentiation parameters of mature macrophages. Moreover, when infection occurs in immature cells, transformation will be triggered only when these cells acquire some of the differentiation markers.

Recently, it was observed that transfection of fibroblasts with DNA from AMV and that of its associated virus resulted in morphologically transformed cells which are able to form colonies in semi-solid media. Preliminary data indicate the presence of a protein homologous to that of v-myb, a protein which might be at

the origin of this rare transformation event (62).

The oncogenic properties of E26 virus differ considerably from those of AMV. This virus induces a mixed erythroid and myeloid leukemia with a predominance of the former. In addition, E26 virus causes a similar leukemic response in the Japanese quail, whereas AMV does not (63). In vitro, E26 virus is able to transform macrophages as well as immature cells of both the erythroid and myelomonocytic lineages. Furthermore, transformation of quail fibroblasts by E26 virus has been reported (64), but still awaits confirmation. It is not known which of the two oncogenes carried by the E26 genome, v-myb and v-ets, is responsible for the respective leukemia.

The dual oncogenic response of E26 virus has been the subject of several recent studies performed in vitro (65,66). They have indicated that infection of bone marrow cells with E26 virus induces transformation of both erythroid- and myeloid-committed cells. A larger number of target cells was found in early embryonic tissues compared with those in adult bone marrow. This observation correlates with a high percentage of immature progenitors of the erythroid and myelomonocytic lineage found in the primitive streak and the 12-somite stages. Upon infection, three different types of colonies were obtained, namely pure erythroid, pure myeloid, and a mixed colony containing cells of both lineages. Even after several passages, cultures derived from mixed colonies were shown to contain a self-replicating bipotent target cell.

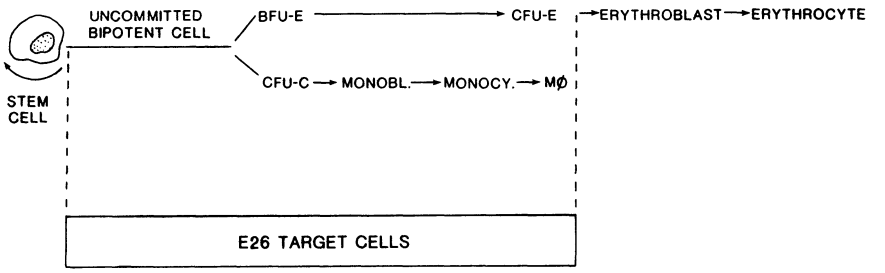

Figure 1. E26 virus interference with two hemopoietic progenitor cell populations.

In conclusion, E26 virus is able to infect and transform a bipotent (uncommitted erythroid-myeloid) hemopoietic cell as well as a myeloid- and erythroid-committed progenitor cell. Our data also showed that E26-transformed erythroid cells, derived from embryonic tissues, tend to escape the block in differentiation; hence hemoglobinization of more mature erythroid cells occurs. This is in contrast with E26-transformed erythroblasts derived from adult bone marrow, which remain immature and therefore do not exhibit any further change in their transformed phenotype. Further work is in progress to examine this question. A model describing the possible pathway of E26 virus infection of cells from both erythroid and myelomonocytic lineages is presented in Figure 1.

5.3. Erb-containing viruses: AEV

AEV causes erythroblastic leukemia and sarcomas in susceptible birds in a short period of time. AEV transforms cultures of chicken fibroblasts and hemopoietic precursor cells of the erythroid lineage (67). This virus contains two oncogenes, namely v-erbA and v-erbB, encoding two proteins $p74^{gag\text{-}erbA}$ and $p61\text{-}68\text{-}72^{erbB}$, respectively (68,69). The existence of these two gene products prompted the question of whether one or both proteins are necessary for the full oncogenic expression of AEV in vivo and in vitro. Frykberg et al. (70) and Sealy et al. (71,72) independently constructed deletion mutants either in v-erbA or in v-erbB genes, and the results obtained by these two groups are essentially the same. The data show that the ability of AEV to induce erythroleukemia in chickens and to transform fibroblasts in culture depends on the expression of $p61\text{-}68\text{-}72^{erbB}$ (mutant v-erbA$^-$B$^+$). In contrast, the mutant which is incapable of synthesizing this protein (mutant v-erbA$^+$B$^-$) lacks transforming activity in vitro. The role of v-erbA in leukemogenesis remains to be assessed.

The characterization of AEV target cells for in vitro transformation of bone marrow cells has been reported. Briefly, AEV target cells are recruited within the BFU-E (burst forming unit-erythroid) compartment (73). After infection the maturation of the BFU-E proceeds but it is blocked at the CFU-E (colony-forming unit-erythroid) stage, as described by Samarut and Gazzolo (74) and Beug et al. (75). Indeed the AEV-transformed cells express erythroid markers of these late progenitors but have acquired a self-renewal potential that will impair terminal differentiation. These cells do not synthesize hemoglobulin. Several observations indicate that virus-transformed cells may at time escape the block resulting in partial or total spontaneous differentiation (76,77,78,79). In examining the response of embryonic tissues of different ages to infection with E26 virus, we

have observed that erythroblast-like cells produced from virus-transformed blastoderms showed a definite level of spontaneous differentiation up to the terminal stage of erythrocytes (see above). These studies prompted us to re-examine the transforming activity of AEV in embryonic tissues and the data obtained may be summarized as follows (80):

(a) In the embryonic tissues examined (primitive streak, 12 somites, yolk sac at different ages) the target cells for AEV were not in the BFU-E compartment. The embryonic target cell probably resides either in an earlier compartment just preceding the BFU-E stage or within the CFU-M compartment, or both.

(b) All embryonic transformed colonies obtained after infection with wild-type AEV contain a high percentage of hemoglobinized cells.

(c) Colony-derived transformed cells display CFU-E markers, but unlike normal CFU-E they have acquired self-renewal potential while retaining some differentiating ability. Subcloning of these transformed cells results once again in partially hemoglobinized colonies.

A schematic model for AEV interactions with target cells is presented in Figure 2. The upper section of this scheme illustrates the erythrocytic differentiation pathway characterized by the sequential expression of specific antigens expressed during maturation: immature antigen (Im), brain antigen (Br) and hemoglobulin (Hb). The signs (+) or (-) refer to the expression of markers or their absence, respectively. The lower part of the scheme represents the identification of target cells belonging to two different compartments: (a) the BFU-E in the bone marrow, and (b) the CFU-M or the pre-BFU-E or both, in the embryo. Target cells from both compartments differentiate to CFU-E-like transformed cells after infection. In the case of bone marrow, the CFU-E are blocked at this stage, while undergoing self-renewal. By contrast, embryo-derived CFU-E-like transformed cells partially escape the block (horizontal dotted line) to terminal differentiation (triangle).

Spontaneous differentiation has been observed by Graf and Beug (81), when transformation was induced by infection with a v-erbA deletion mutant (v-erbA$^-$ B$^+$). They suggested that terminal differentiation is probably due to the absence of the v-erbA gene. Our results, however, indicate that self-renewal, together with differentiation, occurs with wild type AEV when this virus transforms embryonic erythroid cells. It remains to be proven whether embryonic hemopoietic cells provide a different environment which may affect the full oncogenic expression of the virus.

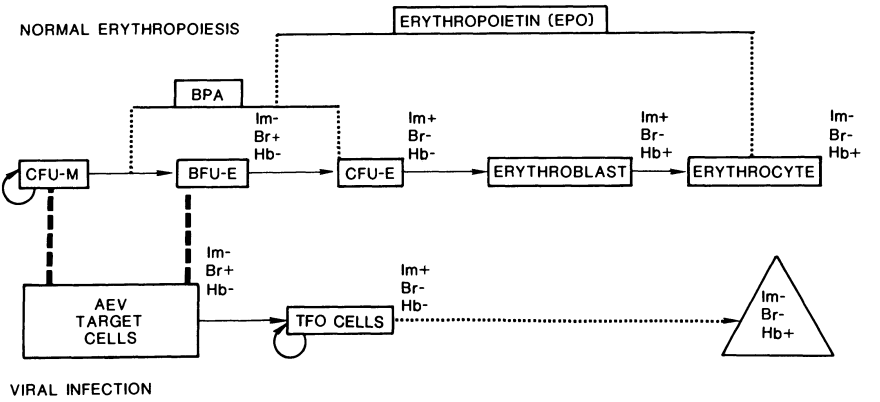

Figure 2. AEV interference with erythroid differentiation pathway.

Although we seemingly have a better knowledge of the target cell for each virus, and have improved our methods for detecting differentiation markers in transformed cells, we are still far from fully understanding how viral gene products interfere with cell differentiation. However, it is well documented that not all defective leukemia viruses follow similar patterns in their interaction with cells from the hemopoietic system. In the case of AEV and AMV, for instance, it seems that these viruses can express their transforming potential only when the infected cell has reached a certain stage along its differentiation pathway. Conversely, in the case of E26, cells at all stages of differentiation are competent for transformation. Finally, the spontaneous differentiation observed in embryonic transformed cells adds a new dimension to the understanding of virus-cell interactions. Many questions remain unanswered, two of which are particularly pertinent. Is the presence of an as-yet-unidentified differentiating factor interfering with the full viral expression in the embryo? Or, is the absence of a regulatory factor lacking in the embryonic tissue, but present in the adult tissue, preventing the full transforming activity of the virus? Answers to these questions will help clarify the complex problem of how disruption of the hemopoietic program occurs.

6. CONCLUSIONS AND FUTURE TRENDS

In the last 2 to 3 years research in the field of avian retroviruses, specifically concerning cell interactions with sarcoma and acute leukemia viruses, has again provided new insight into the mechanisms of oncogenesis. Progress has been made by consolidating previously proposed explanations and by re-evaluating some of the easily accepted dogmas. This review has therefore attempted to emphasize some of the present problems which will stimulate further work and hopefully, in the near future, clarify some as yet poorly understood problems. It is now well established that viral oncogenes interfere with basic mechanisms of cell growth and metabolism and are implicated in the multistep process leading to malignant cells (1,2). New evidence has been acquired showing that these sequential events are under the influence of both the products of the oncogenes and of the cell regulatory processes linked to differentiation.

Recent information collected during studies of cell-virus interactions suggests that at least four steps may be distinguished following virus infection. These steps are: (a) Immortalization; (b) Transformation (as determined by in vitro studies); (c) Tumorigenesis (as determined by in vivo studies); (d) Metastasis or Invasiveness. It has been postulated that these events occur sequentially. This assumption is not always confirmed by experimental data, since results obtained with infected neuroretinal cells indicate that proliferation is not always a prerequisite of transformation and/or tumorigenesis (25). Therefore, the link between proliferation and transformation of differentiated cells in general, and of hemopoietic cells in particular, needs to be analyzed further. Tumor formation needs further investigation. Virus inoculation into susceptible animals always leads to the formation of either solid tumors or leukemia, whereas tumor induction was never obtained when non-producer virus-transformed cells were injected in immuno-incompetent embryos (82). This observation suggests that the transformed cells may respond to regulatory signals of the host and be induced to differentiate.

Two major tasks are still challenging the cell biologist. On the one hand we need more systematic studies in the area of regulation of cell proliferation and differentiation. On the other, the mechanism of the metastatic processes remains to be elucidated since it is not yet clear how tumor cells escape the immune defences of the organism. It is conceivable that these cells have acquired the ability to escape because specific alterations occurred within the cellular gene.

ACKNOWLEDGMENTS

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9

THE PATHOGENESIS AND PATHOLOGY OF NEOPLASMS CAUSED BY AVIAN LEUKOSIS VIRUSES

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1. INTRODUCTION

The subfamily Avian Oncovirinae of the family Retroviridae is composed of avian leukosis viruses (ALVs), which transform target cells slowly, and avian sarcoma viruses (ASVs) and avian (acute) defective leukemia viruses (DLVs), which transform target cells relatively rapidly. Before this subdivision was made, the entire group was called avian leukosis/sarcoma viruses, avian type C viruses, avian oncorna and avian oncoviruses. The subdivision is used in this monograph to facilitate the discussion of the genomic structure of the avian retroviruses in other Chapters. It should be recognized, however, that the pathologic expression of the various avian retroviruses is variable and depends on many factors other than belonging to ALV, ASV, or DLV. For example, field and laboratory isolates of avian retroviruses induce various leukemias, but it is not clear what proportion of the leukemias are caused by ALVs or DLVs or even ASVs. Also, nothing is known of the genomic structure of the field viruses that cause the occasional outbreaks of sarcomas, erythroblastosis, myelocytomatosis or other tumors.

This Chapter will focus on the pathogenesis and pathology of the avian leukoses, which are caused by strains of ALV, and of leukemias, which are caused by strains of ALV, and of leukemias which are caused by laboratory strains of ALV and DLV. The pathogenesis and pathology of avian sarcomas caused by strains of ASV are discussed in Chapter 7 of this monograph.

With the advent of highly effective vaccines against Marek's disease (1,2), neoplasms caused by ALV and related production losses are sometimes the most important economic constraints to poultry production. Erythroblastosis, myeloblastosis and sarcomas are less common but may, on occasion, devastate flocks.

In laboratory inoculation trials in newly hatched chickens, ALVs produce a great variety of neoplasms. For reasons that are only partially known and will be discussed, only a few of the types of neoplasms occur commonly in flocks of commercial chickens. This review will focus on the neoplastic conditions that occur commonly in field flocks. It will cover briefly the virus classification, and describe factors affecting the pathogenesis and pathology of the major neoplasms which occur in commercial chickens. For a more extensive description see (3).

2. VIRUS CLASSIFICATION

2.1. Virus groups and subgroups

The avian leukosis and sarcoma viruses have in common a group-specific internal antigen which was first identified by complement fixation using mammalian (hamster) antisera (4). Seven subgroups are recognized on the basis of (a) viral interference, (b) viral neutralization, and (c) host range in genetically susceptible or resistant cells (5,6). ALV, DLV and ASV subgroups A through E occur in chickens, and to date, subgroups F and G have only been found in pheasants. Only subgroups A and B, and possibly E, are of major economic importance in commercial poultry production (7). The viral envelope contains glycoproteins that determine the subgroup specificity of the virus. Within each subgroup are viruses that vary greatly in degree of pathogenicity and nature of tumor produced. Lymphoid leukosis (LL) is the most common pathologic expression. Viruses from more than one subgroup may be identified from diseased birds.

2.2. Distinguishing between avian leukosis and avian sarcoma viruses

Under conditions approximating the optimum dose and route of exposure, the ALVs cause predominantly leukoses, DLVs cause predominantly leukemias, and ASVs cause predominantly sarcomas. However, when conditions are manipulated (see later), there is considerable overlap because each virus can be induced to cause predominantly the other tumor. In addition, highly cloned avian retrovirus preparations have been shown to induce multiple types of tumors (8,9).

In general, ASVs replicate more rapidly than ALVs and cause a more rapid visible transformation and progression of tumors, both in vivo and in vitro. In chicken embryo fibroblast cultures, which are most commonly used for assay, ALVs and DLVs do not usually cause visible transformation whereas ASVs regularly do. This property of ASVs to transform cells rapidly in cell culture is used in methods to detect ALVs, to determine their subgroup, and to determine

whether the chickens from which the cells are derived are genetically resistant or susceptible to infection (10). The predominant target cell in vivo for transformation by ASVs is the fibroblast, though ASVs have been shown to cause transformation of many different types of cells. Transformation often occurs in a short time (7 to 10 days) in susceptible chickens and progression of the tumor and metastasis are rapid.

The various strains of ALV and DLV usually do not transform chicken embryo fibroblasts. However, some DLVs, under specialized culture conditions, transform specific target cells. Thus, focus assays for avian myeloblastosis virus (AMV), avian erythroblastosis virus (AEV), and some other DLVs have been developed (11,12). In these instances the viruses transform their target hematopoietic cells.

The following generalizations about the pathologic expression of the various avian retroviruses provide a framework for further discussion. The target cells for transformation by ALVs and DLVs are immune responsive and blood forming cells, whereas for ASVs they are fibroblasts. The incubation period for leukoses is longer than for sarcomas. Many laboratory strains of ASV and all DLVs are defective in their replication and lack the ability to produce the envelope proteins. Their subgroup is determined by the helper ALV used for their rescue and propagation. These viruses contain an oncogene responsible for the acute neoplasia. Some strains of ASV produce pocks on the chorioallantoic membrane of embryonated eggs, whereas ALVs do not. ASVs have been shown to cause neoplasms in a variety of animals including mammals, whereas all ALVs are host specific for chickens.

3. FACTORS AFFECTING THE PATHOGENIC RESPONSE

3.1. Virus strains and oncogenic spectrum

When a virus is isolated from a tumor and passaged a few times in susceptible chickens, it is often referred to as a strain. An extensive list of strains and some of their characteristics have been published (13,14). The virus strains are usually obtained from the tumor after which it was named and usually, particularly in early passages, produce a similar kind of tumor. Thus, Rous sarcoma virus (RSV) was isolated from a sarcoma and induces predominantly sarcomas, and AEV was isolated from a bird with erythroblastosis and induces predominantly erythroblastosis. However, strains often cause more than one kind of tumor. If conditions such as the dose of inoculation, the route of administration, the age at exposure and genetic make-up of the host are manipulated, a variety of tumors can be produced and the predominant tumor may change. For example, selection of virus

donors with hemangiomas resulted in a larger proportion of birds with this tumor than in previous passages, and eventually a virus that produced predominantly hemangiomas was obtained (15). The types of tumors produced under specified conditions are characteristic of the virus isolate or strain and are referred to as the "oncogenic spectrum" of the virus (see Fig. 1). Conditions that affect the oncogenic spectrum include the strain of virus, the dose of virus, the route of inoculation, the age of the host, the genetic make-up of the host, the sex of the host, and various environmental factors. These will now be discussed in turn with emphasis on those conditions that lead to the most commonly observed tumors in the field.

3.2. Dose of virus

The dose of ALV, DLV or ASV administered greatly affects the incubation period for tumor formation and the nature of the tumor produced. Thus, high doses of certain ALV strains result in predominantly erythroblastosis, which occurs in 2 to 3 months, whereas low doses result in predominantly LL, which occurs in 5 to 9 months (15). When a high dose of virus is given, all birds may succumb from erythroblastosis within a month or two and there may be no survivors to develop LL. In contrast, low doses of the same virus may cause no erythroblastosis, presumably because the dose is below the threshold to permit infection and transformation of the target erythroblastic cells and all affected birds may develop LL. In general, when other conditions are optimal, a virus given at a high dose causes the tumor after which the virus is named. Thus, RSV induces sarcomas, AMV causes myeloblastosis and AEV causes erythroblastosis. At high doses some other tumors appear such as nephroblastomas and osteopetrosis. All these viruses given at a lower dose cause LL. Under natural conditions, exposures are usually at relatively low doses and therefore LL is the most common neoplasm seen. Because the efficiency of horizontal transmission by natural routes is low, very low doses of virus, as may often occur in field situations, may not be able to infect chickens and so may not cause disease at all. In addition, as with most viruses, disease symptoms will appear in only a proportion of established virus infections.

3.3. Route of exposure

Routes which allow high doses of virus to get to the target cells for transformation usually cause more tumors or tumors of the type induced by high doses of virus. Thus, subcutaneous and intramuscular injection of ALV tends to

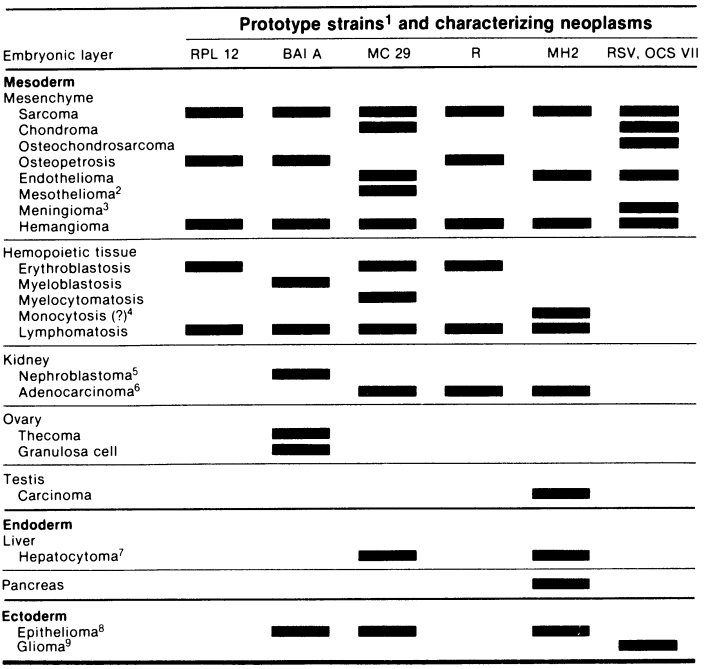

Figure 1. Oncogenic spectrum of selected strains of ALV, DLV and ASV. Black bars represent a response of the type (Beard and Raven Press, by kind permission).

1. RPL12: Regional Poultry Laboratory strain at 12; BAI A: Bureau of Animal Industry strain A, myeloblastosis; MC29: myelocytomatosis; R: erythroblastosis; MH2: Mill Hill strain 2; Murray-Begg virus; RSV: Rous sarcoma virus; OCS VII: osteochondrosarcoma (Tytler).
2. Metaplastic epithelial and chondromal derivatives of peritoneal, pericardial, and epicardial squamous mesothelium.
3. Growths of meninges of mesodermal derivation induced in chickens by intracerebral inoculation of RSV and other sarcoma strains. Neurogenic growths of ectodermal origin are not produced in the chicken.
4. Leukemia of not fully identified cells associated with MH2 infection.
5. Highly complex spectrum of adenoma and carcinoma of glomerular and tubular structures, mesenchymoma, osteoma, chondroma, keratosis, and spindle cell sarcoma.
6. Spectrum includes cystadenoma and tubular and glomerular adenocarcinoma and carcinoma with occasional chondroma.
7. Hepatic growths of multiple variety, trabecular carcinoma, adenocarcinoma, mosaic type growths, hepatobiliary tumors,

hemorrhagic carcinoma, rifted hepatoma, chondroma, and sarcoma derived by transformation of hepatic cells in birds infected with strain MC29 virus. Adenocarcinomas and solid carcinomas of hepatocyte derivation were induced by the MH2 agent.
8. Squamous-cell carcinoma of skin.
9. Astrocytoma, ependyoma, oligodendro-glioma, ganglioglioma.

cause sarcomas because the virus has better access to the target fibroblasts. Intravenous injection of ALV tends to cause erythroblastosis and myeloblastosis because the virus has better access to the hemopoietic target cells. Under natural conditions exposure is likely through the natural openings by droplets in the eyes and lungs and orally through feed and water. Exposure through breaks in the epidermis (cuts or defeathered follicles) may also occur. These routes are not as efficient in infecting the target cells as introducing virus parenterally. Thus, under natural conditions, the most common tumor is characteristic of exposure under laboratory conditions to a low dose of ALV, i.e., LL. Once again, repeated passage by a particular route can change the oncogenic spectrum of a strain as has been elegantly demonstrated (16,17). Nothing is known about the natural epizootiology of ASVs and DLVs in field flocks.

3.4. Age of host at exposure

In general, younger hosts are more susceptible to tumor development than older ones. Embryos are even more susceptible than hatched chickens. The resistance to tumor development, when virus exposure is by natural routes, develops very much faster than resistance when virus exposure is by parenteral routes (18). Thus, by 3 weeks it is very difficult to induce LL with most viruses when chickens are exposed orally or intranasally, though some LL can be induced by intravenous inoculation. Under natural conditions, it is likely that most chickens which develop LL or other tumors are infected congenitally, though some may be infected by contact transmission shortly after hatching.

In genetically susceptible chickens, resistance to virus infection may also increase with age. This has been referred to as type II resistance in contrast to resistance to tumor development or type I resistance (19). Type II resistance develops more slowly than type I resistance. In genetically susceptible chickens of 2 months of age or older, exposure to ALV results in lower viremias of shorter duration and, consequently, little or no shedding in the egg (20,21). It is not known whether these types of resistance affect induction of LL by DLVs or ASVs. The genes which determine resistance to virus infection or tumor development are described in Chapter 3 of this monograph.

3.5. Genotype of host

There are two levels of resistance to tumor development, namely, at the level of infection of the cell and at the level of cell transformation (22). Cells may be resistant to infection with viruses of one subgroup. For example, C/A cells are resistant to infection by subgroup A viruses. Also, C/AB cells are resistant to infection by viruses of both subgroups A and B, and C/O cells are susceptible to viruses of all subgroups. Chickens with a genotype A^rA^r are thus C/A and are resistant to development of tumors by subgroup A viruses. The frequencies of alleles that encode cellular susceptibility and resistance to infection by ALV, DLV or ASV vary greatly among commercial lines of chickens (23,24). However, most flocks have a relatively low level of this type of resistance.

At the second level of resistance, cells may be resistant to transformation by ALV or ASV and probably also DLV. Thus, genetic resistance to LL is not dependent on the cellular elements of the immune system but is dependent on the ability of the bursal target cell to become transformed (25). The low frequency of lymphomas observed in the field might suggest that this type of genetic resistance is common among commercial flocks. Little is known about its mode of inheritance, but it should be realized that other factors such as age at virus exposure are involved also.

3.6. Sex of host

Females and castrated birds of both sexes are more susceptible to LL than are males (26). Males are more susceptible to osteopetrosis.

3.7. Environmental factors

Many early attempts at identifying environmental factors such as temperature, humidity, and source of feed, which affect the occurrence of neoplasms were unsuccessful. However, environmental factors which deplete the bursa of Fabricius clearly reduce the incidence of LL and may increase the incidence of osteopetrosis. Thus, pathogenic strains of infectious bursal disease virus (IBDV) reduce the incidence of LL (27). The widespread occurrence and early infection of many flocks with this disease agent may account for the low level of LL in flocks known to be heavily congenitally infected with ALV. Attenuated IBDV vaccine strains which do not deplete the cells in the bursa of Fabricius, however, have no effect on reducing LL (28).

4. CLASSIFICATION OF LEUKEMIAS AND LEUKOSES

Ellerman (29) was one of the first to distinguish between erythroid ("intravasculare Leukose"), myeloid ("myeloische Leukose"), and lymhpoid ("lymphatische Leukose") leukosis. For a comprehensive review of the classification of these diseases, see (3). The classification of the tumors produced is based on the nature and origin of the cells involved, the blood-forming cells. There are tumors of red and white blood cell lineage. In tumors of red blood cell lineage, the cells are transformed and the normal development of the cells is arrested at the stage of the erythroblast and therefore the disease is called erythroblastosis. Some viruses transform cells of the myeloid lineage and arrest their maturation at the myeloblast stage, i.e., before granules develop. The leukemia so produced is called myeloblastosis. Other viruses transform the cells and arrest their development at a stage when the granules are fully developed and, in these instances, the tumors are aleukemias and are called myelocytomatosis. Thus in erythroblastosis and myeloblastosis leukemias predominate, whereas in myelocytomatosis aleukemia predominates. Some viruses of this group cause tumors of the lymphoid cells of B-cell lineage. The cellular transformation and arrest of development and maturation usually occur at the point of transition from lymphoblasts to plasma cells, i.e., just before or at the beginning of IgM production (30). Most tumors are composed of IgM-producing cells, though some tumor cells do not produce IgM either because transformation occurred before the cells acquired this ability or the ability was lost after transformation and proliferation. The aleukemic form is most commonly seen in the field. No ALV or DLV has been described which transforms cells of the T-cell lineage.

5. OTHER TUMORS AND TUMOROUS CONDITIONS

Sarcomas are tumors of connective tissue cells. Those viruses that induce sarcomas rapidly, for example RSV, usually induce myxosarcomas which are characteristically filled with slimy and tacky myxomatous material. Those with longer incubation periods often produce fibrosarcomas which often contain collagen. The oncogenic spectrum of many viruses includes hemangiomas, hepatomas, nephroblastomas, and adenocarcinomas of the kidney, but they are usually less common in the field. Very occasionally a high incidence of one of these tumors will occur in a flock, though there are no satisfactory explanations why. Many strains of ALV and DLV induce osteopetrosis, and, for some virus strains, this is the predominant or only lesion (31).

6. NON-NEOPLASTIC CONDITIONS

High levels of infection or infection at a very early age in the laboratory with most strains of ALV likely cause some degree of reduced growth and stunting of chickens. Anemia, hepatitis, immunodepression, hypothyroidism, and obesity have been described (32,33,34,35,36,37). In most instances, the specific lesions appear to be more common with a particular strain of virus, however, some of the lesions, e.g., anemia and stunting, are common for many different strains. It is unclear whether similar conditions occur under field situations. It is likely that in field situations non-neoplastic conditions are not as frequent since the majority of chickens are exposed to a lower dose of virus and at older age. However, there is a consistent, though small, reduction in growth rate of broilers (38) and an increase in non-neoplastic mortality, reduced egg production, reduced fertility, and reduced hatchability in layers (39).

Microscopic lesions have been described in many of the visceral organs of chickens one to 10 weeks of age infected with ALV. The lesions consist of discrete foci or larger diffuse areas of lymphoblasts and lymphocytes and sometimes even germinal centers. At their peak, the lesions may be visible grossly in the spleen, heart and testis. Microscopically they have been described in the visceral organs, particularly liver, heart, testis, thyroid, pancreas and dorsal root ganglia (32,33,40). The lesions are likely inflammatory and may be related to the clinical signs such as hypothyroidism and obesity.

7. TRANSPLANTABILITY AND DEVELOPMENT OF CELL LINES

Sometimes when naturally occurring tumors are triturated and inoculated into susceptible young chickens, tumors with the same characteristics as the donor tumor develop rapidly. Such tumors occur even when the recipients are obviously of a different genotype than the donor. It is impossible to determine on gross or microscopic examination whether such tumors are virus-induced or are transplants. Tumor transplants often occur when the conditions of trituration allow live cells to survive and be injected into the recipient. Tumor transplants are usually associated with a shorter incubation period, often occur at the site of inoculation, and often occur in a different tissue distribution than the original tumor. All of the tumors induced by ALV, DLV and ASV, except osteopetrosis, have been transplanted in this manner. For example LL, which takes over 3 months to develop as a primary tumor and in which the initial lesion is always in

the bursa of Fabricius, when transplanted can yield tumors in 10 days with lesions at the site of inoculation and often no lesions in the bursa of Fabricius (3). Histologically, transplanted tumors are generally more uniform and their cells are more anaplastic or primitive than the primary tumors from which they originate.

Tumor cells generally do not survive in vitro for long periods under conditions usually used for virus isolation. However, cells transformed in vivo can yield, on careful cultivation, cell lines that can be propagated indefinitely, e.g., lymphoblastoid cell lines from LL. Also, some cell types cultured in vitro can be transformed into stable cell lines, e.g., erythroblasts and myelobasts (12,41).

8. LYMPHOID LEUKOSIS

8.1. Pathogenesis

Under both natural and experimental conditions it is very rare to see LL in chickens under 14 weeks of age. Peak mortality usually occurs at 20 to 24 weeks of age, and declines thereafter, though some cases may occur at any time between 14 weeks and depopulation of the flock. Because of this long incubation period, LL is never a problem in broilers, fryers or roasters. It may be a problem in flocks of meat-type breeders and mature egg-type chickens.

Chickens are usually infected with virus congenitally or shortly after hatching (Fig. 2) (42). The meconium of congenitally infected chicks may contain large amounts of virus, and egg fluids, saliva and faeces frequently contain virus as well. It is likely that sexing and vaccination procedures play a role in disseminating the virus. There is a primary multiplication resulting in a viremia which occurs at about the same time as, and may be associated with, many of the non-neoplastic lesions. Establishment of viremia may be delayed by maternal antibody. The virus may persist in white blood cells for prolonged periods (43). Neoplastic changes can first be observed in individual bursa follicles as early as 4 weeks after experimental inoculation at one day of age. By 7 weeks, most chickens may have one or more abnormal follicles (44) (see Fig. 3). However, in many chickens the follicular lesions regress likely as a result of the immune response (Fig. 2). In some chickens, the tumor progresses. T-cell immunosuppression has been demonstrated (45). Metastasis of the proliferating B-cells to other organs may occur. Under field conditions and laboratory conditions where low virus doses transform few target cells, tumors are usually monoclonal (see Chapters 4 and 7).

Chickens dying of LL have gross or microscopic tumors in the bursa in almost

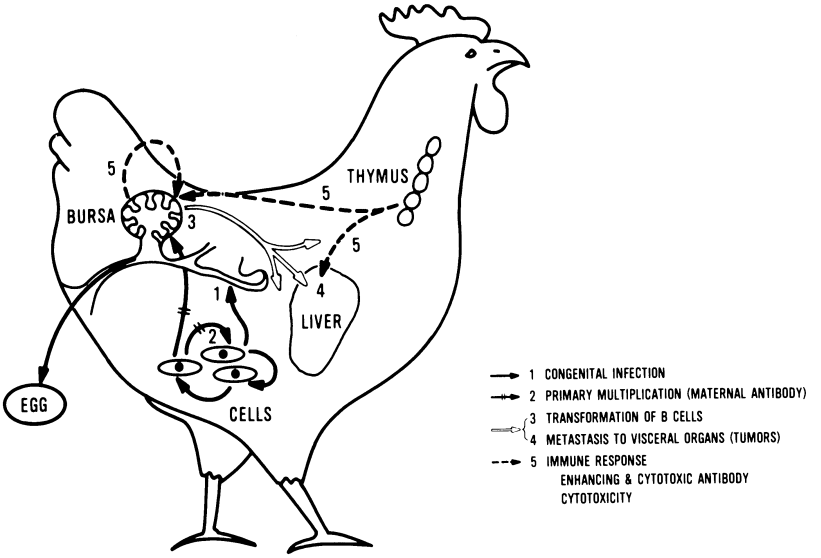

Figure 2. A model illustrating the pathogenesis of lymphoid leukosis.

every case (44). Death results from organ dysfunction. Grossly visible tumors may develop in the bursa, liver, ovary, spleen, kidney and other visceral organs. It appears that the target cell for transformation is a post-bursal stem cell (46) which must be genetically susceptible to infection (25). Any treatment that destroys the target cell prior to transformation or peripheralization effectively prevents the development of LL. Thus, surgical bursectomy between one day and 5 months of age (47), treatment of embryos or young chicks with androgens or androgen analogs (48), chemical bursectomy (46), and infection with IBDV (27) prevent the development of LL. In each instance, except possibly the androgen analogs, the side effects are detrimental and the treatments would not be an economically feasible method for LL control (see also Chapter 13).

Recent molecular biological studies indicate that lymphomagenesis is a multistage process, probably involving more than one single transformation event (37,49,50) and at least two genes, c-myc and B-lym (51). During viral integration into the host genome, the strong viral promoter gene integrates adjacent to the host c-myc gene which is present in all cells in all animals. It is a counterpart of a gene first identified in myelocytomatosis virus MC29. The viral promoter induces

transcription of the host oncogene (c-myc) located downstream from the insertion site. Thus in the B-cell, the c-myc gene is activated. The role of B-lym, the second transforming gene, is less clear. It is possible that the activated c-myc gene and the B-lym gene act at different stages of the multistage process of neoplastic transformation of B-cells (52). The products of these genes are responsible for both neoplastic transformation and arrest in maturation. The arrest in maturation results in an interference with the normal intraclonal switch of B-cell immunoglobulin production, from IgM to IgG (49). Promotion of host oncogenes may be induced by other viral promoters, such as reticuloendotheliosis virus (REV), or very rarely, may occur spontaneously (50,53). Because the maturation of the transformed cells is arrested, LL tumor cells have IgM on their surface and not IgG or IgA (46). The IgM may be produced in excessive amounts, particularly late in the disease. Under experimental conditions where high doses of virus are injected into susceptible chicks, multiple transformational events occur, neoplasms are polyclonal and the IgM may be heterogeneous (54). Under other conditions, such as low virus dose which is common in the field, or such as low infectivity of target cells which may occur with suboptimal routes of infection or partially resistant cells, only a single transformational event occurs and the neoplasms are monoclonal. If a limited number of transformational events occurs but one occurs earlier than the others and metastasizes more rapidly and extensively than the others, then the neoplasms will also appear to be monoclonal.

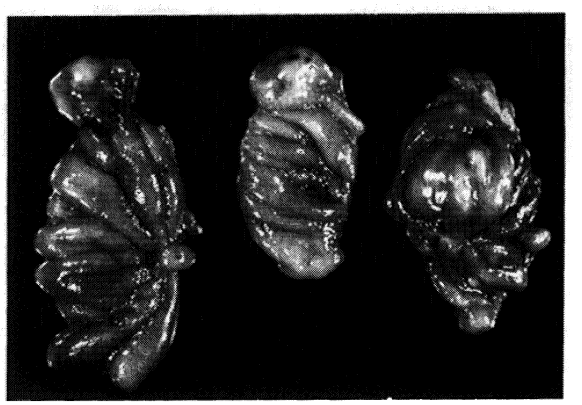

Figure 3. Primary LL tumors in the bursa of Fabricius. (Photograph courtesy of A.M. Fadly).

8.2. Signs

There may be no outward signs in individual chickens observed before affected chickens die. Signs that do occur, such as ruffled feathers, dullness, anorexia, anemia and emaciation (32,33,34,35,36,37) are not specific for ALV infection. Abdominal or cloacal palpation of the bursa often reveals the characteristic tumor, and often the nodular liver can be detected. In advanced stages an enlarged abdomen and penguin-like stance may be observed. Once clinical signs develop, the course is usually rapid. The effects of ALV infections on production and mortality on a flock basis are described in Chapter 12.

8.3. Gross pathology

At autopsy, the most visible tumor is that of the liver (Fig. 4). Tumors may also occur in the spleen, kidney, lung, gonad, heart, bone marrow and mesentery. On careful examination (sometimes microscopic examination is necessary), the bursa of Fabricius is almost always involved. Tumors are usually soft, smooth, and glistening, and on cut surface are creamy white. Seldom they are fibrous or gritty. They may be diffuse, nodular or miliary, or a combination of these forms.

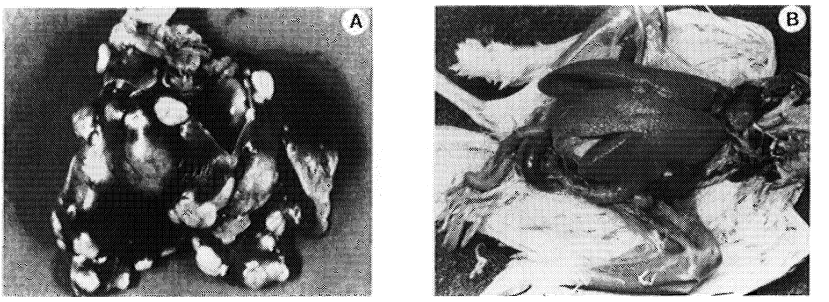

Figure 4. A) Focal and B) diffuse lesions of lymphoid leukosis (Photographs courtesy N.F. Cheville).

8.4. Histopathology

All tumors appear to have arisen from focal metastatic points. Even in organs appearing diffusely involved when examined grossly, the microscopic pattern is one of coalescing foci (Fig. 5). Often the parenchyma of the organ is displaced and compressed by the rapidly expanding foci of lymphoid cells which are often surrounded by bands of fibrous tissue. The foci often resemble large germinal centers. The tumors are composed almost exclusively of large lymphoblasts which

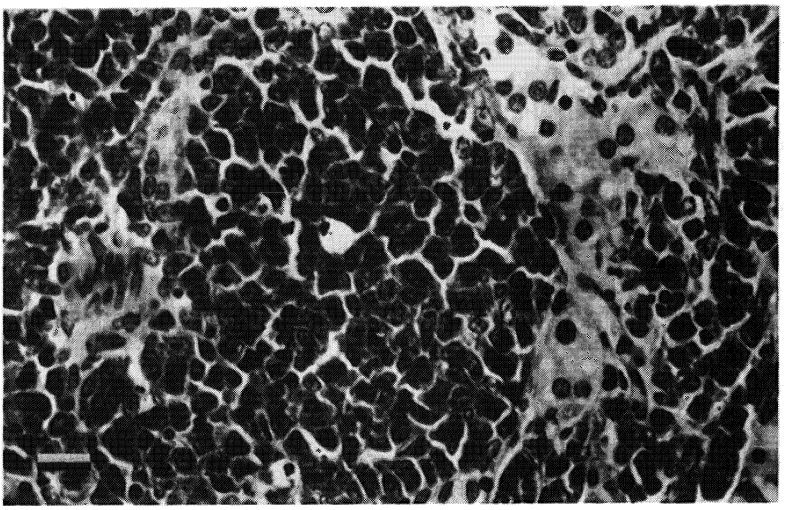

Figure 5. Liver with focal lymphoid leukosis tumor of uniform, large, anaplastic lymphoblasts with multiple nucleoli. Bar = 20 μm. (Photograph courtesy L.N. Payne).

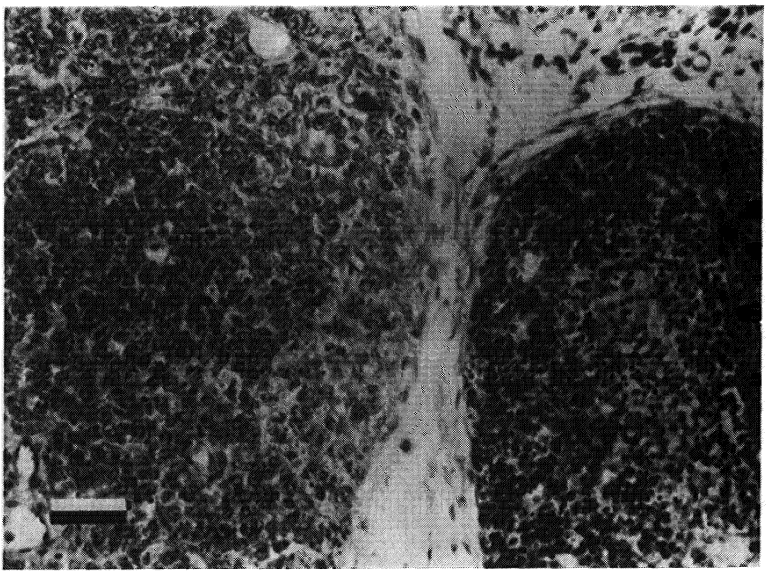

Figure 6. Bursa of Fabricius with follicle at left packed with large uniform lymphoblasts characteristic of lymphoid leukosis and follicle at right containing normal lymphoid cells. Bar = 80 μm. (Photograph courtesy N.F. Cheville).

are usually fairly uniform in size (Fig. 6). They have a slightly basophilic cytoplasm and a large vesicular nucleus in which there are one to three large conspicuous acidophilic nucleoli. At times there may also be a number of smaller non-neoplastic lymphocytes present which appear to have infiltrated the tumor mass.

9. ERYTHROBLASTOSIS

9.1. Pathogenesis

Under natural conditions erythroblastosis usually occurs between 3 and 6 months of age. Experimentally, inoculation of high doses of virulent virus into young susceptible chickens may induce disease as early as 7 days after one-day-old inoculation. Disease symptoms are already apparent at the time of hatching when embryos are infected by in ovo inoculation. In field flocks, it is common to encounter isolated cases of erythroblastosis. Outbreaks with larger numbers of chickens affected at one time are very rare.

The target cell for transformation is the erythroblast which occurs inside the blood sinusoids (intra-vascularly) in the bone marrow. On transformation of the target cell, continued differentiation is blocked and the cells continue to multiply, spilling into the circulation and causing an erythroblastic leukemia. Often there is also transformation at sites of extramedullary hemopoiesis such as in the liver and spleen. Many ALVs, and probably DLVs, also cause an anemia which may aggravate the condition of the host.

9.2. Signs

The earliest signs of lethargy and weakness may be followed by emaciation and diarrhea and are not specific. As the condition progresses, the comb and shanks may be pale or may become cyanotic, depending on whether erythroblastosis or anemia predominates. Often there may be hemorrhage from a feather follicle or a wound. With advancing erythroblastosis, the blood has a characteristic cherry red color.

9.3. Gross and histopathology

The characteristic gross lesion is a diffuse enlargement of the liver and spleen which have a characteristic cherry red to dark mahogany color and are soft and friable. Often there is pulmonary edema, hydropericardium and fibrinous ascites. Petechial hemorrhages are common and profuse hemorrhage may occur.

The bone marrow in the long bones is characteristically very soft or watery and dark cherry red. If the anemia predominates, visceral organs may be atrophied and pale.

The sinusoids of the liver are usually filled and dilated with erythroblasts (Fig. 7). Erythroblasts can be seen in the capillaries of all organs. In the bone marrow, there are usually sheets of homogeneous erythroblasts, with small islands of myelopoietic activity and little or no adipose tissue. The erythroblasts seen in sections and smears in all organs are always entirely intravascular. Unlike hemorrhagic anemia, there are usually erythrocytes and erythroblasts present with very few intermediary stages. The erythroblast has a large round nucleus with very fine chromatin and one or two nucleoli. The abundant cytoplasm is characteristically basophilic and may have some hemoglobin precursors. It usually has a characteristic perinuclear halo and sometimes vacuoles and fine granules. In blood smears the cells are often irregular in shape and have pseudopodia.

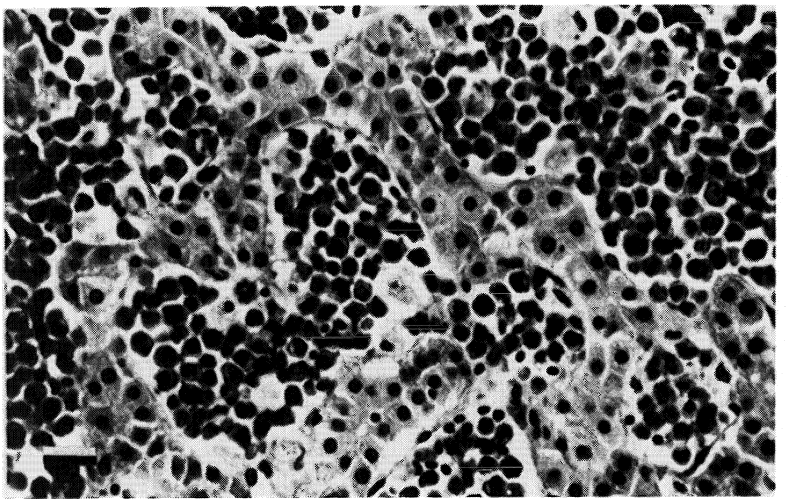

Figure 7. Liver with intravascular erythroblastosis. Sinusoids are packed with erythroblasts which have a coarse nucleus, immature hemoglobin in the cytoplasm and frequently a perinuclear halo. Bar = 20 μm. (Photograph courtesy L.N. Payne).

10. MYELOBLASTOSIS

10.1. Pathogenesis

Myeloblastosis occurs very rarely under field conditions. Isolated cases are likely to occur before 6 months of age.

The target cell for transformation is most likely the hemocytoblast in the extrasinusoidal spaces in the bone marrow. The transformed cells fail to differentiate and continue to proliferate, resulting in multiple foci of myeloblasts. The cells grow rapidly, overtake the normal bone marrow elements, and spill over into the sinusoids resulting in leukemia and invasion of other organs.

10.2. Gross and histopathology

The early signs of myeloblastosis are not specific and are similar to those of erythroblastosis. The course is highly variable. The liver and spleen are enlarged, friable, and grayish. The surface of the liver often has areas that are normal in color, gradually changing to grayish tumorous areas. The spleen and kidneys may be similarly involved. The bone marrow characteristically is firm and gray to reddish gray.

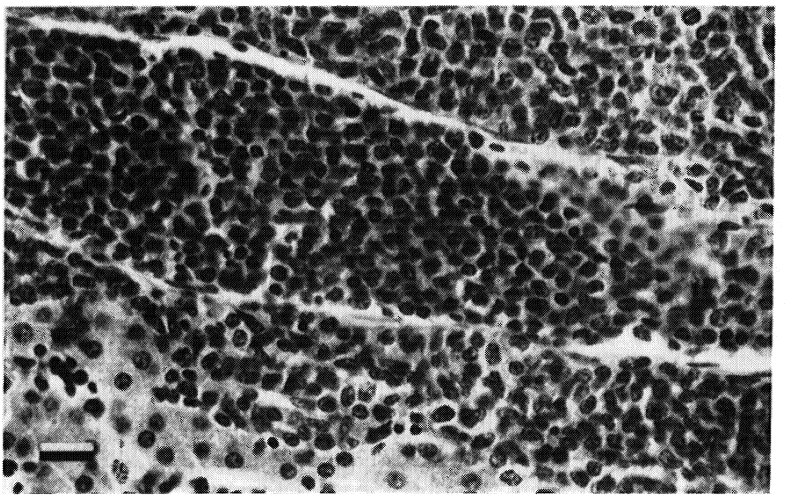

Figure 8. Liver with intra- and extra-vascular myeloblastosis induced by BAI strain A. Sinusoids and extra-sinusoidal spaces packed with myeloblasts with fine chromatin network and pale cytoplasm. Bar = 20 μm. (Photograph courtesy L.N. Payne).

Microscopically there is a massive invasion of the parenchymatous organs with myeloblasts which accumulate both intra-vascularly and extra-vascularly (Fig. 8). Accumulations are particularly extensive around the portal tracts in the liver. In the bone marrow, myeloblasts occur both intra- and extra-vascularly, but the greatest activity is extra-vascularly in the extra-sinusoidal spaces. Myeloblasts are large cells with a slightly basophilic cytoplasm and a large nucleus, containing one to four acidophilic nucleoli which do not stain prominently, and a fine chromatin network. Often myeloblasts comprise over 75% of cells in the blood. They can be readily distinguished from myelocytes which have many large acidophilic granules.

11. NEPHROBLASTOMATOSIS

11.1. Pathogenesis

Tumors are most frequently found in birds, 2 to 6 months of age, dying of other causes. Rarely do they appear to be the primary cause of death.

Nephroblastomas likely originate from embryonic tissue in the kidney. After virus infection and transformation, the epithelial cells and the stroma proliferate and differentiate. Epithelial cells may differentiate into glomeruli, tubules or keratinized epithelium and stroma may differentiate into sarcomas, cartilage and bone. Anaplasia of epithelial cells may result in sheets of cells with no tubular organization. Blocked tubules result in cysts.

11.2. Gross and histopathology

There are no signs that are clinically characteristic of the disease. Sometimes the abdomen is distended or may feel pressurized due to the large mass of tumors present. However, lesions are often first detected at autopsy. Tumors vary from small nodules embedded in the kidney tissue to large lobular masses (Fig. 9) which replace most of the kidney tissue or which are suspended below the kidney and may be connected to the kidney by only a thin fibrous stalk. Tumors are often cystic and usually involve only one kidney. On cut surface epithelial whorls, cartilage and sometimes bone may be seen.

There is enormous variation in cell type and composition in these tumors. Epithelial structures vary from glomeruli and tubules to distorted tubules (Fig. 10) and cysts, to sheets of irregular undifferentiated cells with no tubular organization. Also epithelial cells may differentiate into keratinizing stratified squamous epithelial structures known as "pearls". The stroma may form sarcomas, cartilage or bone.

Figure 9. Nephroblastoma in a 16-week-old chicken inoculated with a field strain of ALV at hatching. (Photograph courtesy A.M. Fadly).

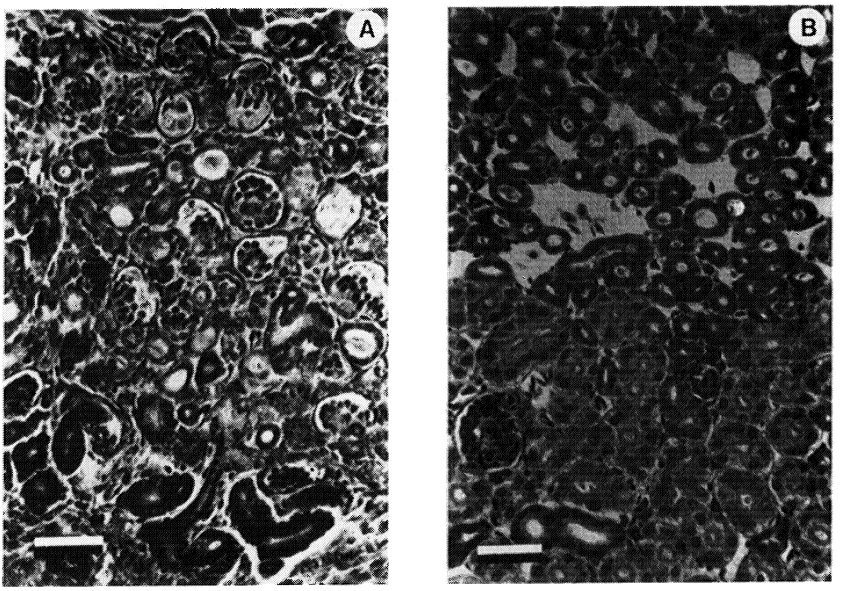

Figure 10. A) Nephroblastoma from a 3-month-old chicken infected as a 10-day-old embryo with myeloblastosis-associated virus (MAV-2). Note disorganized tubules and primitive-appearing nephrons. B) Normal kidney tissue from an unaffected portion of kidney adjacent to the nephroblastoma shown in a). Bar = 30 μm (Photographs courtesy of R.E. Smith).

12. OSTEOPETROSIS

12.1. Pathogenesis

This disease is most frequently seen in broilers on the processing line and in 8 to 12-week-old egg-type chickens. Experimentally, the disease can be induced in embryos and may occur at any time thereafter. The lesion is basically a proliferative or hypertrophic one which is considered by many to be neoplastic. After virus infection, the osteoblast precursor cells and osteoblasts multiply rapidly and quickly become osteocytes embedded in bone. Osteoclasts are more numerous in diseased bone than in normal bone (31). Thus the disease appears to be a neoplastic transformation of osteoblasts. Progression of the disease is severely aggravated by depletion of the humoral immune response by bursectomy or immunosuppression by ALV (36).

Figure 11. Chicken on left has osteopetrosis and was inoculated with ALV at one day of age. Both birds are 7 weeks old. (Photograph courtesy of V.L. Sanger).

12.2. Signs

Sometimes the first signs in affected birds are reluctance to stand or walk. More commonly, the first signs in broiler chickens are the hardened and thickened bones discovered at the processing plant. In egg-type chickens, a uniform or

irregular thickening of the diaphyseal or metaphyseal regions can be seen (Fig. 11). On palpation the affected areas are unusually warm. Birds with advanced disease have characteristic "bootlike" shanks. Affected chickens are usually stunted and pale and walk with a stilted gait or limp.

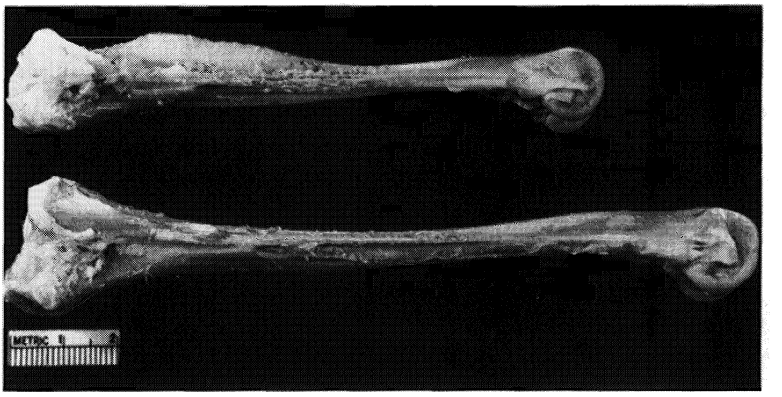

Figure 12. Tibia (top) from 60-day-old chicken with osteopetrosis. The other bone is from a normal chicken of the same age. (Photograph courtesy of V.L. Sanger).

12.3. Gross and histopathology

The first visible signs are usually a nodular growth on the femur (Fig. 12) or humerus of affected chickens. Lesions are usually bilaterally symmetric. The bone is spongy in early cases and rock-hard in advanced cases. Proliferation of the bone may be so extensive as to obliterate the marrow cavity (Fig. 13). Osteopetrosis and LL frequently occur together in the same bird. When LL is not present there is usually a general atrophy of the visceral and lymphoid organs, particularly the spleen which may be unusually small. Anemia is usually present in addition, and may be severe.

The periosteum over the lesion is greatly thickened and has a large number of basophilic osteoblasts (Fig. 14). The Haversian canals are larger and irregular in shape and the lacunae are more numerous, larger and have a different orientation than normal bone. Osteocytes are more numerous than in normal bone and are large and eosinophilic, in contrast to the new bone surrounding them which is basophilic and fibrous. Often lesions are focal and in cross section affect a sector of the bone.

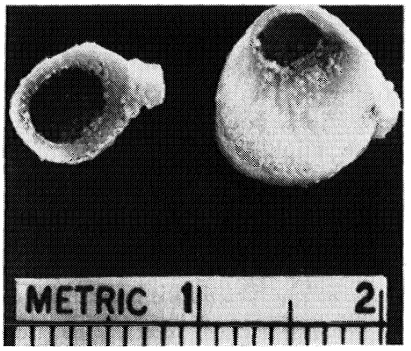

Figure 13. Cross sections of the tibia with osteopetrosis (right) and normal tibia (left) shown in Fig. 12. (Photograph courtesy of V.L. Sanger).

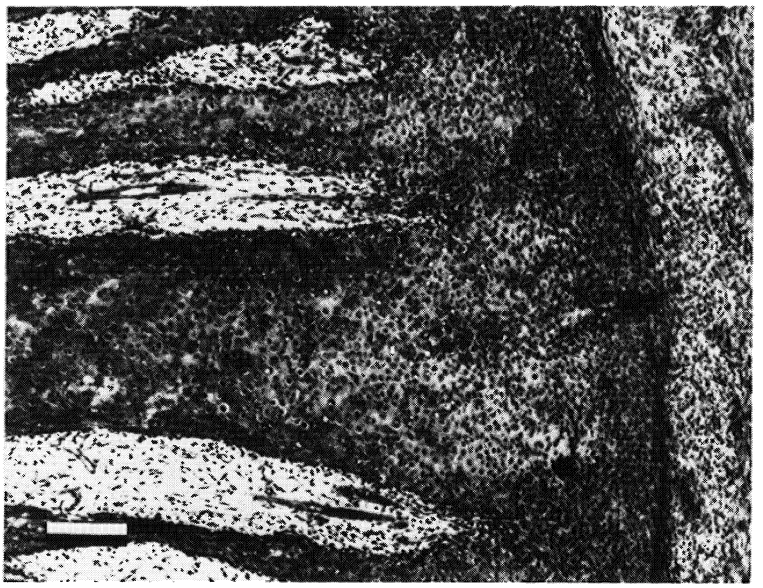

Figure 14. Osteopetrotic bone induced by MAV-2(0). The periosteum is thickened, and the vascular spaces are reoriented into a radial pattern. Bar = 200 μm. (Photographs courtesy of R.E. Smith).

13. CONCLUDING REMARKS

ALV infections are widespread in commercial flocks. ASVs and DLVs likely also occur in field flocks though there are no serologic tests which distinguish between ALVs, ASVs and DLVs. Economically important viruses of this group belong to subgroups A and B, and possibly E. The pathogenic response is not determined by the subgroup but rather by the virus strain; dose of virus; route of exposure; and age, genotype and sex of host. Non-neoplastic conditions such as reduced growth and stunting are probably more important economically than the tumors induced. Lymphoid leukosis is a tumor of the B-lymphoid cells. Erythroblastosis and myeloblastosis are tumors of the red and white blood forming cells. Nephroblastomas are tumors of the kidney. Under certain conditions, each of these tumors can be transplanted from chicken to chicken and cell lines can be developed from them. Osteopetrosis is a proliferative condition of the bone which is most common in male chickens, which have a depressed humoral immune responsiveness.

Unquestionably, LL is the most common in the field and is often confused with Marek's disease. Even though almost all flocks are infected with ALV, the incidence of LL is usually low. The incidence of ASV and DLV in field flocks is unknown. The reasons why LL does not occur in flocks that are known to be heavily infected with ALV are not completely known. It is likely that factors that affect the target cells, such as IBDV infection, greatly affect the occurrence of LL. Thus, the occurrence of significant LL outbreaks is unpredictable, but when such outbreaks occur they can devastate an egg-producing flock.

Most progressive poultry producing companies have active programs of ALV reduction. The adverse economic effects of both non-neoplastic and neoplastic conditions will be reduced though LL may not be completely eliminated as it has been reported in virus-free chickens (51).

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10

DIFFERENTIAL DIAGNOSIS OF LYMPHOID LEUKOSIS

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1. INTRODUCTION

Lymphoid leukosis (LL), a lymphoid neoplastic disease of chickens, is caused by viruses of the avian leukosis/sarcoma virus group. The avian leukosis viruses (ALV) belong to the subfamily Oncovirinae of the family Retroviridae (1). LL is characterized by a relatively long incubation period that is rarely less than 16 weeks. Proof that the target cells for transformation by ALV are the lymphocytes in the follicles of the bursa of Fabricius is well documented (2,3,4). Transformed bursa cells metastasize to the liver, spleen and other visceral organs leading to death of the host around the onset of sexual maturity or later. Diagnosis of LL on the basis of gross and microscopic changes is possible in most cases in which the bursa is involved. If typical LL bursal lesions are not present, it is important to make a differential diagnosis between LL and other neoplastic and non-neoplastic diseases that may be confused with it.

Neoplastic diseases that can be confused with LL are Marek's disease (MD), reticuloendotheliosis (RE), erythroblastosis, myeloblastosis and myelocytomatosis (1). Several non-neoplastic diseases should also be considered in the differential diagnosis of LL. Of these, the most important condition is the granuloma seen in chronic bacterial and fungal infections. These granulomatous lesions should be easily differentiated from the neoplastic lesions of LL on the basis of microscopic examination of affected tissues.

Of all the neoplastic and non-neoplastic disease conditions that can be confused with LL, emphasis will be given only to MD and RE. These three neoplastic diseases (LL, MD and RE) are recognized as separate entities because of their etiologic distinctness: LL, caused by ALV; MD, caused by avian herpesvirus serotype-1; and RE, caused by reticuloendotheliosis virus (REV), a retrovirus which is distinguishable from that of the ALV group. Despite this etiologic distinctness, the pathological manifestations of LL, MD and RE can be, in some cases, difficult to differentiate. MD is by far the most common neoplastic disease usually confused with LL. In contrast, REV-induced lymphomas are not known to be common in chickens (5). However, lymphomas similar to those induced by ALV have been described in chickens experimentally inoculated with non-defective strains of REV (6,7). Further, there is considerable serological and virological evidence of REV infection in chickens (5). As a neoplastic disease of chickens, LL is usually associated with infection with exogenous ALV. However, low incidence of lymphoid tumors pathologically typical of LL has been reported in breeding flocks maintained free of exogenous ALV (8,9).

A lymphoid neoplasia, lymphoproliferative disease, caused by yet another avian retrovirus unrelated to ALV or REV has been described in turkeys. To date, this neoplastic disease condition has not been reported in chickens (10).

2. CLINICAL SIGNS

The clinical signs of LL are not specific. The comb may be pale, shriveled and occasionally cyanotic. Emaciation and weakness occur frequently. Enlargement of the liver and bursa sometimes can be detected on palpation. Flock history may aid in the diagnosis of LL, since the disease almost exclusively occurs in chickens older than 16 weeks of age. Mortality usually peaks between 24 and 40 weeks of age.

In MD, involvement of the peripheral nerves may lead to partial or complete paralysis of extremities. Blindness may result from the involvement of the iris.

Although paralysis may be present in a few chickens in a flock, not every chicken is paralyzed. Because the clinical signs and gross lesions of MD often occur in chickens younger than 16 weeks of age, age of the flock at the onset of the disease may aid in differentiating MD from LL. However, MD is known to affect chickens of all ages. Other factors such as MD vaccination programs and sanitary practices should also be of importance. Lack of vaccination, improper use of vaccines or poor management practices may lead to outbreaks with MD. Because field strains of MD virus (MDV) can cause immunodepression, mortality from severe atrophy of lymphoid organs may precede mortality from tumors; however, this is rare in chickens hatched with maternal antibodies to MD. Most chickens affected with MD are depressed and dehydrated.

Because RE is not a naturally occurring disease in chickens, the clinical signs associated with this disease complex were described primarily in laboratory studies in which chickens were experimentally inoculated with REV or in studies that described chicken flocks vaccinated with REV-contaminated vaccines (5). The clinical signs of RE in chickens largely depend on the strain of virus involved. Non-defective strains of REV are capable of inducing a runting disease syndrome or chronic lymphoid neoplasia. Defective strains of REV can induce an acute reticular cell neoplasia. The clinical signs of REV-induced runting syndrome and acute reticular cell neoplasia have recently been reviewed (5). Clinical signs exhibited by chickens with lymphoma induced by non-defective REV could be similar to those of LL or MD, depending on the latency and site of lymphomas. Clinical signs in chickens that develop REV-induced visceral and bursal lymphomas after a long incubation period are similar to those of LL. In contrast, clinical signs in chickens that develop REV-induced non-bursal lymphomas, with nerve involvement, after a short latency may appear similar to those of MD.

3. ETIOLOGY

Virus-induced lymphomas in chickens are caused by two families of viruses, the retroviridae (ALV and REV) and the herpesviridae (MDV). A virus that induced visceral lymphomas in chickens, later called lymphoid leukosis virus, was first isolated in 1946 (11). The virus was soon recognized as a member of the avian leukosis/sarcoma virus group. In the early 1960s, MD was recognized as a separate entity from LL when visceral lymphomas accompanied by neural lesions became prevalent in broiler chickens (12). In 1968, MD was shown to be caused by a herpesvirus (13,14). The REVs were first isolated from turkeys in 1958 (15). Later

they were recognized as retroviruses distinct from the ALV group (5). A turkey isolate of REV (strain T) has been shown, following rapid passage in chickens, to consist of a replication-defective transforming virus that causes acute neoplasia and of a non-defective associated helper virus that causes immunosuppressive runting syndrome (16). The ability of the non-defective T strain of REV to replicate in cell culture and its inability to induce acute reticular cell neoplasia (due to lack of v-rel oncogene) distinguish it from the defective T strain of REV (5). All other known REV isolates appear to be similar to the non-defective T strain of REV. The immunosuppressive effects of non-defective strains of REV has recently been reviewed (5,17). Both B- and T-cell functions are severely suppressed in chickens inoculated with these non-defective strains of REV. It was only recently that these strains were also shown to be capable of inducing lymphoid neoplasms in chickens (5,6,7,18).

The structural components of the avian retroviruses and their replication were recently discussed in detail (1,5,19). Morphologically, these viruses are classified as C-type particles. MDV is a cell-associated herpesvirus and has a chemical composition typical for herpesviruses (20). In productive infection, cells are lysed and virions are released. In non-productive infection, cells remain intact and can become transformed to produce MD tumors (20). Since both ALV and MDV are ubiquitous, all commercial flocks and many chickens in a flock are probably infected with these viruses. Thus, isolation of ALV or MDV from a tumor or from a tumorous chicken does not necessarily indicate a causal relationship between the virus and the tumor. Except for testing chickens maintained under pathogen-free conditions, the demonstration of ALV or MDV has little or no diagnostic value. A number of test procedures for the detection of ALV and MDV have recently been reviewed (21,22). Unlike ALV and MDV, REV is not ubiquitous; thus, the demonstration of REV or antigen should be of significant diagnostic value. Tests for detecting REV have been described in detail (23).

4. PATHOGENESIS

4.1. Transmission

Congenitally infected chickens are considered the primary source of contagion. ALV infections in chicken flocks are mainly maintained via congenital transmission from laying hen to progeny (1). Many congenitally-infected chickens become immunologically tolerant to the virus and remain viremic for life. Congenitally-infected hens consistently shed virus into the environment and

transmit virus to a major part of their progeny. Males appear to have no role in congenital transmission of ALV (1). Viremic tolerant chickens or chickens exposed to virus early in life are the most likely to develop LL. Chickens exposed to ALV by contact can vary greatly in the frequency of birds that become permanently viremic and lack antibody or those that lose viremia and develop antibody.

MDV is recognized in almost all chicken flocks. The virus is readily transmitted horizontally to adjacent chickens, either by contact or it can be carried in dander or dust particles. The major source of infection is thought to be the cell-free virus in the feather follicle epithelium (20). Congenital transmission of MDV has not been detected and is not a factor in virus spread (20).

Although REV infection is not common in chicken flocks, the demonstration of REV antibody and isolation of REV confirm its existence (24). Several laboratory studies have demonstrated both contact and congenital transmission of REV, however, the efficiency of transmission appears to be low (5). To date, contact transmission seems more important than congenital transmission in maintaining REV infection. However, in the field, the source of virus for contact exposure remains unclear.

4.2. Target cells

Evidence that the B-cell in the bursa of Fabricius is the target cell for transformation by ALV is well documented (2,3,4). Interference with the normal development of the bursa by chemicals, hormones, viruses or surgery has been shown to significantly reduce or eliminate LL tumors (2,3,4,25,26).

There is considerable evidence that the target cell for transformation by MDV is a T-cell (20). Unlike LL, elimination of the bursa-dependent system appears to have no influence on the development of MD lymphoma (27,28,29,30); whereas, severe depletion of the T-cell has been shown to result in significant reduction or elimination of MD lymphoma (31).

Although there is histologic evidence that the predominant cell in the acute neoplasia induced by defective strains of REV is a mononuclear cell of the reticuloendothelial system, the identity of such cells remains equivocal. Neonatal bursectomy or thymectomy did not influence the incidence of these acute RE tumors (5). In contrast, there is evidence that the target cell in bursal-associated lymphomas induced by non-defective strains of REV after a long latency is a B-cell (6,32,33). The identity of the target cell in REV-induced non-bursal lymphomas has not been determined.

4.3. Lymphomagenesis

LL tumors start in the bursa of Fabricius. Lymphomas begin with preneoplastic lesions in the bursa referred to as transformed follicles. These transformed follicles can be seen microscopically as early as 4 weeks following infection with ALV at hatching (3,34,35). After a relatively long incubation period, transformed bursa cells metastasize to the liver, spleen and other visceral organs leading to the death of the host around the onset of sexual maturity. Recent studies have suggested that this long latency in LL is a property of target B-cells and is unrelated to maturational events of the host physiology (36). The molecular events leading to transformation of target B-cell by ALV have recently been discussed in detail (37). It is now believed that transformation of bursa cells induced by ALV is initiated by integration of a provirus near the host oncogene, c-myc, thereby activating the expression of the oncogene and triggering the transformation process.

Three phases of MDV infection have been described: (1) early lytic infection, (2) latent infection of lymphocytes, and (3) proliferative phase involving lymphoid and reticular cells (20). The lymphoproliferative phase may progress to MD tumor development. Unlike LL, MD lymphomas are composed of a mixture of neoplastic, inflammatory and immunologically active cells. Factors that regulate the molecular events in MD lymphomagenesis are poorly understood.

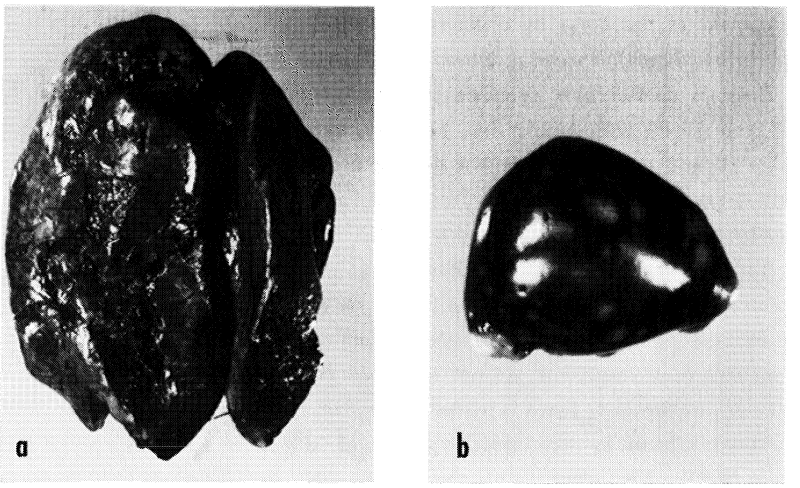

Figure 1. LL. Diffuse lesions in liver (a) and nodular lesions in spleen (b). Organs obtained from a 20-week-old chicken inoculated with ALV at hatching.

Recent studies have suggested that the biologic and molecular mechanism involved in the pathogenesis of REV-induced bursal-associated lymphomas are identical to those of LL (33,38).

5. GROSS PATHOLOGY

As stated earlier, diagnosis of LL on the basis of gross pathological changes is possible in most cases in which the bursa of Fabricius is involved. Grossly, LL nodules can be seen in the bursa of chickens older than 16 weeks of age, and in some of those killed at younger ages. Organs that are most frequently affected in LL include bursa, liver and spleen; other visceral organs are also affected but to a lesser degree. LL tumors are soft, grayish-white in color, diffuse or focal (Fig. 1). If typical LL bursal lesions (Fig. 2) are not present, LL and MD cannot be distinguished by gross examinations of the visceral organs. In such cases, the history, symptoms, gross and microscopic lesions and cytology all need to be considered in coming to a diagnosis of LL.

Unlike LL, gross lesions of MD can be and are often present in chickens

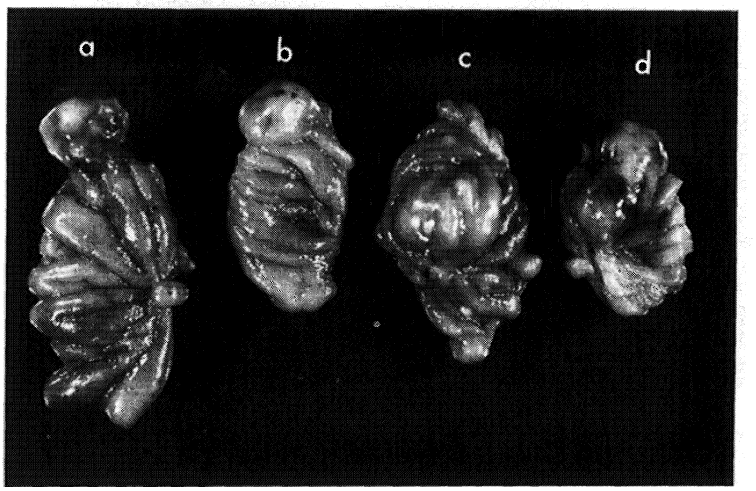

Figure 2. LL lesions in bursa of Fabricius from 16-week-old chickens inoculated with ALV at hatch. Distinct nodular tumor (a,b) and multiple tumors (c,d).

younger than 16 weeks of age. Grossly, nerve lesions are the most common findings in chickens affected with MD (20). The affected nerves, notably the N. vagus, the brachial and sciatic nerves, appear enlarged and are often oedemateus. The affected portion of the nerve may be two to three times that of normal size. There is also a loss of cross-striations and gray-yellow discoloration of the affected nerves. Solid MD lymphomas may occur in one or more of the following organs: lung, heart, mesentery, kidney, gonad, liver, spleen, pancreas, proventriculus, muscle and skin including comb (20,39). The gonads, especially the ovaries, are the most often affected organs.

Grossly, visceral lymphomas of MD, with the exception of the bursa of Fabricius, are similar to those found in LL. Tumors of the bursa are rarely seen in MD. In such cases, however, the tumors appear as diffuse thickening rather than the nodular lesions seen in LL. This diffuse thickening is due to the interfollicular infiltration by MD tumor cells. Both nodular and diffuse lesions can be found in various organs of chickens affected with MD.

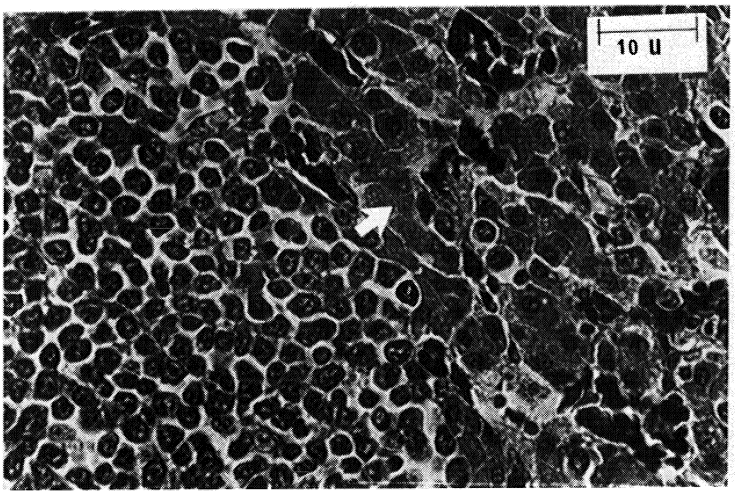

Figure 3. Microscopic lesions in liver from a chicken that died with LL at the age of 20 weeks pi, following ALV infection at hatching. There is a compression of hepatic cords (arrow). Also, note homogeneity of tumor cells.

The gross lesions of RE have recently been reviewed (5). Of the various pathological manifestations that can be induced in chickens by inoculation of REV, only the bursal-associated lymphomas can be confused with LL. The gross pathology of bursa-dependent lymphomas induced by non-defective strains of REV

are identical to those of LL. These REV-induced lymphomas resemble those seen in LL on the basis of organ distribution, pathology, bursa dependency, latency, surface IgM production and activation of c-myc (6,18,32,33,38). Thus, REV-induced bursa-dependent lymphomas can not be differentiated from those induced by ALV. Non-bursal lymphomas induced by non-defective REV have recently been characterized. Grossly, these lymphomas resemble MD since they can be seen in various visceral organs, as well as in the peripheral nerves, but not in the bursa (40).

6. MICROSCOPIC PATHOLOGY

Microscopically, LL tumors are focal and multicentric in origin. The tumor cells displace and compress the cells of the organ rather than infiltrating between them. The tumor cells are primarily immature lymphocytes (lymphoblastic) which are characterized by morphologic homogeneity (Fig. 3). These cells have a poorly defined cytoplasmic membrane, large cytoplasm and vesicular nucleus (1). LL tumor cells stain red with the methyl green pyronine stain because of the abundant amount of RNA in their cytoplasm. The demonstration of pyroninophilic cells in the bursal follicles has been used in the early detection of LL (3,35), since

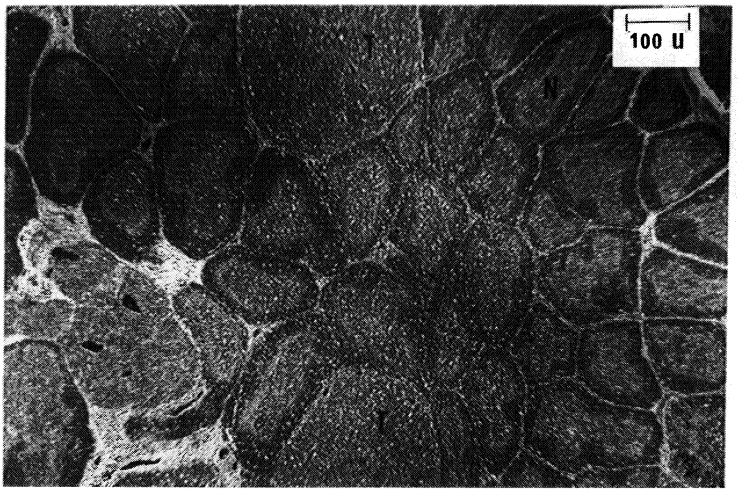

Figure 4. LL. Microscopic appearance of multiple transformed follicles in bursa of Fabricius. Transformed follicles (T) are clearly distinguishable from normal follicles (N). Also, note the intrafollicular infiltration of bursal follicles with tumor cells.

the bursa is the organ in which microscopic lesions of LL can be detected as early as 4 weeks of age. Further, the demonstration of pyroninophilic cells in wet fixed smears of fresh specimens has been used to differentiate LL from MD (41). In LL, the tumor cells are uniformly pyroninophilic, whereas only a few cells are pyroninophilic in MD tumors. The intrafollicular infiltration of bursal follicles with lymphoid tumor cells is pathognomonic for LL (Fig. 4). Recently, intracytoplasmatic viral matrix inclusion bodies accompanied by some swelling in myocardial cells have been demonstrated in adult chickens naturally infected with ALV (42).

Unlike LL, microscopic lesions of MD are characterized by the morphologic heterogeneity of neoplastic lymphoid cells (20). Small, medium and large lymphocytes, plasma cells and lymphoblasts may be present in peripheral nerves or visceral organs. This morphologic heterogeneity of lymphoid tumor cells (Fig. 5) is an important factor in differentiating MD from LL in the field. Neoplastic lesions of MD are rarely detected in the bursa. However, in such cases, infiltration of bursal follicles with lymphoid tumor cells is interfollicular rather than the intrafollicular infiltration commonly seen in LL. In most field cases, microscopic demonstration of heterogeneous lymphoid tumor cells in peripheral nerves should be considered pathognomonic for MD since nerve involvement in LL is absent (1,20).

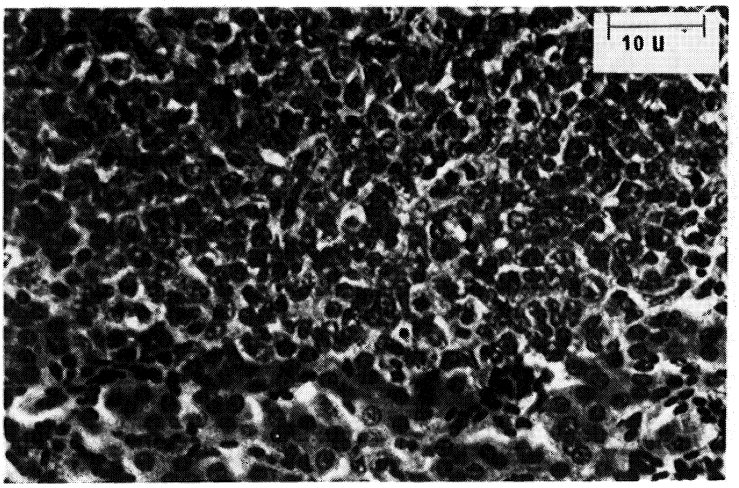

Figure 5. Microscopic morphology of MD lymphoma in liver of a White Leghorn layer hen affected with MD. Note marked infiltration by pleomorphic lymphocytes.

Depending on strain of virus, inoculation of chickens with REV can result in several distinct microscopic lesions in affected organs. Microscopic lesions of REV-induced acute neoplasia of the reticularendothelial system and acute runting syndrome have recently been reviewed (5). These lesions are very characteristic and are not commonly confused with those of LL. In contrast, the microscopic pathology of REV-induced bursal-associated lymphomas is identical to that of LL (Fig. 6). Microscopic bursal lesions, similar to those seen in LL, can be detected in chickens at 13-15 weeks of age, inoculated with non-defective REV at hatching (6,33). Microscopic pathology is of no value in differentiating these REV-induced bursal-associated lymphomas from LL. Further, by experimental inoculation of REV non-bursal lymphomas are induced, with microscopic lesions resembling MD, but these have been shown to lack the pleomorphic lymphocyte populations characteristic of an MD lymphoma (40). However, because REV-induced lymphomas do not naturally occur in chickens, a field outbreak with lymphomas should be provisionally attributed to LL or MD.

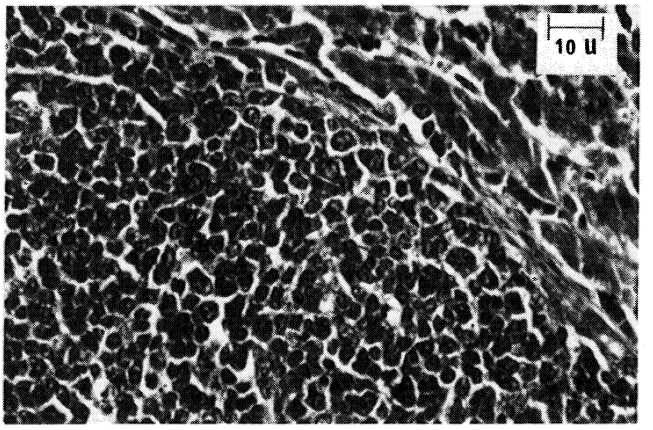

Figure 6. Microscopic morphology of REV-induced bursa-dependent lymphoma in liver obtained from a 24-week-old chicken inoculated with virus at hatching. Note the uniform lymphoblastic morphology of tumor cells (by courtesy of R.L. Witter).

7. SEROLOGICAL AND IMMUNOLOGICAL TECHNIQUES

As stated earlier, both ALV and MDV are ubiquitous viruses. Thus, except for testing chickens maintained under pathogen-free conditions, the demonstration of ALV or MDV antibody has little or no diagnostic value. The virus neutralization

test is the most sensitive test available for detection of ALV antibody (1). Because most field strains of ALV do not produce morphologic changes in cell culture, Rous sarcoma virus (RSV) pseudotypes are used in the virus neutralization test. Further, because ALVs are divided into five subgroups (A,B,C,D and E) (1), the diagnosis of ALV infection by virus neutralization test requires the use of RSV pseudotypes representing all subgroups. However, RSV pseudotypes of subgroups A and B are the most commonly used viruses in the virus neutralization test (21), since most field isolates of ALV belong to subgroups A or B.

Several serologic procedures for the detection of MDV antibody have recently been reviewed (22). Agar gel precipitation, immunofluorescence and virus neutralization tests are the most commonly used procedures for detection of MDV antibody. Recently, an enzyme-linked immunosorbent assay (ELISA) for the detection of MD antibodies has been described (43).

Unlike LL and MD, the demonstration of REV antibody should be of significant diagnostic value, since REV-induced neoplasia is not known to be common in chickens. In fact, the diagnosis of RE should not be based on only the gross and microscopic lesions, but also on the demonstration of REV or antibody (5). Virus neutralization, immunofluorescence, agar gel precipitation and ELISA have been used successfully for demonstrating REV antibody (18,23,44,45).

The demonstration by immunofluorescence of specific cell surface antigenic markers on the tumor cells is considered the best method to establish a definitive diagnosis of field cases of lymphoid tumors that could not be readily diagnosed on the basis of clinical signs and gross and microscopic lesions. During the last decade, several immunological studies of LL and MD lymphomas have demonstrated that: (1) the predominant cell in LL tumors is a B-cell; (2) IgM is expressed on the surface of LL tumor cells; (3) the predominant cell in MD is a T-cell; and (4) a Marek's disease tumor-associated surface antigen (MATSA) is expressed on the surface of a limited proportion of MD tumor cells (1,20). Most recently, an IgM-producing B-cell has been shown to be the predominant cell in REV-induced bursa-dependent lymphomas (6,32). The identity of the target cell in non-bursal RE lymphomas remains unclear, although recent studies have excluded B-cell markers from such lymphomas (40).

Differential diagnosis of LL and MD, on the basis of the presence of specific cell surface antigenic markers, by indirect immunofluorescent staining of tumor cells has been described in detail (46,47). Briefly, a single cell suspension prepared from fresh tumor tissue is reacted for 30 min with specific sera raised against B-cells, IgM, T-cells or MATSA. After washing, the cells are reacted for another

30 min with appropriate fluorescein isothiocyanate-conjugated anti-immunoglobulin. The stained cells are washed and then examined for specific fluorescence on their surface. Using this technique, LL tumors are characterized by a predominance of B-cells (90% or more) and IgM marker. In contrast, MD tumors are characterized by a predominance of T-cells (approximately 70%) and the presence of MATSA on 0.5-35% of tumor cells (20). Recently, a highly specific high titer monoclonal antibody against MATSA has been developed and proved to be of considerable diagnostic value (39,48). The use of indirect immunofluorescent staining of tumor cells is of no value in differentiating REV-induced bursal-associated lymphomas from LL, since the tumor cell in both lymphomas is a B-cell and expresses IgM. However, this technique should be valuable in differentiating LL from REV-induced non-bursal lymphomas, since the latter lack B-cell markers (40).

Table 1. Summary of the most useful criteria to differentiate lymphoid leukosis (LL) from Marek's disease (MD) and reticuloendotheliosis (RE) in chickens.

	Lymphoid Neoplasm[a]			
	LL	MD	RE	
			Bursal lymphoma	Non-bursal lymphoma
Nerve lesions	-	+(-)	-(+)	-(+)
Bursa lesions	+(-)	-(+)	+	-
Homogeneity of tumor cells	+	-	+	+
Predominance of B-cell and IgM markers[b]	+	-	+	-
Predominance of T-cell markers and presence of MATSA[b]	-	+	-	?

a) LL, lymphoid leukosis; MD, Marek's disease; RE, reticuloendotheliosis; + = present; - = absent; ( ) = rarely or occasionally; ? = not known.
b) Determined by indirect immunofluorescent staining of tumor cells.

Recently, molecular biological studies of LL tumors have shown that expression of c-myc oncogene is activated in the majority of tumors examined (37,49).

Based on this finding, the detection of altered c-myc oncogene has been used to confirm the diagnosis of LL in the field (E.J. Smith, unpublished data). This criterion has no value in differentiating LL from REV-induced bursal-associated lymphomas, since the molecular events leading to transformation in both lymphomas are identical (38). However, detection of integrated provirus with ALV and REV probes can be used to demonstrate the viral etiology of lymphomas. Whether detection of alteration of c-myc can be used to differentiate LL from MD is not known. Table 1 summarizes the most useful criteria that can be used to differentiate LL from MD and RE.

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11

LABORATORY DIAGNOSTIC PROCEDURES FOR DETECTING AVIAN LEUKOSIS VIRUS INFECTIONS.

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1. INTRODUCTION

Procedures for detecting infections with avian leukosis virus (ALV) have evolved from research on the properties of virus particles, cellular biology of virus multiplication, viral oncogenesis, response of the host to infection and on the epizootiology and control of lymphoid leukosis (LL) in chickens. Most of the test procedures have been based on the inter-relations found to exist between strains of avian sarcoma virus (ASV), which are rarely detected in chickens under natural conditions, and ALVs which are widespread in commercial poultry. Details on the structure of avian retroviruses and their interactions with cells in either in vitro or in vivo environments are given in other Chapters of this volume and general principles and techniques in diagnostic virology have been published (1). The intent herein is to review information on methods that have proven useful in detection of ALV and which may provide background for the development of more efficient procedures.

Although there are a number of ASVs, the strain referred to here is the Bryan high titer strain of Rous sarcoma virus (RSV). ALV and RSV are morphologically identical retroviruses whose genomes include env genes, which code for subgroup- or type-specific antigens; gag genes which encode internal group-specific antigens (gsa); and, pol genes which code for reverse transcriptase. ALV lacks an oncogene, whereas RSV possesses the src gene that encodes for the capacity to transform cells in vivo and in vitro. The gsa is common to all strains of ALV and ASV and comprises p27, p19, p15, p12 or p10, where p represents protein and the

accompanying figure represents molecular weight in kilodaltons (2,3). Since p27 is the most abundant protein in the virus particle (4), it is most widely used as the representative gsa.

RSV can transform cells but is replication-defective, in that it lacks the env gene and requires the contribution of helper ALV to produce the subgroup-specific antigens in the envelope (5). Consequently, RSV and helper ALV have the same subgroup-specific antigens (6). Helper virus found in stocks of RSV is called Rous associated virus (RAV) and these are laboratory strains of ALV. RAV-1, RAV-2, RAV-49, RAV-50 and RAV-0 are prototype viruses of subgroups A,B,C,D and E, respectively. The RAV acronym is parenthetically added to identify subgroups of RSV. For example, RSV(RAV-1) is an RSV of subgroup A. Defective RSV in cultures free of ALV is designated RSV(-) (7,8).

ALV and RSV can be conveniently cultivated in chicken embryo fibroblasts (CEF) and viruses of the same subgroup have the same host range. Phenotypes of cells susceptible to all subgroups are referred to as C/0 whereas designations C/A, C/B, C/C, C/D and C/E indicate cellular resistance to subgroups A,B,C,D and E, respectively. Cells may be resistant to a combination of subgroups. Subgroup A viruses have a broader host range than other subgroups in that they more readily infect other avian cells, such as quail cells. ALVs of subgroups A,B,C and D are exogenous viruses and cause LL and other neoplasms in chickens (5,7,9). Subgroup A virus infections are widespread in commercial flocks, subgroup B viruses are less frequently detected, and subgroups C and D have only been detected in Finland (10,11,12). Subgroup E viruses are not known to be oncogenic and although the genome of that virus is present in most chickens, it is usually only partially expressed (13).

An objective of this Chapter is to give an overview of methodology for detecting ALV infections and to indicate factors that influence sensitivity and specificity of tests. Interpretation of results and application of procedures are also considered. Details for conducting those tests which are most frequently employed, are given in the appendix.

2. COLLECTION AND HANDLING OF SPECIMENS

The likelihood of detecting virus, viral antigen or antibody in specimens is influenced by many factors including age at time of exposure (14). Chickens horizontally infected with ALV usually develop titers of virus neutralizing antibody which persist throughout life (15,16,17). Even in chickens with high levels

of neutralizing antibody it is frequently possible to isolate virus from white blood cells, spleen, oviduct and other tissues (14,18,19). Maternal antibody is transferred through yolk but according to Rubin et al. (15) the titers in progeny are 1/10th to 1/100th of that in the hen. Congenitally infected chickens may become immunologically tolerant in that they are persistently viremic and do not develop antibody to the subgroup of virus that initiated infection (15,20). Immunological tolerance can be induced by inoculating chicks up to 2 weeks of age with virus (21). Virus can usually be detected in any tissue from such chickens and from excretions as indicated below (14,22,23).

2.1. Specimens for detection of antibody

Serum, plasma and egg yolk are suitable as specimens for assessing the humoral immune response to infection. Blood can be treated with heparin at a level of 60 IU per ml or with 10% v/v of a 3.5% sodium citrate solution (24). If serum is to be collected, the tubes should be tilted to allow maximum surface for clotting. Serum and plasma should be inactivated at 56°C for 30 min. Antibody has been extracted from egg yolk by mixing equal volumes of yolk and phosphate buffered saline (PBS) and then adding two volumes of chloroform and thoroughly mixing at hourly intervals for 4 hours. The preparation is centrifuged and the antibody for harvest is in the upper clear saline layer (25,26,27).

2.2. Specimens for detection of ALV

Ideally specimens should be collected in sterile containers. Those expected to contain high levels of bacteria such as meconium or swabs from the vaginal, cloacal and oral cavities should be immersed in 1 ml of media containing up to 1000 IU of penicillin, 1000 μg of streptomycin, 100 μg of gentamicin and either 50 μg of fungizone or 50 IU of mycostatin (24). Since ALV is heat-sensitive, it is essential that specimens be collected on melting ice and be stored at temperatures below -70°C.

White blood cells can be separated from anti-coagulant-treated whole blood by low speed centrifugation. Plasma containing white blood cells is harvested and the cells are washed in culture medium (28). To facilitate gradient centrifugation, heparinized blood can be layered over a mixture of Ficoll (synthetic polymer of sucrose) and sodium metrizoate (29). When serum is to be tested as a source of virus, the clotting process can be accelerated by collecting approximately 5 ml of blood in tubes containing 0.1 to 0.2 ml of 25% heat-inactivated chick embryo extract and the blood is placed in an icebath when clotted (28).

Feather pulp from live birds, and liver and magnum of oviduct from freshly killed birds are rich sources of virus (23,30). These tissues can be added to tubes and after storage at -70°C the fluids can be assayed for virus (19,30). The titer of virus in the suspending medium can be increased by preparing a 10% homogenate and subjecting it to three cycles of rapid freezing and thawing (24). Grinding tissue may not be necessary for virus detection and is impractical if large numbers of specimens are being processed (19).

Albumen is collected by punching a hole near the small end of the egg and drawing off the thin albumen with a needle and syringe or by breaking the shell and collecting with a microtiter pipette. Virus may survive for more than 2 weeks in the albumen of eggs stored at 8°C. However, the virus is not stable under these conditions and thus it is desirable to collect albumen within a few hours of oviposition and immediately assay or store specimens at -70°C (31,32).

2.3. Specimens for detection of group-specific antigens

Specimens for detection of group-specific antigen (gsa) are usually collected in the buffer that is used for a particular test. Those for enzyme-linked immunosorbent assay (ELISA) are collected in PBS whereas those for the complement fixation (CF) test are collected in barbital or veronal buffer. Feather tips and other tissues can be suitably ground with an instrument such as a PT10 sawtooth probe generator (Brinkmann Instruments, Rexdale, Ontario). The homogenate can then be sonicated or freeze-thawed three times to release antigen (19). Egg albumen requires no special preparation but is easier to work with after having been diluted 2- to 4-fold in buffer. The gsa in albumen was shown to be stable for at least 63 days at 8°C (31).

3. IN VIVO ASSAY OF RSV AND ALV

In genetically susceptible chickens, RSV induces tumors at the site of inoculation and will also induce focal lesions on the chorioallantoic membrane (5,7). ALV strains vary in virulence and in vivo assay is essential for their characterization. In natural field outbreaks, cases of LL first occur as chickens approach sexual maturity whereas erythroblastosis, caused by the same virus, occurs at an earlier age (see Chapter 9). An in vivo assay system, in which chicks are inoculated at one-day-old, requires 270 days to assess susceptibility to development of LL. An assay based on the erythroblastosis response requires 43 or 63 days when the inoculum is administered respectively to embryos or one-day-old chicks (24,33).

4. IN VITRO ASSAY OF RSV AND ALV

Many variables inherent in the in vivo assay of avian retroviruses are eliminated by tissue culture procedures. Viruses are most conveniently cultured in CEF (34,35,36). A culture medium, which has been successfully used in a number of studies, consisted of a mixture of equal parts of medium F10 and medium 199 supplemented with 5% tryptose phosphate broth (TPB) and 2 to 4% calf serum (8,37). Penicillin and streptomycin, the combination of which is active against a broad spectrum of Gram-positive and Gram-negative bacteria, are added at rates up to 100 IU or 100 μg per ml of medium, respectively. Mycostatin is active against yeasts and molds and 40 IU per ml is included in media. Other commonly used antibiotics are gentamicin, active against Gram-positive and Gram-negative bacteria; polymyxin, active against Gram-negative bacteria; amphoterricin B, active in yeast control and terramycin, which is active against Mycoplasma organisms (35). Agar overlay media consisted of the same mixture of medium F10 and medium 199, supplemented with 5% calf serum and containing 0.9% purified agar (8).

Sensitivity of all tests involving the use of tissue culture are influenced by susceptibility of the cells to the virus. For example, true C/O cells or cells susceptible to all but subgroup E viruses are not readily available in all laboratories and consequently cultures are frequently prepared from pools of embryos, some of which are not susceptible to all subgroups. Variability can be reduced by storing stocks of cells in liquid nitrogen and pretesting aliquots for susceptibility. A convenient system is to grow primary cells in roller bottles, harvest within 3 days and freeze them in culture medium containing 5 to 10% dimethylsulfoxide (DMSO) and 15% calf serum. Secondary cells can then be used in virus assays.

4.1. Cytopathogenic effect of ALV

For practical purposes, field isolates of ALV are non-cytopathogenic since no prominent morphological alterations have been associated with these viruses in CEF maintained for up to 18 days. However, infected cells may grow in a disorganized pattern and become granular and refractile (38). Laboratory strains are more likely to produce morphological alterations and after 18 days post-inoculation (pi) with such viruses, contact inhibition may increase and cells may become epithelioid and grow more rapidly than fibroblasts (39).

Laboratory strains of ALV, such as RAV-1 and RAV-2, may under certain

conditions cause plaque formation (40,41). Subgroups B and D induced plaques in several lines of CEF, whereas subgroup A virus only made plaques in cells of the C/C phenotype. Temperature of incubation, incorporation of natural red dye in the second agar overlay culture medium and timing for this overlay were all important to the development of plaques (41). Fetal calf serum and certain batches of newborn calf serum were inhibitory to plaque formation (40,41). The addition of DMSO was also inhibitory (40). This technique may be useful for assessing amounts of ALV in stocks of defective RSV.

4.2. Transformation of cells by RSV

In contrast to ALV, RSV readily transforms CEF cultures. One virus particle is capable of transforming one cell into a new and stable cell type (33,42). While differences in the morphology of transformed cells are dependent on the RSV genome, in general the cells are rounded and refractile, lose contact inhibition, multiply and become multi-layered. Foci of such cells are usually evident within 3 to 5 days pi (22,33,43,44,45). There are, however, variations in the degree of infectivity of the various subgroups. RSV stocks of subgroup A usually attain titers 10- to 100-fold higher than viruses of subgroups B and C (46).

Many additives to culture medium influence the outcome of infection and some that have practical application in conducting test procedures are considered here. TPB greatly enhanced morphological transformation when added to culture medium (47,48). In contrast, fetal calf serum suppressed transformation when incorporated in agar overlay medium (49). The polyanion heparin inhibited infectivity of RSV of subgroup B, but not subgroups A and C (50,51). The synthetic polycations diethylaminoethyldextran (DEAE-D) and polybrene enhanced infectivity of subgroups B,C,D and E, but did not influence subgroup A (46,51). To explain these findings it was suggested that both viruses and cells have negatively charged surfaces and that the polycations neutralize the electrostatic repulsion whereas polyanions cause it to increase (51). The infectivity of subgroups B and C can also be increased by an "enhancing factor" released from cells chronically infected with ALV of subgroup A (RAV-1) (52,53). The factor acted on both cells and virus and increased adsorption of virus to cells and focus formation. It was postulated that the factor may have consisted of fragments of surface membrane that contained adsorption sites for virus. This effect was additive to that of DEAE-D (53). Infectivity was reportedly increased by incubating the virus with heterologous antiserum (46,54). Another factor which enhanced the multiplication of ALV subgroups B,C and D was found in the saline fraction of chloroform-

extracted chicken egg yolk. Since the egg yolk was free of antibody to ALV, it was suggested that the mechanism of action was different from that achieved with antiserum (27). Other factors that increased focus formation were raising the temperature from 37°C to 41°C and lowering the concentrations of Ca^{++} and Mg^{++} (48).

5. PRODUCTION OF VIRUS STOCKS AND ANTISERUM

5.1. Production of RSV

Virus stocks have been produced from wing web tumors that developed following inoculation of chickens with RSV. Virus yield is dependent on the dose used to induce tumors and yields decline after 3 weeks pi as antibody titers develop and cellular immune functions cause tumor regression (7,33). As in the case of in vitro studies, yields of subgroup B virus were usually lower than those with subgroup A. Yields of RSV can be increased by inoculating chickens that are immunologically tolerant to ALV of the same subgroup. Chickens can be rendered tolerant by embryo inoculation or by congenital transmission of ALV (20,55,56). Calnek (57) propagated virus in wing web and breast muscle of 3- to 5-week-old chickens. Tumors harvested 6 days pi were mixed 1:10 on a wt/vol basis with citrate buffer containing 0.001% hyaluronidase. The preparation was incubated 1 hour at 37°C with agitation and was then homogenized and centrifuged. Supernatant fluid was stored as stock virus. Consistent with the finding that individual clones of RSV produced 10 to 100 times as much ALV as RSV, stocks of RSV prepared from tumors were also found to contain 10 times as much ALV as RSV (33,58).

Stocks of RSV can also be produced by inoculating CEF known to be free of ALV with a high dose of RSV. The following day growth medium is replaced by medium containing half the percentage of agar routinely used in an overlay. Three days later, or when the majority of cells are transformed, the soft agar overlay is poured off and the cells are subcultured and maintained under liquid medium. Spent culture medium harvested 1 to 3 days after subculture is stored as virus stock (L.B. Crittenden, personal communication). Another in vitro approach is to produce stocks of virus in cultures of non-producer (NP) cells, transformed by RSV, which require a helper ALV for the production of complete infectious virus (33). Cultures of chicken embryo NP cells have been produced from a single focus of RSV-transformed cells that escaped infection with helper virus. To achieve this it has been necessary to suppress the growth and spread of helper virus by addition

of antiserum to medium and by co-cultivation of susceptible chicken cells with resistant duck embryo fibroblasts (59). The Japanese quail embryo fibroblast cell line, designated R(-)Q, is composed of NP cells transformed by an RSV which lacks envelope glycoprotein, gp85 (60,61). Quail cells are susceptible to subgroups A and E, but are resistant to subgroups B and C (8). When cultures of NP cells are inoculated with ALV to which they are susceptible, they produce RSV with subgroup-specific antigens of the helper. A single infectious unit of ALV is sufficient to activate production of RSV of the same subgroup (62). If the NP cells are true C/0 cells they could be activated to produce any of the subgroups of RSV by the addition of the appropriate ALV. Some stocks of chicken NP cells were found to produce subgroup E virus, RSV(RAV-0) (63). If the latter cells were used for producing stocks of subgroups A,B,C, or D, it would be necessary to passage the stock on C/E cells to eliminate subgroup E virus. Likewise, passage of any of the subgroups of virus in selectively susceptible cells can be used as a purification procedure.

5.2. Production of ALV

Virtually all tissues from chickens that are congenitally infected with ALV contain virus which can be extracted as described above for virus stock. Dougherty and DiStefano (23) detected 10^5 to 10^7 infectious units of virus per gram of tissue from various organs harvested from 42-week-old chickens. Comparable titers were detected in tissues from 19-day-old embryos. Egg albumen from infected hens is a convenient source of virus that requires a minimum of processing. Albumen from eggs stored for one day at 8^oC contained up to 10^5 infectious units of virus per ml (31).

Stocks of ALV can be produced in vitro by inoculating selectively susceptible CEF cultures and building up the level of infection by subculturing or by transferring fluids from old cultures onto freshly prepared cultures that are in an active state of growth. Alternately, cell cultures can be prepared from congenitally infected embryos. Culture fluids can be harvested as virus stock.

5.3. Production of antiserum

Virus neutralization tests indicate that RSV and its own helper ALV have identical subgroup-specific antigens but there may be immunological differences between RSV and ALV of the same subgroup. Nevertheless, neutralization of RSV was shown to be an acceptable indicator of anti-ALV activity (15,33). Subgroup-specific antiserum to RSV has been produced by inoculating 4- to 6-week-old

chickens in the wing web or breast muscle with a dosage sufficient to induce tumors that can subsequently be rejected. Chickens that have rejected tumors can be hyperimmunized by administering a second and much higher dosage of virus (54). Antiserum of comparable specificity has also been produced by intravenously inoculating 4- to 6-week-old chickens with a high dose of ALV.

Antibody to gsa has been produced by inducing tumors in hamsters or pigeons with RSV (64,65,66). An alternate approach that has resulted in greater yields of high titered polyclonal antibody, has been to immunize rabbits with chromatographically purified p27 antigen from avian myeloblastosis virus (67). Monoclonal antibodies against p27 have also been produced by hybridoma cell lines. The spleen cells from BALB/c mice for fusion with myeloma cells were from animals immunized with the following: RSV-transformed mouse cells (68), RSV of the Schmidt-Ruppin strain (69) or with purified avian myeloblastosis virus (70). Five non-overlapping antigenic determinants have been defined within p27 (69).

6. TESTS BASED ON INTERACTION BETWEEN ALV AND RSV

6.1. Resistance inducing factor test

The resistance inducing factor (RIF) test is of historical interest as it was the first in vitro method for detecting infection with ALV and was applied in producing the first flocks of chickens free of the virus (38,71,72). Protocols for conducting the test have been included in other reviews (5,33,35). While the test has been superceded by methods that are more convenient to apply, the principles and basic procedures are reviewed here because they can be applied in conjunction with other techniques. Rubin (38) demonstrated that cells infected with ALV were resistant to RSV of the same subgroup. This interference was associated with the interaction of the virus envelope with receptor sites on the cell surface (9,73,74). To render cells fully resistant would probably necessitate that all receptor sites on the surface of the cell would have to be covered with virus particles but not all of these particles would have to be infectious. Steck and Rubin (74) used the terms early and late interference for convenience in discussing the RIF phenomenon. Early interference was established by inoculating cultures with specimens containing 1 to 10 infectious units of ALV per cell and challenging 1 hour later with RSV (74). There was an increase in susceptibility to RSV when challenge followed ALV inoculation by 5 to 36 hours, presumably because ALV had entered cells and was no longer intimately associated with receptor sites on the cell membrane. This was followed by an increase in resistance which was likely due to

production of new virus particles that had associated themselves with receptor sites. The phenomenon of early resistance could be exploited as a method for detecting ALV and for comparing the biological properties of viruses. In contrast to early interference to RSV, which was associated with the adsorption of the ALV inoculum to the cell membrane, late interference was largely induced by ALV released from cultured cells subsequent to inoculation with low levels of virus.

For the RIF test, susceptible CEF are cultured for 1 to 3 weeks pi in order to build up levels of ALV prior to challenge with RSV. While RIF-infected cultures have been reported to be 4,000 to 10,000 times more resistant than uninfected controls, cultures are considered positive if there is at least a 10-fold reduction in numbers of RSV foci in ALV-infected as compared to uninfected cultures (33). The RIF test is useful for identifying subgroups of ALV. The test is highly specific and there is no evidence that unrelated viruses could induce resistance to RSV (74). Furthermore, RIF failed to induce resistance to other cytopathogenic viruses (75).

6.2. Non-producer cell activation test

The non-producer cell activation test, known as the NP test (59), is based on the rescue of RSV from NP cells. It is as sensitive as other tests described herein for detecting ALV, and detects all subgroups of exogenous virus. In the NP test, specimens are inoculated onto cultures of C/O cells transformed by RSV but that are free of exogenous helper viruses. Some lots of NP cells produce RSV(RAV-0) but this does not affect the test if the assay phase is conducted on C/E cells (63). Since NP chicken cells are difficult to produce, a useful modification is to replace these cells with R(-)Q cells (8,76). Since quail cells are resistant to ALV of subgroup B, it is necessary to co-cultivate C/E and R(-)Q cells in order to detect all subgroups of virus. For the mixing phase, C/E cells are plated and inoculated with specimen material and three days later R(-)Q cells are added. After 9 days of co-cultivation, cell-free fluids from these cultures are inoculated on C/E cells.

6.3. Phenotypic mixing test

Details for conducting the phenotypic mixing (PM) test are in appendix 11.1. The test is more convenient to apply than other tests for ALV and this accounts for its widespread use (63). In the mixing phase, C/O cells are inoculated with RSV(RAV-0) and with a specimen which could contain ALV of subgroup(s) A,B,C and/or D. The resultant pseudotypes of RSV would be the same as the helper viruses in the specimen and are detected by assaying fluids from these cultures on

C/E cells. Depending on the availability of cells of different phenotypes, the test can be altered to detect selected subgroups of ALV. For example, to detect ALV of subgroup B, specimens could be inoculated along with RSV(RAV-1) in the mixing phase and the assay for phenotypically mixed virus could be on C/A cells. Likewise, by inoculating C/0 cells with RSV of subgroup B in the mixing phase and then assaying on C/B cells, the test could be used to detect subgroups A,C,D and E. To detect the endogenous subgroup E virus (RAV-0), C/0 cells could be inoculated with RSV(RAV-1) and RAV-0 and fluids from these cultures could be assayed on C/A cells (63).

To compare the sensitivity of tests conducted at different times, controls should include cultures inoculated with serial dilutions of various subgroups of ALV. Negative controls should include uninoculated cultures and cultures inoculated solely with the subgroup of RSV that was used in the mixing phase of the test. While ALV is the most prevalent retrovirus in commercial flocks of chickens, ASV or REV could be present and could phenotypically mix with RSV (5,77).

6.4. Assays for endogenous ALV and chick helper factor

Crittenden et al. (8) adapted the NP test for the detection of RAV-0. Turkey embryo fibroblasts (TEF) co-cultivated with R(-)Q cells are inoculated with specimen material and 9 days later cell-free fluid is harvested for virus assay. Since the R(-)Q cells and TEF are also susceptible to ALV of subgroups A and D and to some subgroup C viruses, fluids from the first phase of the test are assayed on both C/0 and C/E cells. If foci develop on C/0 but not on C/E cells, the specimen is positive for endogenous but not for exogenous ALV. An alternate procedure is to inoculate TEF or susceptible CEF in parallel with C/E cells. After 9 days freeze-thaw cells and test for gsa in supernatant fluid by ELISA as described in section 7.2. Positive tests are those in which antigen is detected in extracts from the susceptible cells but not from the resistant C/E cells.

Chick helper factor (chf) is reported to be an autosomal dominant genetic determinant and represents the endogenous expression of subgroup E envelope glycoprotein in the cell membrane. The chf phenotypically mixes with the envelope glycoprotein of ALV or RSV that exogenously infects the cell and the result is production of fully infectious subgroup E virus (5,8,78). Whole blood or culture cells to be tested for the factor are mixed with R(-)Q cells and are then assayed on C/0 and C/E cells. Foci on C/E cells indicate contamination with exogenous ALV whereas foci on C/0 cells but not on C/E cells is evidence of chf or RAV-0. Thus, to determine if chf is present, it is also necessary to assay for

RAV-0 (8). Another approach is to inoculate susceptible cells, suspected of containing chf, and that are free of RAV-0, with subgroup B RSV of the Prague strain. Subsequently, cell-free supernatant fluid from these cultures is assayed on quail embryo fibroblasts that are resistant to subgroup B but susceptible to subgroup E. Foci in quail cells indicates that complementation of the viral genome with chf resulted in production of infectious subgroup E virus (78).

7. TESTS FOR ALV BASED ON DETECTION OF GROUP-SPECIFIC ANTIGENS

In addition to tests discussed below, soluble gsa can also be detected in tissue extracts by radioimmunoassay (79), immunodiffusion (80) and by autoradiographic procedures for detecting incorporation of ^{14}C amino acid into viral proteins (81).

7.1. Complement fixation test

The original complement fixation test for ALV (COFAL) (64) is an indirect method which detects all subgroups of exogenous virus in specimens. The procedure includes inoculating specimens onto CEF and subsequently testing extracts from cells for gsa. Usually several cell culture passages during 2 to 3 weeks are required for antigen build-up and the freeze-thaw method is used to release antigen from cells. Cells utilized in the COFAL test should be free of endogenous gsa but should be susceptible to all subgroups of virus (82). Extracts of inoculated and uninoculated cells are tested in parallel. Detailed protocols for conducting the test in microtiter plates have been reported (24,64). The test is specific for gsa of the leukosis/sarcoma viruses and does not detect REV antigens. COFAL is more difficult to perform than PM tests but the procedures are of comparable sensitivity (36,59).

A direct approach for detecting ALV infection in live chickens is to conduct direct complement fixation (CF) tests on egg albumen (31,37,76) or feather pulp (30,83). Egg albumen is particularly suitable as it is not anticomplementary and is less likely than other specimens to contain detectable levels of antigen of endogenous origin. The prozone phenomenon occasionally results in false negative readings but this is not a problem when albumen is diluted 4-fold (32).

7.2. Enzyme-linked immunosorbent assay

Protocol for conducting the enzyme-linked immunosorbent assay (ELISA) is given in appendix 11.2. ELISA is a versatile test procedure for detecting ALV infections. Reagents usually include IgG fractionated from sera against p27 and a

conjugate of anti-p27 IgG coupled to horseradish peroxidase (84,85,86,87). To reduce non-specific activity, conjugate may be diluted with normal serum. The test can be read photometrically and lends itself to computerized analysis of results. For example, standard error can be calculated for replicates in different quadrants of the plate (88). General information on the reagents, conjugation procedures and data manipulation for the enzyme immunoassay has recently been reviewed (89). Details for detecting gsa in polystyrene microtiter plates have been reported (84,85,86,87). Procedures include pre-coating wells with a purified preparation of anti-p27 IgG. These sensitized plates can then be stored several weeks at 4°C. Test plates can be washed in PBS containing either Tween 80 or Tween 20 (wash buffer) and specimens are added to individual wells. Subsequent addition of anti-p27 IgG horseradish peroxidase conjugate is preceded and followed by incubation and washing procedures. A substrate/chromogen mixture is added and plates are agitated during incubation to facilitate chromogen conversion. The wavelength selected for photometrically measuring absorbance is dependant on the chromogen used (89). A major concern has been non-specific activity. For example, wells at the edge of the plate may stain non-specifically. This prompted the recommendation to fill outside wells with buffer and to use the 60 interior wells for the test (1). The problem may also be reduced by maintaining a constant temperature throughout the plate during the test. One precaution is to avoid stacking plates. Also there may be less non-specific activity with monoclonal than with polyclonal antibody (70). Controls include negative specimens, serial dilutions of known positive antigens and wells coated with IgG but not containing antigen.

ELISA is more sensitive than CF for detecting gsa and can be used to detect antigen in specimens such as vaginal/cloacal swabs, that are frequently anti-complementary (14,84,85,86,87). ELISA can also be used in conjunction with tissue culture procedures for ALV assays and sensitivity has been comparable to that based on the phenomenon of phenotypic mixing. Cultures of a susceptible phenotype are inoculated with specimens suspected of containing ALV and the cultures are maintained for 9 days (83).

The presence of endogenous gsa in host tissues limits the usefulness of serological tests for accurately diagnosing infections with exogenous viruses. Determining titers of gsa in specimens can be useful since concentrations of antigen are usually higher in exogenous than in endogenous infections (28,83). It is also of diagnostic significance that some monoclonal antibodies against p27 have a greater affinity for antigen of exogenous than for antigen of endogenous origin (L.F. Lee, personal communication).

8. DETECTION AND DISTRIBUTION OF VIRAL ANTIGENS IN HOST TISSUES

In contrast to the CF test and ELISA, which detect soluble antigen, it appears that the following immunocytochemical procedures only detect antigen that is intimately associated with virus particles (19,90,91,92,93,94) or with virus matrix inclusion bodies (93). Distribution of virus and viral antigen is also of diagnostic significance.

8.1. Immunofluorescence

Methodology for preparing immunofluorescent conjugates to detect gsa in tissues has been described (19,95,96). In ALV-infected chickens concentrations of gsa and virus particles is usually confined to specific sites within organs (19). For example, in splenic tissue the stained gsa is associated with numerous intercellular virus particles interspersed among reticular cells of the sheathed capillaries, whereas in the magnum of the oviduct it is largely associated with virus particles lining the basal lamina of glands and the luminal border. Stained antigen in Lieberkühn glands from various regions of the gut is intercellular and the distribution is comparable to that in albumen secreting glands of the oviduct. However, fewer virus particles are usually detected in the gut compared to the oviduct. Frequently there are extensive concentrations of virus particles in myocardium (19). Liver is noteworthy as it had been shown by the COFAL test to be a rich source of virus (23) and yet no major concentrations of virus particles or immunofluorescent antigen are found in that organ (19).

8.2. Immunoperoxidase anti-peroxidase staining method

Details for immunoperoxidase anti-peroxidase (PAP) staining are in appendix 11.3 and 11.4. Sternberger's PAP staining procedures for light and electron microscopy have been applied to detect subgroup-specific (97) and group-specific antigens of ALV (93,94,97). The distribution of antigen in oviduct (Fig. 1) and other tissues stained by the PAP method is the same as detected by the immunofluorescence procedure (93,94). For light microscopic studies (94), antigenicity is best preserved by fixation of tissue sections in a 1:1 mixture of absolute acetone and absolute ethanol or in modified Bouin's fluid and embedding in low melting point paraffin (54°C). Non-specific staining associated with the PAP procedure is reduced by treatment of deparaffinized sections with 3% H_2O_2, 10% egg albumen and 3% normal swine serum. Immunohistochemistry includes treatment with specific rabbit anti-p27 serum, swine anti-rabbit serum as a

bridging layer and PAP soluble complexes. Immune complexes are marked by treatment with H_2O_2 and diaminobenzidine. After counter-staining, the sections are mounted to make them permanent (93,94).

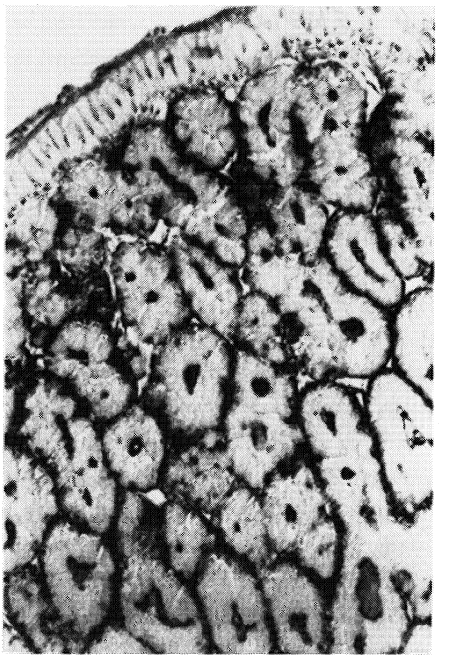

Figure 1. Magnum of oviduct. Diffuse positive immunoperoxidase reaction for group-specific antigen in interstitial tissue around tubules and in glandular lumina. Magnification X90. From (94), by kind permission.

For electron microscopy the procedure includes brief fixation in a mixture of aldehydic fluids with picric acid and dehydration in alcohols followed by embedding in an acrylic resin. Sections, mounted on 200 mesh nickel grids, are successively treated with rabbit serum against p27, swine anti-rabbit immunoglobulin and PAP. As in light microscopy, staining is achieved by treatment with H_2O_2 and diaminobenzidine and with 4% OsO_4 to increase electron density of their reaction product. The technique has been applied to study the genesis of viral matrix inclusion bodies which are largely confined to myocardium of adult chickens, naturally infected with ALV (93) (Fig. 2).

8.3. Protein A-gold method

Results with the protein A-gold staining technique (Fig. 3) and the PAP method were similar but an advantage of the protein A-gold method is that it does not obscure background around the antigen. Furthermore, no etching is necessary as the protein A-gold complexes will only attach at the surface of the section. Since the marker binds to the Fc portion of any fixed immunoglobulin, treatment of sections with normal serum is not suitable as a control. One control is to demonstrate that attachment of conjugated protein A-gold to the Fc portion of the anti-p27 immunoglobulin can be blocked by treatment with unconjugated protein A. Another is to demonstrate that when specific antiserum is omitted, the protein A-gold does not attach to viral antigen. Procedures followed are in appendix 11.5 (93).

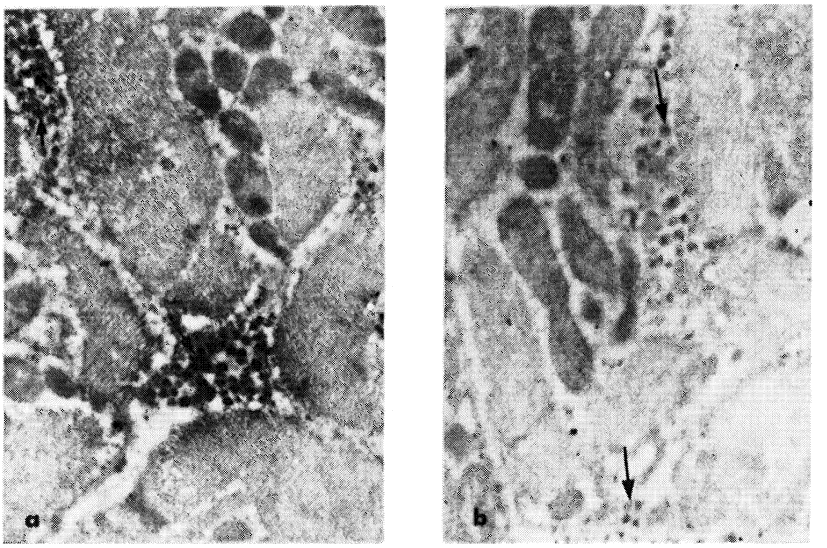

Figure 2a. Myocardium. Virus particles (arrows) stained with immunoperoxidase. Magnification X20,000.

Figure 2b. Myocardium. Virus particles (arrows) in a similar preparation treated with control serum. Magnification X20,000.

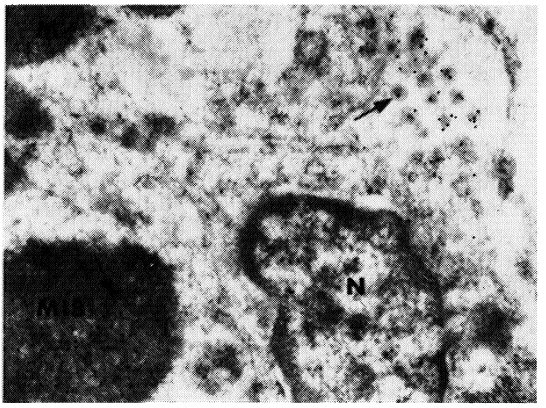

Figure 3. Myocardium stained with Protein A-gold. Gold particles associated with a matrix inclusion body (MIB) and with virus particles (arrows). Nucleus (N); Mitochondria (M). Magnification X42,500.

8.4. Light microscopy

The viral matrix inclusions of ALV can also be demonstrated in histological sections of myocardial tissues stained with Giemsa (Fig. 4). The inclusions are round or spindle shaped, stain basophilic and are irregularly distributed in the myocardium. Elongated inclusions run parallel to the long axis of the cell and are often two to three times longer than the cell nuclei (93).

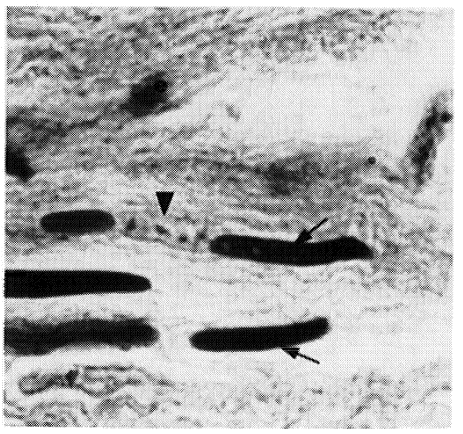

Figure 4. Histological section of myocardium stained with Giemsa. Basophilic intracytoplasmic matrix inclusion bodies (arrows). Nucleus (arrow head); erythrocyte (e). Magnification X320.

9. DETECTION OF ANTIBODY AGAINST SUBGROUP- AND GROUP-SPECIFIC ANTIGENS

9.1. Virus neutralization

The virus neutralization test is the standard procedure for detecting subgroup-specific antibody to ALV. Usually a constant amount of virus is reacted with varying dilutions of serum. As previously noted, various treatments can enhance infectivity of the virus. The starting dilution of the heat-inactivated serum is usually 1:5 or 1:10 because undiluted preparations cause non-specific inhibition. The virus-serum mixture is usually incubated for 40 min at 37°C, prior to assaying for residual virus on susceptible cells. One assay method is to drain cultures to be inoculated and then to add just enough inoculum to cover the cells. One hour later the inoculum is removed and the cultures are overlaid with agar (22,27). Another approach is to add virus directly to liquid media covering the cultures and then to replace this medium 18 hours later with an agar overlay (33).

Calnek (57) simplified the test for neutralizing antibody by conducting assays in multi-well plates. Since microscopic changes could not be evaluated in his plates, the indicator of infection was color change associated with increased metabolism in cells transformed by RSV. The test is now conducted in microtiter plates and infectivity is assessed by microscopic examination for transformation of cells (J.L. Spencer, unpublished data). Details are in appendix 11.6.

While neutralization of virus can be evaluated by inoculation of the chorioallantoic membrane of embryos and wing web of chickens, these are less sensitive than neutralization tests in cultured cells (5,15).

9.2. Other tests for antibody detection

Since virus neutralization tests are time consuming and require facilities for tissue culture, there continues to be a need for alternate test procedures for detecting subgroup- and group-specific antibody. Enzyme immunoassay techniques have been reported to detect higher titers of subgroup-specific antibody than the virus neutralization procedures (98,99). For the ELISA, plates are sensitized with a purified RSV (98) or RAV (99) antigen. The indirect peroxidase method of Mizuno and Keigo (100) uses ALV-infected CEF fixed on coverslips as antigen. These tests have not been evaluated for detecting antibody to natural infection with ALV. An immunodiffusion test for detecting subgroup-specific antibody has been described and more work is needed to assess the efficacy of the technique (101).

Since gsa is an internal viral antigen, the likelihood of it stimulating production of antibody is probably limited. However, such antibody has been detected by immunohistochemical methods (102) and by an ELISA blocking technique in sera from some chickens (84). The test is conducted as for the direct ELISA except that after plates are sensitized with IgG they are reacted with a solution of avian myeloblastosis virus protein prior to being treated with anti-gsa serum from chickens and then with conjugated IgG.

10. CONCLUDING REMARKS

Progress has been made in developing rapid and sensitive techniques for detecting ALV infections in chickens. However, these techniques have largely been based on detection of virus or viral antigen rather than on detection of antibody. Enzyme immunoassay has proven particularly useful in detecting gsa. Modifications to the test and the production of improved reagents, such as monoclonal antibodies, have been shown to increase its specificity and sensitivity. There is an urgent need for serological procedures to distinguish exogenous from endogenous ALV infections. Rapid tests for subgroup- and group-specific antibody, which are not dependent on assay in living cells, would greatly facilitate disease control programs. To improve laboratory diagnostic procedures there is a critical need for information on host-virus relationships. While a great deal is known about the virus there is less known about its replication in various tissues and organs of the living host. For example, results of virus isolation procedures suggest that virtually every cell in some chickens is infected with ALV and yet electron microscopy and immunohistochemical studies demonstrate that production of complete virus particles tends to be localized to specific sites within certain organs. Thus improvements in laboratory procedures are needed not only for detecting infection but also for elucidating pathogenesis and immune mechanisms of the host.

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11. APPENDIX

11.1. PHENOTYPIC MIXING TEST

For both the mixing and assay phases of the test, cultures of secondary CEF are prepared from cells recovered from storage in liquid nitrogen. Cells are cultured in equal parts of F10 and medium 199 supplemented with 4% calf serum, 5% TPB, 100 IU of penicillin, 100 µg of streptomycin, 40 µg of mycostatin and 2 µg of DEAE-D per ml (see section 4). Approximately 600,000 cells are plated per 35 mm plastic Petri dish. Procedures are similar to those reported by Okazaki et al. (63).

Mixing phase

Day 1. To a suspension of C/O cells is added sufficient RSV (RAV-0) to induce approximately 1000 foci per culture. Approximately 3 hrs after plating, cultures are examined to ensure that cells are viable. They are then inoculated with 0.1 to 2.0 ml of specimen material. For highly contaminated specimens such as cloacal swabs, the level of penicillin and streptomycin in the culture medium is increased 5-fold for at least a 24-hr period.

Day 2. Culture medium is changed and the level of calf serum is reduced to 2%.

Day 5. Culture medium may be harvested and stored at -70°C (useful in case cultures subsequently peel) and fresh medium is added to cultures.

Days 6 or 7. Culture medium is harvested and added to that stored on day 5. This preparation is then centrifuged at 2000 rpm for 10 min at a temperature of 4°C. The cell-free supernatant fluid serves as an inoculum for the next phase.

Assay phase

The second phase is performed in C/E cells.

Day 1. Approximately 3 hrs after plating, the cells are inoculated with 0.5 ml of supernatant fluid from the previous phase.

Day 2. Eighteen hrs pi liquid medium is replaced with agar overlay medium (see section 4). Additional medium is added at 1 to 3 day intervals.

Day 7. Cultures are examined for foci.

Controls. In both phases of the test at least one culture is uninfected. In the mixing phase at least one culture is infected with RSV(RAV-0) only, while others are inoculated with 10-fold serial dilutions of ALV of subgroups A,B,C and D.

11.2. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Specimen diluent

Phosphate buffered saline (PBS, pH 7.5)
NaCl, 8.0 g
KCl, 0.2 g
KH_2PO_4, 0.12 g
Na_2HPO_4, 0.91 g
Distilled water to 1000 ml.
Sterilize at 18 pounds pressure for 35 min and store at 4°C.

Wash solution

Phosphate buffered saline with Tween 80 (PBS-Tween, pH 7.4)
NaCl, 8.0 g
KH_2PO_4, 0.2 g
Na_2HPO_4, 1.2 g
KCl, 0.2 g
Tween 80, 0.5 ml
Distilled water to 1000 ml.

Diluent for pre-coating antibody
Tris (hydroxymethyl aminomethane)-HCl buffer (0.01 M, pH 9.0; 100x)
Tris-HCl, 24.2 g
Distilled water, 70 ml.
Adjust to pH 9 with concentrated HCl and then add distilled water to bring volume to 100 ml.

Conjugate.

Horseradish peroxidase conjugated to rabbit anti-p27 IgG.

Substrate buffer (0.05 M citrate, pH 5.0)

Citric acid, 9.6 g
Distilled water, 1000 ml.
NaOH, sufficient to adjust pH.

Substrate for horseradish peroxidase conjugate

Citrate buffer, 100 ml.
2,2'- azino-di-(3-ethylbenzthiazoline sulfonic acid), 2.5 ml; H_2O_2 (3%), 0.5 ml.

Test procedure

Pre-coat flat bottom wells of polystyrene microtiter plates with 200 µl of rabbit anti-p27 IgG diluted in 0.02 M Tris-HCl buffer (pH 9.0) to give an end product of 1 µg per ml. Store plates at 4°C for 18 hrs to 3 wks.

Automated and semi-automated equipment is available for washing plates. To wash manually, invert test microtiter plates containing pre-coat reagent over a sink, shake out contents and then rap the inverted test plate sharply on a flat surface covered with a clean paper towel and blot excess fluid. Fill all wells with PBS-Tween and allow to stand for 3 min. Shake out wells, rap on a paper towel

and blot dry. Regardless of equipment, wash wells 3 times and refill the wells after the third wash. Overflow of wells should be avoided.

With a vacuum system remove PBS-Tween from wells.

Add 50 µl of control and test antigen per designated well and incubate for 30-60 min. For this and subsequent incubations, plates are covered with a loose fitting lid and are held at room temperature.

Wash 3 times as in step 2.

Dilute conjugate according to manufacturers directions in PBS-Tween (wash solution) containing 5% swine serum and add 50 µl of diluted conjugate per designated well.

Incubate 2 hrs.

Wash 4 times as in step 2. The last wash is left in wells for 10-15 min before emptying the plate and proceeding.

Add 100 µl of freshly prepared substrate to each well and mechanically shake plates for 30 min.

The end result is a quantifiable chromogenic product that is expressed in optical density units. Alternately, the intensity of the reaction can be scored visually.

Positive antigen controls. Positive specimens are selected to give varying degrees of positivity.

Negative antigen controls. Specimens devoid of antigen should give little or no color change.

Conjugate controls. No test antigen is added to wells that are otherwise treated as are antigen controls.

Plate controls. No test antigen or conjugate is added to wells that are otherwise treated as are antigen controls. These wells serve as blanks for the automatic plate reader.

11.3. PAP FOR LIGHT MICROSCOPIC STUDIES

Thin (about 2 mm) slices of tissue are fixed for 30-60 min in a 1:1 mixture of absolute acetone and absolute ethanol or for 2 hrs in modified Bouin's fluid (picroformol with 1% acetic acid). The acetone-ethanol mixture gives stronger staining of gsa.

After fixation with modified Bouin's the tissues are dehydrated with rising concentrations of ethanol. Those fixed in acetone-ethanol are directly immersed in absolute ethanol. Tissues are then immersed for up to 1 hr in Histoclear (National Diagnostic Somerville, New Jersey).

Embed tissues in low melting point paraffin (54°C). 5 µm thick tissue sections are mounted onto glass slides and are quickly deparaffinized with xylene and are transferred through baths of 95% alcohol, 75% alcohol and water.

Tissue sections are treated for 5 to 10 min with 3% H_2O_2 to reduce endogenous peroxidase and are then rinsed in PBS.

Treat sections with a 10% solution of egg albumen for 30 min to further reduce non-specific staining. Excess albumen is removed by shaking the slide and this is followed by immersion of sections for 30 min in 3% normal swine serum.

Rabbit anti-p27 serum is applied to the slides for 1 hr in a humidified environment. Slides are then washed for 2 min in each of three baths of PBS. Control sections treated with negative rabbit serum are washed in separate baths.

Swine anti-rabbit serum (1:10) is applied to sections for 30 min. Slides are washed for 2 min in each of two baths of PBS followed by 2 min in a Tris bath. Excess buffer is removed by shaking.

Sections are treated with the PAP conjugate (Dako Corporation, Cedarlane Laboratories Ltd., Hornby, Ontario) for 30 min at 21°C and are then washed for 2 min in each of three baths of Tris buffer.

Sections are treated with freshly prepared and filtered preparation of 0.05% diaminobenzidine hydrochloride and 0.01% H_2O_2 in 0.05 M Tris buffer (pH 7.6) for 3 min and are then washed for 5 min in distilled water.

Counter-stain with hematoxylin or 1% methyl green, wash in distilled water and then dehydrate in 70%, 95% and absolute ethanol. Clear in xylene and mount.

11.4. ELECTRON MICROSCOPIC PAP METHODS

Specimens are fixed for 60 min in a mixture of 1-2% paraformaldehyde, 0.2-0.5% glutaraldehyde and 0.5% picric acid in 0.1 M PBS, pH 7.3.

Wash for 2 hrs in PBS; dehydrate for 2 hrs in increasing concentrations of ethyl alcohol.

Embed in soft LR White acrylic resin (London Resin Company, Basingtoke, Hampshire, England), polymerize at 55°C in gelatine capsules or in Lowicryl K4M (Balzers Union AG, Balzers, Lichtenstein), polymerize at -30°C by UV light.

Mount silver to pale gold sections on 200 mesh nickel grids and etch for 2-5 min in a solution of 1 vol of 2% NaOH in absolute ethyl alcohol and 1.5 vol. of distilled water. Wash with Tris saline.

Sections on grids are treated face down on droplets of sera put on strips of parafilm. Rabbit serum against p27, diluted in Tris saline (pH 7.6) and containing 1% normal swine serum is applied first.

Next swine antirabbit immunoglobulin diluted in Tris saline and then PAP containing 1% normal swine serum are applied. Sections are treated for 5 min before and after the two immunological steps with heat-inactivated swine serum diluted 1:30 with Tris saline (pH 7.6).

After PAP preparations are rinsed in 3 changes of Tris buffer and washed for 3 min with 0.0125% of 3.3' diaminobenzidine tetrahydrochloride (Polysciences Inc., Warrington, Pa., USA) containing 0.0025% H_2O_2 in Tris buffer and are then stained in 4% OsO_4 for 30 min and are weakly counterstained with lead citrate and uranyl acetate.

11.5. PROTEIN A-GOLD METHOD

Preparation of tissue sections mounted on nickel grids is as for the PAP staining method. Etching is omitted.

Sections covered for 10 min with filtered 0.5% bovine serum albumen in PBS (PBS-BSA) or 1% ovalbumen in PBS and blotted (must not dry).

Treat tissues face down on droplets of rabbit anti-p27 serum for 2 hrs at 21°C, rinse and wash for 5 min with PBS-BSA and treat for 1 hr at 21°C with protein A-gold (Protein A G10 EM-grade, Janssen Pharmaceutica, Life Sciences Division, Products Division, Toronto, Ontario, Canada). Rinse and wash in three changes of PBS-BSA (gently stir). Rinse with Millipore filtered distilled water and counterstain with uranyl acetate and lead citrate.

Prepare controls: One preparation is only stained with protein A-gold for 1 hr at 21°C. A second preparation is treated with specific antiserum followed by washing the sections and blocking the Fc portions of bound immunoglobulins with protein A for 1 hr at 21°C, rinsing and washing with PBS-BSA and staining with protein A-gold.

11.6. Virus neutralization

The test procedures which follow are suitable as a flock screening procedure and may not detect low levels of antibody. Culture medium is the same as used for the PM test.

Day 1. 40 µl of medium is dispensed in wells of a 96 well microtiter plate and to this is added 10 µl of heat-inactivated test serum (starting dilutions of serum are usually 1:5 of 1:10). 50 µl of culture medium containing sufficient RSV to induce 20 to 30 foci per well (when treated with negative control serum) is next added. Plates are then placed on a shaker installed in a 37°C incubator and are agitated for 40 min. About 45,000 susceptible CEF suspended in 100 µl of culture medium are next added.

Day 2. Eighteen hrs after plating cells the liquid culture medium is replaced with agar overlay medium. Additional overlay medium is added at 1 to 3 day intervals.

Day 7. Cultures are examined for foci.

Controls Positive and negative chicken serum and also calf serum are used in lieu of test serum.

12

INFLUENCES OF AVIAN LEUKOSIS VIRUS INFECTION ON PRODUCTION AND MORTALITY AND THE ROLE OF GENETIC SELECTION IN THE CONTROL OF LYMPHOID LEUKOSIS.

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1. INTRODUCTION

Although lymphoid leukosis (LL) was first described in 1868 (1), the economic significance of the disease has only recently been recognized. Mortality associated with LL tumors, the most common visible manifestation of avian leukosis virus (ALV) infection, is low and occurs mostly in older hens (2,3,4,5,6). Therefore, it was generally believed that the economic damage caused to the poultry industry by ALV infection was small, even though there was some suspicion, based on industrial observations that ALV infection impaired production traits (7). Higher mortality from ALV was observed only rarely, in flocks with a prevalence of congenitally infected chickens (8,9). The situation changed after it was shown that

G.F. de Boer (ed), Avian Leukosis.

non-neoplastic effects of ALV infection result in serious economic losses. Indications of such effects appeared in 1978 (10) and 1979 (11), followed by assessment of the size of such effects in White Leghorns in 1980 (12). Subsequently, several more studies, discussed in detail below, further elucidated the changes in performance associated with ALV infection.

The following aspects of economic damage caused by ALV infection will be examined: (a) frequency of ALV infection of birds as affected by the bird's genotype and the status of the population with regards to selection for production traits; (b) influence of ALV infection on production and mortality; (c) influence of ALV infection on variation and heritability of economically important traits; and (d) influence of ALV infection on genetic gains in populations under selection. The economic benefits expected from a reduction in the frequency of ALV infection or its complete eradication will be discussed along with the future role of genetic resistance to ALV in achieving and maintaining a poultry industry free of ALV.

2. FREQUENCY OF ALV INFECTION

The magnitude of overall effects of ALV infection depends to a large extent on the frequency of birds infected with ALV in the population. Environmental effects on the spread of ALV infection are discussed, along with other epizootiological phenomena, in Chapter 3 of this monograph. Therefore, only the influence of host genotype (genetic resistance of susceptibility to ALV infection) and of genetic selection for production traits on the frequency of ALV infection in populations will be considered here in more detail.

2.1. Effects of the host genotype on susceptibility to ALV infection.

As discussed in Chapter 3, genetic aspects of resistance to ALV infection at the cellular level are well understood (13). Resistance to ALV infection is highly specific for a virus subgroup and is inherited in a simple, Mendelian fashion (14). Investigations conducted in 1969 showed that most White Leghorn chickens are susceptible to infection with subgroups A and B of ALV while resistance seems more frequent in the meat-type and, at a somewhat lower level, in brown-egg laying birds (15). Later reports confirmed that the frequency of cellular resistance to ALV infection is very low in White Leghorns (11) while the low frequency of ALV infection observed among commercial parent stocks of meat-type chickens supported the earlier suggestion that they tend to be more resistant (16,17). Reasons for this difference between the frequencies of cellular resistance to ALV

in the two production types are not understood. Egg-type and meat-type stocks were originally synthesized from several breeds and the synthetic populations, from which today's commercial stocks originated, may have overlapped in terms of the original breeds used. For example, Leghorns may have contributed to the gene pool of meat-type stocks because they were used to introduce the gene "I" that inhibits the formation of feather pigment. Despite such overlaps, the frequencies of ALV resistance between the production types appear to differ dramatically. These differences need to be further examined in view of possible future efforts to increase resistance to ALV infection in commercial stocks.

The main difference between meat-type chickens and White Leghorns is the rapid growth and body conformation of the former, and the high egg production and commercially acceptable egg size and egg quality of the latter. They also differ in egg color. For example, a genetic association of resistance to ALV infection with rapid growth or association of susceptibility with high egg production could have resulted in the differences in resistance. Resistance or susceptibility to ALV infection is believed to be a matter of presence or absence of suitable ALV receptors on chicken cells that allow attachment and penetration of the virus (14). Genetic linkage or pleiotropy involving the receptor and production-related genes could explain the associations. For example, ALV and a hormone could share a cell receptor and this would result in pleiotropy. Unfortunately, direct scientific evidence on the genetic association of ALV resistance and production traits is not available. German researchers (18) developed Leghorn sublines, differing in resistance to ALV infection by selection on the basis of tumor formation after inoculation of the birds with Rous sarcoma virus (RSV) of subgroups A and B. Three sets of such lines, each set originating from a different genetic base population, were later shown to differ significantly in egg production rate (19). Unfortunately the birds in this test were naturally exposed to ALV, and therefore the effects of ALV infection and the possible genetic differences in egg production potential associated with resistance or susceptibility to ALV were impossible to separate from the effects of ALV infection itself.

2.2. Effects of genetic selection for production traits on frequency of ALV infection

Several studies of the frequency of ALV infection in selected and unselected strains demonstrated that the frequency of ALV-infected birds or ALV shedders was higher in unselected control populations than in populations subjected to selection for desirable performance (11,12,20). The reason for the different

frequencies is that individuals infected by ALV have a lower performance and thus have a greater chance of being eliminated in selection. This reduces the rate of congenital transmission of ALV in populations under selection. For example, in a study involving six high egg production-selected and three unselected control strains of White Leghorns over 2 consecutive years (12), the average incidence of ALV infection was 18.5% among 984 females of the control strains and 3.9% among 2799 females of the strains selected for high egg production and related economically important traits. The frequency of ALV infection was also compared between the females selected as breeders and the remaining females. Within the control strains, where female breeders were chosen at random, this difference was not significant, while the frequency of ALV infection was significantly lower among female breeders than among the remaining birds of the selected strains (12). The ALV infection status of males was found to be similar to that in corresponding groups of females (21). The incidence of ALV in cloacal swabs was 25.8% among 97 male breeders of unselected control strains, and 0.6% among 168 selected male breeders from strains selected for high egg production (22).

In another study involving over 1500 hens (20), the incidence of congenital infection with ALV was 5.2% among single cross progeny of selected strains, compared to 13.3% among the progeny of an unselected control strain. In the same study, the incidence of horizontally infected hens was similar in both the control strains (7.5%) and crosses of selected strains (8.8%). Consequences of such differences in the frequency of infection with ALV due to genetic selection for production characters will be further discussed later and the frequency of birds infected with ALV at various stages of propagation of commercial stocks from elite stocks down to commercial hybrids will be given special attention.

3. EFFECTS OF ALV INFECTION ON PRODUCTION AND MORTALITY

ALV infections can result in economic damage to poultry producers by (a) causing mortality from tumor development, and (b) by causing other damage to the host including mortality due to non-neoplastic effects of ALV. The mechanisms of the non-neoplastic effects of ALV are not well understood. It has been suggested that retroviruses, such as ALV, evolved from moveable genetic elements similar to those found in corn, bacteria, yeast and Drosophila (23). Retroviruses may in fact be the moveable genetic elements in vertebrate cells and may be able to transpose without an RNA intermediate, even though such transposition has not as yet been detected (24). Similar to the damaging effects of

moveable genetic elements in Drosophila (25), integration of DNA of retroviral origin (provirus) in the host cell genome may negatively affect cell functions. For example, an experimental insertion of a retrovirus into a mouse germ line resulted in a lethal mutation (26).

ALV was until recently believed to cause no cytopathic effects other than virus budding on the cell surface (27,28). However, intracytoplasmatic viral inclusion bodies were recently found in hearts of adult hens infected with ALV (29). Another slowly transforming avian retrovirus, reticuloendotheliosis virus (REV) is known to cause cytopathic effects in chick embryo fibroblasts (30). There is also evidence that these effects are not caused by integration of the provirus into the cell genome but may be due to synthesis of a viral gene product, or entrance of virus into cells (31). These investigations suggest possible mechanisms through which the non-neoplastic effects of ALV infection may be induced. ALV has been observed in a multitude of tissues (32) including endocrine organs and organs essential for immune response (33,34,35) and this may explain the wide-ranging impairment of performance in economically important traits of infected chickens. As discussed in detail in Chapter 7, ALV can directly suppress responsiveness of lymphocytes to antigenic and mitogenic stimuli, probably through the inhibition of interleukin-2 by T-helper cells. Such immunosuppression has been observed in pre-leukemic chickens. Therefore this mechanism may be one of the causes of the reduced performance and mainly of increased overall mortality in chickens with subclinical ALV infections. In this context, the recently reported high degree of homology among the avian v-myc, human c-myc DNA coding sequences (see Chapter 2), and that coding for interleukin-2 (36) may be of significance.

Various methods for the detection of birds infected with ALV or birds that shed the virus into the environment are dealt with in detail in Chapter 11. The discussion of the reduction in production performance associated with ALV infection that follows, summarizes data from numerous studies. The common denominator of these investigations is that they dealt with birds positive in tests detecting ALV or ALV-gs antigens and measured the changes in performance of these birds, compared with birds that were negative in the same tests. However, it should be kept in mind that the various authors used different laboratory test procedures and made sometimes use of different test materials for detecting ALV infection or shedding. Therefore, the sensitivities and the predictive value for congenital virus transmission of the tests were variable.

3.1. Influence of ALV on mean performance of chickens

ALV can affect production traits and mortality from causes other than LL only in birds that are susceptible to infection. The size of these effects may be influenced by type of infection (congenital or horizontal), age at exposure, and genetic make-up of the birds, as well as other factors. Data on the reduction in performance of egg production chickens that was associated with ALV infections are summarized in Table 1. Similar data concerning performance of meat-type birds are in Table 2. In all instances only differences considered significant by the authors were included in the tables. Overall, the observations of the various authors are quite consistent, considering that genetically different populations were tested under various circumstances and locations. Different rates of horizontal transmission of ALV are an indication of differences in experimental conditions under which the effects of ALV on production traits were studied. Rates of horizontal infection with ALV depend on the initial frequency of congenital infection, rearing environment, and other factors. As mentioned above, the incidence of horizontal infection was about 8% both in crosses of Leghorn strains selected for high egg production and in unselected control strains (20). In another study involving 13 commerical stocks of egg production chickens, 9% of embryos were found to be infected with ALV. By 7 weeks of age, 28% of the flock was viremic, indicating that 19% of the birds were horizontally infected (6).

In this connection it should be noted that the effects of rearing environment on the frequency of ALV shedders may be less pronounced than earlier suggested (40). It was recently shown that the method of rearing (cages vs. floor) had no effect on the frequency of ALV shedders and that differences between the performance of birds reared in cages or on the floor were generally much smaller than those between ALV shedders and non-shedders within the same populations (38). In another study, incidence of LL was 8.4% among 83 birds without maternal antibody, reared on rubber mats, while no LL was observed among 90 similar birds reared on wood shavings (G.F. de Boer, personal communication). The size of differences between the performance of ALV shedders and non-shedders was similar, regardless of whether they were reared in cages or on the floor (38). During the breeding period, when critical horizontal infections occur, plastic or rubber mats used in some flocks for covering wire floors of cages may make floor and cage rearing conditions similar to each other and this may, at least partly, explain the similar rates of horizontal ALV infections between flocks reared on floor or in cages.

Data in Table 1 document that ALV infection is associated with undesirable

Table 1. Reduction in performance of egg production chickens, associated with ALV infections.

Production trait	Age of birds or production period	Reduction in performance[a]	Ref.
Age at sexual maturity (days)		+3	(12)
		+15	(37)
		+6	(38)
Egg production per hen-housed	to 497 days	-25 to -30	(12)
	to 40 weeks	-18	(37)
	to 65 weeks	-23	(37)
	to 371 days	-29	(38)
Survivors' egg production	to 497 days	-17 to -24	(12)
	to 371 days	-17	(38)
Egg production rate (%)	to 497 days	-4.2 to -5.1	(12)
	to 371 days	-6.2	(38)
	7 mo period	-14 to -14.3	(39)
	8 mo period	-8	(19)
Egg weight (g)	at 240 days	-1.3	(12)
	at 40 weeks	-3.0	(37)
	at 280 days	-1.0	(38)
	at 371 days	-0.2	(38)
	at 450 days	-1.1 to -1.7	(12)
Egg specific gravity ($(-1)\times10^{-4}$)	at 240 days	-16 to -18	(12)
	at 450 days	-18 to -14	(12)
Fertility (%)		-2.4	(12)
		-2.0 to -4.4	(39)
Hatchability from fertile eggs (%)		-12.4	(12)
		-5.2 to -25.3	(39)
Mortality from all causes (%)	approx. 250 to 497 days	+5.5 to +14.8	(12)
	to 8 weeks	+4.7 to +17.6	(39)
	8 month laying period	+6	(19)
Body weight (g)	at 40 weeks	-19	(37)
	at 371 days	-17	(38)
	at 8 weeks	-82 to -120	(39)

[a] Means of test-positive minus test-negative birds. For (19): difference between lines selected for susceptibility or resistance to ALV infection. For (39), body weight: difference between progeny of dams positive and negative for ALV shedding.

changes in the performance of egg production chickens in economically important traits. In addition to the traits listed in the tables, albumen quality and percentage of eggs with blood spots were also compared between ALV shedder and non-shedder birds (12,20) and the differences observed were generally small and mostly insignificant. Despite the relative consistency of the results, significant interactions of ALV status and strain of chickens were observed, indicating differential responses of strains to ALV infection (12). For example, the size of

Table 2. Reduction in performance of meat-type chickens, associated with ALV shedding.

Production trait	Age of birds	Reduction in performance[a]	Ref.
Age at sexual maturity (days)		+3	(17)
Hen-housed egg production	to 385 days	-10	(17)
	to 315 days	-28	(17)
Survivor egg production	to 385 days	-8	(17)
		-3	(17)
Egg weight (g)	at 240 days	-0.3	(17)
Mortality (%)	from approx. 260 to 497 days	+2.3	(17)
	from 42 to 315 days	+29.0	(17)
Body weight (%)	at 42 days	-5	(17)
	at 4 weeks	-1.4 to -3.5	(16)
	at 7 weeks	-1.7 to -2.3	(16)

[a] Mean performance of test-positive birds minus test-negative birds.

the reduction in hen-housed egg production associated with ALV infection varied from a small, non-significant difference, to a reduction by 54 eggs, among the genetic strains of Leghorns tested in one study (12). Comparison of Table 2 with Table 1 shows that the negative influence of ALV infection on sexual maturity, egg production, egg weight, and mortality in meat-type chickens is similar to that found in White Leghorns. In addition, growth rate is also somewhat reduced in ALV test-positive birds. It is quite likely that adult body weight of meat-type breeders is also reduced since such reduction was observed in White Leghorns (Table 1).

As shown in Tables 1 and 2, several studies investigated the influence of ALV infection on mortality from all causes and found significantly increased mortality of ALV infected birds in both the rearing and adult periods. It is important to note that the increased mortality was due not only to higher mortality from LL but also to elevated mortality from reproductive disorders, fatty liver syndrome, and

Marek's disease (MD) (12,17). Mortality from LL often remained very low and in one instance, when mortality among ALV test-positive birds was 15.4%, compared to 5.0% among the negative birds, no mortality was associated with LL tumors in the entire population of about 2000 birds tested (12). The degree to which ALV infection results in increased mortality seems to depend on the general genetic make-up of the population. The three pairs of strains of chickens mentioned above were selected from three different genetic base populations for resistance or susceptibility to subgroups A and B of ALV. Two of the susceptible strains showed 1.5 to 12.1% higher adult mortality from MD than their ALV resistant counterparts (19). In the third pair of strains, the ALV susceptible strain had a lower MD mortality than the ALV resistant strain (19). Differences among strains of chickens with respect to the changes in their MD resistance that were associated with ALV infection were also observed in another study. Birds of six strains were exposed at 2 weeks of age to MD virus by contact and observed to 8 weeks post challenge. Only in one of the strains did the birds previously infected with ALV show increased susceptibility to MD when compared to ALV test-negative individuals (41).

Age at the time of infection plays an important role in determining the severity of reduction in the birds' performance and viability. Congenital infection with ALV seems to have the most severe effects. Lymphoid tumors were detected in congenitally infected birds about six times more frequently than in those infected by contact (42). When results of an egg production test from 20 to 40 weeks of age were compared between congenitally and contact-(horizontally) infected hens, LL tumor mortality was 18% in the congenitally infected hens, while no such mortality was observed in their contact-infected counterparts and the hens infected by contact produced 28 eggs more per survivor than those congenitally infected (9). Another study found ALV tolerant (congenitally infected) hens to have significantly poorer hen-housed and hen-day egg production than immune (infected) hens (21). Recently, the sizes of the effects of horizontal and congenital infection on performance were directly compared. Congenitally infected, in contrast to horizontally infected shedder hens that survived to 497 days of age, laid up to 5 eggs fewer and up to 1.3 g smaller eggs but matured 5 days earlier (20). An investigation that used experimental infections with ALV after hatching showed that infection prior to 4 weeks of age results in negative effects on egg production, while birds infected at older ages showed no such significant effects (43).

Rate of feathering in chickens is controlled by a single, sex-linked locus with

two alleles. Poultry breeders use these genes to identify males and females among newly-hatched chicks on the basis of feather development. A study of possible association of the feathering phenotypes with ALV infection and egg production indicated that when offspring or dams were of the slow-feathering type, the offspring experienced an increased rate of horizontal ALV infection compared with fast-feathering members of the same families (44). The increased infection rate seems to result from immunological tolerance to exogenous ALV infection induced by (glyco)proteins which are encoded by endogenous viral (ev) genes that are associated with slow-feathering genes (see section 3.4., and Chapter 7).

The reductions of egg production, growth rate, and other economically important traits in birds infected with ALV concern all components of the poultry industry. The overall adverse effects of ALV on performance of a flock depend on the size of the differences between performance of the test-positive hens and test-negative hens, and on the proportion of the flock that is infected with ALV (12). For example, if egg production of ALV-positive hens is reduced by 25 eggs and there are 20% positive hens in the flock, the average performance of the flock would be 5 eggs lower than the performance expected after ALV eradication. In a similar flock with 40% ALV-positive birds, the expected increase in the flock average from ALV eradication would be 10 eggs, etc.

3.2. Influence of ALV on variation and heritability

Increase in the average performance of a flock is only one of the benefits expected from ALV eradication. Additional improvements should result from reduced phenotypic variation, improved accuracy with which the birds' genetic potential can be assessed, and increased genetic gains (45). Besides effects on mean performance, ALV also increases variation in production traits. It was shown that an 8% incidence of ALV infection increased phenotypic variance by 2, 7, 10 and 13% for egg weight, age at first egg, egg production rate, and number of eggs per hen-housed respectively (46). The same study suggested that the expectation of the difference between phenotypic variation in a population free of ALV infection and in a population in which ALV is present is

$$E(\triangle_V) = \frac{n}{n-1} d^2 p(1-p) + pV_d,$$

where n = population size,

d = mean difference between performance, of test-positive and -negative birds,

p = proportion of test-positive birds, and

V_d = variance of d.

Processing and marketing of eggs and poultry meat puts a great deal of emphasis on uniformity of the products because processing lines are increasingly automated and also because of product standards. Thus even a small increase in variability such as that induced by infection with ALV is undesirable. Increased variation is also undesirable in poultry experiments or tests because it reduces the experimenter's ability to detect significant differences. It should be noted that the formula for estimating the increase in variance above (46) is applicable also to other situations where some members of a population are similarly influenced by disease or other factors altering performance.

ALV infection influences not only overall phenotypic variation but also variance components and heritability estimates. Sire variance components for age at first egg, egg number, egg production rate and egg weight were 3 to 18% greater in the population containing 8% ALV test-positive birds than when the data from the positive birds were eliminated (46). Corresponding increases in dam variance components were 5 to 48%, and in individual variance components (within dam, within sire) 2 to 13%. Changes in sire heritability resulting from the changed variance components amounted to -1 to +6%, and similar changes in dam heritability were -2 to +12%. Due to large standard errors of the heritability estimates, the significance of these effects was not established. Nevertheless, the results pointed out the need to consider effects of agents, such as ALV, in designing breeding plans and in data analyses.

3.3. Influence of ALV on genetic improvement of production traits and role of selection for production performance in eradication of ALV.

ALV infection could affect the rate of genetic progress from selection. The infection was shown to reduce performance and, therefore, the genetic potential for performance may not be adequately expressed in birds infected with ALV. Thus the influence of ALV can reduce the effectiveness of genetic selection for high performance. In the literature there is no evidence for a correlation between any production trait and susceptibility to ALV infection, or the size of effects of ALV infection, but it should be noted that no studies directly addressed this problem. The conspicuous susceptibility of certain strains of White Leghorns and relative resistance of some meat-type stocks of chickens to ALV infection, discussed above, may indicate the existence of such a correlation. However, if there are no significant genetic correlations between the birds' potential performance and susceptibility to ALV infection, the infection would only increase environmental variation and thus reduce heritability. Reduced heritability in

populations containing birds infected with ALV has been reported (46) and this reduction would be expected to reduce genetic gains from selection.

There are additional consequences of ALV infection in populations under selection for high performance. Some of the advances in the performance of selected strains, compared to unselected control strains, does not result from genetic gains but is created by a reduction of ALV infection in the selected strains. The size of this "false genetic gain" can be estimated as follows (12): The total selection response ($\triangle_S$) is the difference between mean performance of a selected and a corresponding control strain and can be partitioned as:

$\triangle_S = \triangle_G + \triangle_L$,

where G = genetic gain, and

$\triangle_L$ = "false genetic gain" due to reduction in the frequency of test-positive birds resulting from selection for high performance in a trait affected by ALV.

The size of the two components of $\triangle_S$ can be estimated as:

$\triangle_G = x_1 - x_2$.

$\triangle_L = q_2 (x_2 - y_2) - q_1 (x_1 - y_1)$.

where x = mean performance of test-negative birds,

y = mean performance of test-positive birds,

q = frequency of test-positives.

The subscript 1 corresponds to selected and 2 to unselected control strain.

The development of the relative sizes of $\triangle_L$ and $\triangle_G$ over an extended selection program is illustrated in Fig. 1. As the frequency of test-positive individuals in the selected strain is reduced by their elimination by selection based on performance, $\triangle_L$ increases until it is stabilized. Assuming that q_2 remains constant, $\triangle_L$ would reach its maximum when ALV is completely eradicated from the selected strain ($q_1 = 0$). Thus, relative to $\triangle_S$, $\triangle_L$ will tend to be high at the beginning of a selection program and its relative size will decrease with increasing $\triangle_G$ (Fig. 1). For example in four long-term selected strains and corresponding control strain, $\triangle_L$ relative to $\triangle_S$ was estimated between 4 and 14% for egg production and 3 to 7% for egg weight (12). The percentages of $\triangle_L$ would be expected to be high in the first few generations of a selection study. Therefore, short-term selection studies may give misleading results when agents

similar to ALV are present in the populations studied.

In commercial poultry breeding, genetic selection is applied in primary (elite) breeding stocks. The number of individuals in the elite stocks are in the hundreds or thousands. Two to three generations are usually required for propagation of the

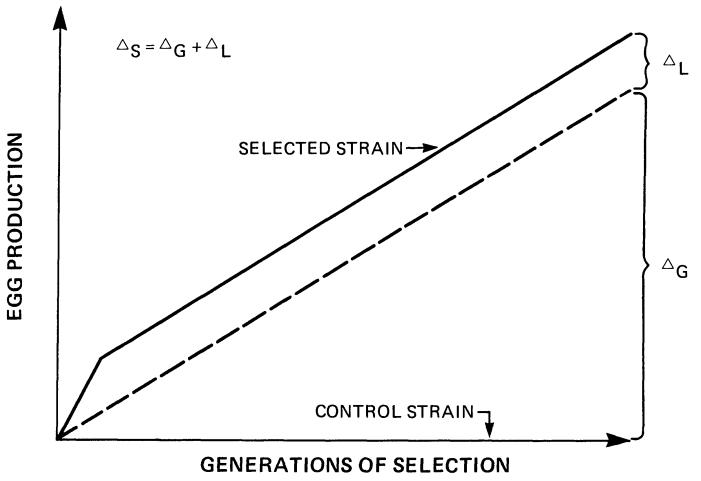

Figure 1. Total selection response ($\triangle_S$), expressed as the difference between the performance of a selected and an unselected control strain, and the size of true genetic gain ($\triangle_G$), and "false genetic gain" ($\triangle_L$) created by reduction in the frequency of test-positive birds, resulting from selection for high performance in a trait affected by ALV.

elite stocks before the final product, the commercial hybrid, is produced in sufficient quantities of hundreds of thousands or millions of birds. The grandparent and parent stocks used in this propagation are under relaxed selection. The possible exceptions are some meat-type stocks in which limited mass selection may continue during the propagation. The fate of the "false genetic gain" under these circumstances is illustrated in Fig. 2. The frequency of ALV test-positive birds will be reduced in the primary breeding stocks by selection for high performance and thus a "false genetic gain", $\triangle_L$, will be established when performance of selected stocks is compared with unselected controls. In the process of propagation under relaxed selection, the frequency of infected birds will again increase and this increase will result in the loss of $\triangle_L$. In practical terms a disappointing performance of the commercial hybrids may be observed when they are compared to the performance of the primary breeding stock. Depending on environmental circumstances, the frequency of ALV infection in commercial hybrid flocks will

vary and, obviously, could reach even higher levels than those in the genetic base. Under such circumstances $\triangle_L$ could reach negative values.

Genetic selection for high egg production when ALV is present in the populations was studied using a computer model to simulate the population

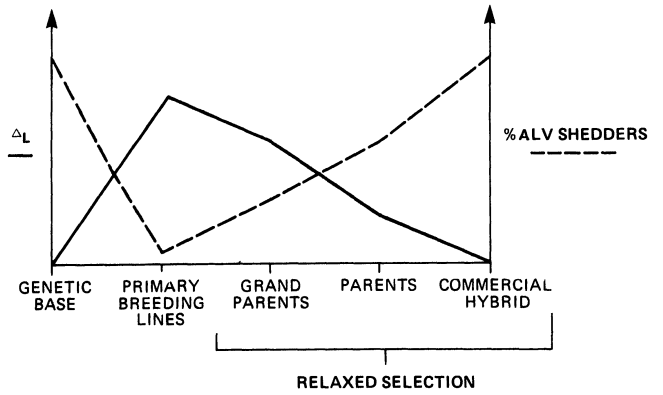

Figure 2. Changes in the size of "false genetic gain" ($\triangle_L$), corresponding to the changes in the frequency of ALV test-positive birds in the process of producing commercial hybrid chickens.

dynamics involved (47). The simulation suggested that selection for high performance in the trait influenced by the pathogen, based on performance of the individuals themselves, would be more effective than selection of entire families, based on family mean performance, in removing the infected individuals from the populations. Overall response to individual selection for high egg production was, under these circumstances, greater than to family selection. Despite such selection, the virus appeared to remain in most populations due to horizontal transmission when the populations were assumed to be housed with infected individuals from other populations. After this source of infection was removed from the simulations, selection for high egg production alone resulted in ALV eradication in about nine generations. The simulation results resembled results of certain experiments that were difficult to explain because they were not consistent with quantitative genetic expectations that did not allow for congenital transmission of non-genetic factors such as pathogens.

These results indicated that the presence of ALV or other similar pathogens in breeding populations needs to be taken into consideration in designing breeding

plans and selection experiments. The greater efficacy of individual selection, as opposed to family selection, in removing the infected selection candidates was also confirmed by practical poultry breeders (J.S. Gavora, unpublished data from commercial breeding companies). Separate housing of selected strains under eradication should improve the efficacy with which selection for high performance, in combination with elimination of ALV test-positive birds, would result in progress towards ALV eradication.

3.4. Effects of endogenous ALV

Besides exogenous ALV infections dealt with up to this point, there are indications that endogenous ALV infections, described in detail in Chapter 5, might affect production traits as well (48). These effects are believed to be rather small and may be either direct or may result from immunological tolerance of chickens expressing ev genes to antigens common to exogenous and endogenous ALV (49). In addition to concerns about possible effects of ev genes on economically important production traits, there are also indications that the presence of the proviral DNA sequences, homologous to the ALV genomic RNA, in the genome of chickens may be an important factor in ALV eradication. A recent study (50) compared the influence of ev gene expression on the response of chickens to inoculation, at one day of age, with ALV. Chickens negative for the ev2 and ev3 genes had a higher incidence of neutralizing antibody and a lower incidence or viremia than semi-congenic chickens positive for the same genes. Chickens of an unrelated line 0, completely lacking any ev genes, had an even higher production of antibody and lower rate of viremia than the semi-congenic chickens. From these results it appears that endogenous ALV may influence the birds' ability to mount an immune response to exogenous ALV. Birds that do not express or completely lack ev genes should, therefore, be easier to select for reduced exogenous ALV transmission because they would be less likely to subsequently transmit ALV vertically. The gene ev21 was recently found to be closely associated with the slow-feathering gene on the sex chromosome Z (51). This may explain the reduced ability of slow-feathering birds to mount immune response against exogenous ALV that results in increased rates of ALV infection (44).

Mortality from a non-neoplastic syndrome, that occurs after RAV-1 inoculation, was shown to be higher in ev2- and ev3-negative chickens than in their positive counterparts (50). Unexpectedly though, no such mortality was seen in the line 0 that lacks ev genes. Also, there was no significant difference among the semi-congenic birds in mortality associated with neoplasms. Therefore, the

effects of ev genes on mortality do not seem to negate the possible advantage from removal of the ev DNA from the genome of chickens. Further research is needed to establish more clearly whether ev genes have significant negative effects on economically important production traits. For this, large numbers of pedigreed birds free of exogenous ALV need to be tested both for the presence and expression of ev genes, and multiple production traits.

4. PROPECTS FOR THE FUTURE

The demonstration of the effects of ALV infections on mean performance, as well as on variation, heritability, and genetic gains, indicated the large economic damage that the presence of the virus is causing to the poultry industry. It is possible that similar situations may exist with regards to other pathogens and in other species of livestock. The losses due to ALV infections provide ample justification for an increased effort by researchers to achieve further understanding of the disease and to develop methods for its control. Eradication of the pathogen from the poultry populations appears to be the most desirable solution. Because of the present structure of the poultry industry, eradication of ALV needs to be accomplished first within the primary breeding stocks that are relatively small in size. Through propagation of these stocks large populations of commercial hybrids will automatically become free of ALV, providing reintroduction of the virus can be prevented.

Successful eradication of ALV subgroups A and B from commercial breeding flocks was reported as early as 1975 (52), but concentrated efforts by many poultry breeding companies, did not commence in earnest until the 1980's, and are currently underway. This effort was made possible by the new developments in the techniques for detection of ALV infection and shedding (53,54) and was motivated by the large economic damage from the infections discussed above. Although none of the companies made written claims that their products are free of ALV, several have announced successful reductions in the frequency of ALV shedders and have unofficially informed their customers that their stock is free, or close to being free, of ALV. Eradication of ALV is further discussed in Chapter 13.

Once eradication of ALV from commercial flocks is successfully accomplished, there will continue to be a risk of reinfection. To achieve permanent eradication, it will be desirable to make chickens genetically resistant to ALV infection. This can be regarded as the second phase of ALV control, and the accomplishment of this goal will become easier, once exogenous ALV is elimi-

nated from the flocks. It has been clearly established that variation in resistance to infection with ALV exists. There is strong evidence that resistant chickens lack specific virus receptors in their cell membranes (14). This mechanism of resistance agrees well with the observation that susceptibility to ALV is a dominant trait because the presence of a single allele coding for the receptor substance renders an individual susceptible to ALV infection. A dominant trait is usually easier to eliminate from a population because, unlike a recessive trait, it is expressed in both homozygotes and heterozygotes. Genetic resistance to infection with ALV and methods for its detection were recently reviewed in detail by Payne (14).

Responses to subgroups A, B, and C of ALV are determined by corresponding autosomal loci Tv-A, Tv-B (55), and Tv-C (56). Resistance to subgroup D viruses seems to be controlled by Tv-B (32). Genetic resistance to subgroup E virus appears to involve two autosomal loci (57), although the existence of one of these was disputed (58). From a practical point of view, resistance to subgroups A and B is most important because subgroups C and D of the virus are very rare. Endogenous subgroup E viruses are produced by the chickens from the proviral DNA that is a permanent part of their genomes. Resistance or susceptibility to ALV infection can be determined by inoculation of chick embryo fibroblasts with RSV of corresponding subgroups that cause neoplastic transformation of the cells. The transformation is observed as discrete foci of tumor cells (59). Resistance can be similarly detected in cultured feather pulp cells (60). Inoculation of RSV onto the chorioallantoic membranes (CAM) of 11-day-old embryonated eggs results, after another 8 days of incubation, in formation of discrete tumors, visible as pocks on the CAM (61). Membranes from resistant embryos respond with fewer or no pocks (62). Tumor incidence or mortality after subcutaneous or intramuscular inoculation (63, 64) or by intracerebral inoculation (65) with RSV can also be used as indicators of resistance to infection to ALV.

Breeding for resistance to ALV infection requires an accurate assay allowing the breeder to clearly distinguish between resistant and susceptible candidates for selection. The method has to be applicable on a large scale and should provide results rapidly because commercial breeders have to select large populations usually within a short time period. Also, the test should not hinder the individual's utilization in producing the subsequent generation. The use of cultured feather pulp cells (60) provides information on the resistance of the selection candidates, but is difficult to apply on a large scale. Other assays mentioned above do not allow utilization of the tested individuals in further breeding and can thus only be

used in testing relatives of the candidates for selection. The best practical approach seems to be their use in progeny testing. The disadvantage of this approach is the need to test several progeny resulting from mating the selection candidate to tester individuals known to be homozygous for ALV resistance. Detection of a single susceptible individual in the progeny of such matings indicates that the tested parent carries at least one allele for susceptibility. The probability of detecting susceptibility increases with the size of the progeny groups tested. Clearly, rapid and reliable methods, allowing direct determination of susceptibility of the selection candidates to ALV, are required for the selection for ALV resistance in commercial poultry breeding. Resistance due to presence of interfering ALV in the tested individuals or tissues needs to be excluded in order to obtain reliable test results. This re-emphasizes the suggestion above that selection for ALV resistance would have the best chance of success when applied in populations free of the exogenous ALV.

As already mentioned, associations between genetic resistance to ALV infection and production traits have not yet been sufficiently investigated. Information about this relationship needs to be obtained before a concentrated effort to make commercial stocks resistant to ALV infection is made by the industry. Finally, it should be emphasized that the recessive nature of ALV resistance dictates that all primary breeding populations used in the production of a commercial hybrid have to be resistant to ALV in order to have a resistant final commercial product. Today's commercial stocks are produced as crosses of a least two but mostly of three or four primary lines (see also Table 2 of Chapter 13) and this makes the achievement of genetic resistance more difficult. The genetic progress towards resistance may be expected to be more rapid in populations such as some meat-type or brown egg producing laying-type stocks, where the initial frequency of the resistant alleles appears higher than in White Leghorns (15).

Another possibility for a rapid introduction of resistance to ALV infections arises from the potential application of molecular genetics and micro-manipulation techniques. Viral genes coding for envelope glycoproteins for subgroup A and B viruses could be inserted into the genome of chickens. The glycoproteins that would be then produced by such engineered chickens may block cell membrane receptors and render the birds resistant to infection (14).

A third phase in dealing with ALV may be the removal of endogenous ALV from the genome of chickens. However sufficient justification for such efforts, which would likely be time consuming and costly, would have to be provided by research results.

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APPROACHES TO CONTROL AVIAN LYMPHOID LEUKOSIS

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1. INTRODUCTION

In the past, the eradication of lymphoid leukosis (LL), the most common pathological manifestation of avian leukosis virus (ALV) infections in chickens,

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could only be pursued by elimination of all diseased birds from a given flock. This approach was only successful if the initial incidence of ALV infection was low. Waters and Prickett (1) obtained an ALV-free flock (line 15^I) at the Regional Poultry Research Laboratory, East Lansing, Michigan, by careful selection and isolation of families having low incidence of the disease. There are a few earlier reports on leukosis control, but at that time the etiology of the Avian Leukosis complex was not clarified, and it is unclear whether the described investigation referred to LL or classical Marek's disease (2).

Control procedures, aimed at the interruption of virus transmission between successive generations, became available after Rubin had discovered the interference phenomenon of Rous sarcoma virus (RSV) multiplication in ALV-infected chick embryo fibroblast (CEF) cell cultures, the resistance-inducing factor (RIF) (3). The RIF test subsequently developed, demonstrated the importance of congenital virus transmission for ALV epidemiology (epiornithology) (4,5,6). The term congenital transmission describes the form of vertical virus transfer in which infection prior to hatching is established by virus released from the infected hen. Congenitally infected birds are able to disseminate or shed large quantities of virus into the environment. The term shedding refers to the release of virus from the host into the environment, including the egg. Congenital transmission describes infection of the embryo and is therefore not synonymous with shedding (see Chapter 3).

For a long time eradication of LL from poultry was considered only to be of scientific interest for avian retrovirus research and to producers of poultry vaccines. Industry showed little interest in LL control because lymphoma formation was usually at a relatively low level with increased losses only on occasion (7,8,9,10,11). In some regions of the world e.g. a few provinces of India, however, LL mortality seems to remain constantly at a high level (12). During the 1970s, a renewed interest in LL control arose after Marek's disease problems had been solved by vaccination and it had been recognized that ALV infection reduced the productive performance in various ways (see Chapter 12). LL eradication became even more important when epidemiological studies suggested that the rates of ALV infection had increased during the 1970s. Studies on the incidence of ALV infections in various poultry flocks in the USA and Israel during the late 1960s reported ALV infection rates not to exceed 30% (7,8). Recent epidemiological studies, performed in the USA, the Netherlands and Australia, indicate ALV infection rates occasionally to be considerably higher (11,13,14,15). In general, the rate of ALV infections in breeding stock is lower than in hybrid end-product

chickens. The incidence of ALV infections is usually higher in laying-type chickens than in meat-type flocks. In Australia, however, the reverse situation seems to occur (13).

Relatively few vaccination studies with avian retroviruses have been performed. Up to date application under field conditions has been seriously entertained only in the Netherlands (16,17), but the interest in LL vaccination is increasing in other parts of the world (18).

2. DETECTION OF CONGENITAL ALV TRANSMISSION

2.1. Virus recovery tests

The accurate identification of hens which congenitally transmit infectious ALV to their progeny is crucial for the success of LL eradication. Test procedures, therefore, have to be evaluated for their predictive value in indicating virus transmission to embryos.

Until the early seventies only two infectivity assays, the RIF test (3) and the complement fixation test avian leukosis (COFAL) (19), were available for the detection of congenital virus transmission. Both tests were too laborious for application in large scale LL control programmes. The assays became less cumbersome after the further development of the non-producer cell activation (NP) test by Rispens et al. (20,21), and of the phenotypic mixing test (PMT) by Okazaki et al. (22). The essentials of these tests were described earlier (23,24). The NP and PM tests were shown to be of equal sensitivity as COFAL. The group-specificity of the COFAL, NP and PM tests for exogenous virus subgroups rendered them more efficient in surveys for infection than the subgroup-specific RIF test. In the early studies aimed at the eradication of LL from breeding flocks the RIF test (6,25) or the NP test (16,17) was employed. The various laboratory techniques are described in detail in Chapter 11.

2.2. Detection of group-specific antigens

The development by Spencer et al. (26) of the (direct) CFT for the detection of group-specific antigens (gsa) made routine testing of large numbers of field samples feasible. Although the virus detection tests remained the most sensitive for the detection of congenital transmission (27,28), the testing of albumen samples for the presence of gsa demonstrated great prospects for LL control. Albumen samples are easily collected and the CFT does not require a virus multiplication step in cell culture, as required for the COFAL. The apparent lack

of sensitivity, mainly resulting from the erratic patterns of congenital transmission, could be compensated by testing larger numbers of samples. The requirement of testing increased numbers of albumen samples by CFT has proven to be particularly desirable when a relatively low incidence of ALV infection has been attained (27).

The sensitivity of gsa detection was increased by the introduction of double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) (29,30,31), but this was accompanied by an increase in "false-positive" reactions due to gsa of endogenous origin (28,37, see also section 2.3). Interference by endogenous gsa was particularly observed in test materials containing cellular material (see Table 1). The sensitivity of a commercial DAS-ELISA kit had probably been reduced (32) to avoid difficulties in the interpretation due to endogenous gsa. The application of monoclonal antibodies directed to different antigenic determinants of ALV-p27 further increased the sensitivity of the DAS-ELISA (33). By comparing the test results of PM tests with those of DAS-ELISA using various combinations of monoclonal antibodies employed for coating of ELISA plates and for the preparation of conjugates, a DAS-ELISA of relatively low sensitivity was selected for the screening of ALV shedding hens. Nevertheless, increased scores of ALV-gsa positive hens were obtained in the field (34).

2.3. Choice of test materials

As methods for the detection of infectious ALV and ALV-gsa became available, test materials from various sources were tested to compare their predictive value in detecting congenitally ALV transmitting hens (3,31,35,36,37). Virus recovery from embryo extracts, prepared from 11-day-incubated fertilized eggs, demonstrated the closest association with congenital ALV transmission (27,31). Testing of albumen samples was second best for the detection of hens which congenitally excrete ALV (28,36). In addition, the presence of ALV-gsa in feather pulp would seem to provide a good alternative test procedure in non-laying birds, although feather testing has only been compared with other test procedures on a limited scale (32,38,39). Initially, testing of vaginal and cloacal swabs by CFT or DAS-ELISA was considered a valuable tool for the identification of hens which congenitally transmit ALV (40,41), but other studies showed that virus shedding via the cloaca was often not synonymous with congenital transmission of ALV (31,35,42,43). The technique of swabbing seems to have some influence. In some laboratories, the impression has been obtained that vaginal swab testing gives a better indication of congenital transmission than testing of

cloacal swabs. Meconium samples from newly-hatched chicks are easily collected, but the results obtained are poorly associated with congenital virus transmission (13,14).

The interpretation of test results became somewhat complicated after the introduction of the DAS-ELISA since this test did not discriminate between gsa of exogenous or endogenous origin. Table 1 gives examples of gsa absorbance scores in DAS-ELISA for various test materials collected from ALV shedding and non-shedding hens of a White Leghorn flock. Vaginal/cloacal swabs and albumen samples obtained from the non-shedding hens yielded ELISA absorbances within the negative range, indicating that very little endogenous gsa was present in these test materials, even considering the fact that the chickens were of the gs^+chf^+ phenotype (28).

Table 1. Range of ELISA absorbances obtained with various materials from two selected groups of hens from a White Leghorn flock.

Materials tested	Originating from	
	19 Non-shedders	18 ALV-shedders
White blood cells	0.170-2.832[a]	0.090-3.897
Vaginal/cloaca swabs	0.080-0.124	0.116-3.350
Albumen samples	0.074-0.164	1.172-2.432
Individual embryos	0.222-3.274	0.042-4.854
Meconium samples	0.084-0.228	0.077-2.300

[a] Range of absorbance at 474 nm
Mean absorbance of negative controls: 0.122 + 0.045 (s.d.), ELISA absorbance $\geqslant$ 0.212 is positive. Data from (28), by kind permission.

Various endogenous viral (ev) genes may be encountered in commercial poultry. Some encode for complete endogenous ALV, others for only one or a small number of structural viral proteins (see Chapter 5). Fortunately, recent studies have demonstrated that gsa levels due to ev gene expression are generally lower than gsa levels originating from exogenous ALV infection (28,37). These differences in gsa levels have made feasible the differentiation between endogenous and exogenous ALV-gsa by testing diluted albumen samples (34,45).

Based on a practical balance which can be achieved between the predictive

value of the test for the detection of ALV transmission and the convenience of collection and processing of test samples, several laboratories have now reached consensus that albumen testing constitutes the most practical approach for screening commercial flocks (27,28,33,36,44).

3. PREVENTION OF HORIZONTAL VIRUS EXPOSURE

Congenitally infected chickens are an important source of infection to uninfected hatch-mates (4,46), particularly if the recipients are devoid of maternal antibodies. Horizontal transmission of ALV occurs especially during hatching and during the early period of rearing. Virus transmission is mediated via allantoic and amniotic fluids, saliva and faeces (4,46) and skin debris (39). ALV transmission can also occur indirectly at handling of chickens, e.g. during vent-sexing and vaccination against Marek's disease (MD), in particular when chickens are devoid of maternal antibody (47). Contact transmission is enhanced by management procedures which lead to intimate contact among young chickens.

Horizontal ALV exposure within the first weeks of life may lead to congenital virus transmission in the laying hen (48). The rate of contact transmission appeared to be lower in chickens reared on wire-mesh floors than on solid floors (10). However, in other studies no differences in congenital ALV transfer in favour of cage rearing were observed (49). The discrepancy between these observations can probably be explained by the common practice of the use of rubber or plastic mats in cages during the first weeks. In the field, horizontal ALV transmission in the circumstances of floor rearing on litter caused a three-fold increase in the incidence of viremia, during the first weeks of life (15). This significant contribution to ALV spread can however be reduced by rearing the chickens in small groups (10,50). On the basis of an epidemiological model it was concluded that ALV infections in flocks would be self limiting if horizontal transmission did not occur (51). The benificial influence of maternal antibodies in recipient chickens exposed to ALV has been evidenced by the delay of antibody development (52) and the lowered proportions of chickens that become viremic (48,53). Hence the induction of maternal antibodies should be considered as an implied goal of vaccination.

Rearing and maintenance of poultry in filtered-air positive-pressure (FAPP) houses can prevent horizontal transmission of infectious agents, in particular for airborne diseases (54,55). However, since virus transmission via small particles in the air represents a minor component of ALV epidemiology, and horizontal

exposure originates mainly from within the flock, FAPP rearing is not a prerequisite for LL control. However, in conjunction with LL control, FAPP house rearing can be utilized to reduce the number of vaccinations against other infectious diseases (56).

4. LL CONTROL BY ELIMINATION OF ALV SHEDDING HENS

The application of the RIF test (3) yielded epidemiological observations which still form the basis of LL eradication procedures. Most virus is released into the environment by congenitally infected hens (4,5), therefore interruption of virus transfer between successive generations of birds provides the best prospects for successful control. Since roosters generally do not contribute to congenital transmission of ALV (4,5,57, Chapter 3), LL control efforts should be performed primarily in basic or primary breeding stock of which the female chickens are used at the grandparent and parent level, designated D and CD in Table 2.

Table 2. General breeding scheme of commercial poultry.

	Basic breeding stocks			
Basic breeding	A	B	C	D
Grandparent	A[a] ♂♂	B[b] ♀♀	C[a] ♂♂	D[b] ♀♀
Parent	AB[a] ♂♂		CD[b] ♀♀	
Final product	ABCD			

[a] Only males are used for producing offspring.

[b] Only females are used for producing offspring.

Commercial chickens are usually produced by poultry breeders and multipliers, according to a general breeding scheme. The final product, a laying or a meat-type chicken, is a hybrid cross of four, sometimes five, closely-bred basic breeding stocks, which have been selected for use either as female or as male breeders.

Hughes et al. (6) were the first to apply the above described principles. They selected ALV-excreting hens using RIF tests on series of embryos obtained from

individual hens. Hens producing ALV-positive embryos were eliminated as breeders and, in addition, eggs for reproduction were only collected from hens with virus-neutralizing antibodies in their serum, an indirect procedure to eliminate immunologically tolerant hens. Zander et al. (25) successfully used similar procedures to eliminate ALV infection from three flocks of breeding chickens. In addition to selection of virus shedders by RIF testing of embryos, they also eliminated potential breeders if they were shown to be viremic. These studies demonstrated that erratic ALV transmission patterns frequently occur. Therefore, negative test results did not always indicate absence of ALV transmission. The predictive value of the identification procedure of ALV excreting hens was improved by the introduction of the NP test (20,21). Since ALV-infected embryos usually contain relatively large quantities of ALV, pools from a series of embryos could be tested without loss of sensitivity (16,17). The Dutch LL control experiments demonstrated that by testing relatively large numbers of embryos, every potential ALV-excreting hen could be detected. As illustrated in the upper-left part of Table 3, virological testing of all embryos collected from three flocks during two periods of 14 days permitted complete eradication of congenital ALV transmission in only one generation (58). Okazaki et al. (50) have confirmed that eradication of ALV shedding could be accomplished through the virological examination of hens.

The breakthrough enabling large scale testing of field samples was provided by the introduction of the CFT for gsa detection in albumen samples (26). As described in section 2.3, the convenience of collection and processing of test samples and the close association with congenital ALV transmission made albumens the test materials of choice. At about the same time the negative effects of ALV infection on several production traits, such as egg production, age of maturity, livability and growth, were recognized (see Chapter 12). This prompted many poultry breeding companies to initiate LL control programmes. Reports of LL control in basic breeding flocks have been presented by De Boer et al. (58), Romero et al. (59), Fadly and Okazaki (60), Payne et al. (41) and Ignjatovic and Bagust (13). A significant reduction of ALV shedding was achieved in all flocks. Eradication, however, was only obtained in those efforts in which albumen samples were tested for ALV-gsa by CFT or DAS-ELISA (35,36,58,59).

In the Netherlands, a total of 60 breeding flocks, mainly flocks used in genetic selection for production traits, have participated in the LL eradication programme. Table 3 summarizes the test results obtained in flocks in which selection for ALV shedding was performed in addition to controlled virus exposure

(vaccination) at 8 weeks of age (see section 5.2). The results obtained in flocks without vaccination are not presented, but were very similar. ALV shedding was eliminated from all flocks provided that a sufficient number of albumen samples were tested. On the basis of observations in experimental laboratory flocks (27) and in the field, it was concluded that elimination of congenital ALV shedding could be achieved by testing at least nine albumen samples. Exceptions to this rule of thumb were observed in three meat-type breeding stocks I, I' (both closely related) and Z of Table 3, probably owing to "false-positive" reactions by endogenous gsa (28,37,66). The LL control efforts failed in one laying-type flock as a result of vertical ALV transmission through the roosters (see section 4.4).

The major poultry breeders in the United States have had LL control programmes in progress for several years. Detailed results, however, are not available since the testing is being performed in private laboratories. Overall results were verbally reported at the Avian Leukosis Symposium in New York, 1983, and in a recent committee report of the American Association of Avian Pathologists (18). In summary, the majority of the breeding lines have responded to testing and selection. ALV shedding of many lines was reduced from initial levels between 10% and 30% to below 2%, but only a few flocks were rendered totally free of ALV infection. Similar results were obtained in Australian commercial breeding flocks in the period 1980-1983. The incidences of ALV shedding were usually reduced to levels ranging from 0 to 5% in a single generation (13).

Evaluation of the results of various laboratories shows that eradication in one or two generations is feasible only if the ALV shedding rate in the original flock is relatively low and if a sufficient number of albumen samples per hen is examined (see section 4.1). It has been advocated that in the final phase of a testing programme efficient methods for detecting antibody, such as the agar-gel precipitation test (AGPT) for detecting subgroup-specific antibody or a blocking ELISA for detecting anti-gsa antibody, should be introduced (42,44,59). We believe, however, that continued testing for gsa with sensitive techniques is preferable, since after low degree virus exposure antibodies may appear relatively late and only in a fraction of the ALV exposed birds. By interrupting the laboratory testing during one generation occasionally a sharp increase of ALV shedding has been observed (W.B. Chase, personal communication).

In the United States, the breeding flocks resisting selection for reduced shedding of ALV are termed "hard lines". Four mechanisms are to be incriminated in the apparent incomplete elimination of ALV shedding in such "hard lines":

Table 3. Congenital ALV transmission in 32 basic breeding flocks which participated in the Dutch experimental LL eradication programme. Only flocks are listed in which vaccination by controlled virus exposure was performed.

Generation No.	Flock A	Flock B	Flock C	Flock D
I	(16)[a] - 13/437[b]	(18) - 22/221	(20) - 29/252	(3) - 1/180
II	(14) - **0/359**[c]	(19) - **0/64**	(20) - **0/358**	(3) - **0/150**
III	(13) - **0/175**	(16) - **0/121**	(1)[d] - **0/300**	(3) - **0/104**
IV	(17) - **0/106**		(4) - **1/350**	
V	(18) - **0/443**		(4) - **0/350**	

	Flock E	Flock E'	Flock F	Flock G
I	(12) - 28/136	(3) - 29/160	(3) - 20/125	(12) - 40/133
II	(9) - 99/430	(6) - **5/278**	(6) - **47/538**	(9) - **70/400**
	(3) - 0/100[f]	(3) - **0/80**	(3) - **0/100**	(3) - **0/94**
III	(3) - **0/270**	(3) - **2/342**	(3) - **26/704**	(3) - **0/204**
		(3) - **0/98**	(6) - **0/130**	
IV[e]		(2) - **5/305**[e]		(3) - **1/154**[e]

	Flock H	Flock H'	Flock H"	Flock I
I	(9) - 3/310	(9) - 0/300	(10) - 1/270	(9) - 5/280
II	(9) - 0/490	(6) - **0/160**	(4) - **0/430**	(5) - **1/230**
III	(2) - **0/426**	(3) - 0/160	(1) - **0/380**	(6) - 7/350
IV	(2) - 0/470	(4) - 0/446		

	Flock I'	Flock J	Flock J'	Flock K
I	(10) - 10/260	(3) - 1/180	(9) - 3/50	(9) - 1/180
II	(4) - **1/410**	(3) - **2/150**	(2) - **0/70**	(3) - **0/70**
III	(4) - **4/300**	(3) - **0/120**	(2) - 0/190	(2) - 0/170

	Flock K'	Flock L	Flock M	Flock N
I	(1) - 1/110	(6) - 0/70	(12) - 0/150	(3) - 4/300
II	(4) - **1/150**	(2) - **0/60**	(3) - **0/120**	(3) - **1/300**
III	(1) - **0/150**	(2) - **0/260**	(2) - 0/208	(3) - **2/212**

	Flock N'	Flock O	Flock P	Flock Q
I	(3) - 1/200	(9) - 2/215	(6) - 0/180	(9) - 3/200
II	(3) - **0/246**	(3) - **0/197**	(3) - **0/169**	(3) - **0/206**
III[e]	(3) - **5/198**[e]	(3) - **0/90**[e]	(3) - **0/155**[e]	

	Flock R	Flock S	Flock T	Flock V
I	(12) - 0/150	(9) - 0/300	(1) - 0/50	(1) - 0/50
II	(3) - **0/120**	(4) - **0/175**	(4) - **0/140**	(4) - **0/140**
III	(2) - **0/208**	(2) - 0/150	(2) - **0/95**	(2) - **0/95**

	Flock W	Flock X	Flock Y	Flock Z
I	(9) - 0/300	(6) - 36/152	(9) - 3/149	(9) - 13/440
II	(6) - **0/325**	(3) - **1/78**	(3) - **0/173**	(3) - **1/290**

[a] Between parentheses number of samples examined per hen. These consisted of embryo extracts in flocks A, B and C (ten generations; boxed) or of individually tested albumen samples (all other generations).

[b] Number of ALV-positive hens/Number of hens tested

[c] "Bold character" means vaccination by controlled virus exposure with ALV of subgroups A and B at 8 weeks of age (see section 5.2).
[d] Total number of egg albumen samples tested per hen. These were examined by CFT (27), except
[e] Five generations which were tested by monoclonal antibody DAS-ELISA combination no. 12 (33,34). The introduction of this test resulted in a slight increase of gsa-positive albumens.
[f] Repeated testing of ALV-negative hens of the same generation.

(4.1) Testing of an insufficient number of albumen samples, or test procedures that are poorly associated with congenital transmission.

(4.2) Horizontal transmission of ALV during the early rearing period.

(4.3) Gsa of endogenous origin.

(4.4) Vertical transmission of ALV by roosters.

4.1. Testing of an insufficient number of albumen samples, or test procedures that are poorly associated with congenital transmission

Failures to obtain complete elimination of ALV-shedding of certain chicken lines, in one or two generations, were mainly due to an insufficient number of test samples examined. Very different patterns of congenital ALV transmission by hens have been observed. For example, during a period of 10 weeks, all eggs from a number of hens known to be ALV excreters were examined. Only about 50% of the albumen samples were gsa-positive and about one-third of the hens in this study produced only occasionally gsa-positive eggs (27). As a result of intermittent virus transmission, the number of albumen samples tested per hen appears more critical than the sensitivity of the assay. The chance of identification of ALV-shedding hens increases with the number of eggs tested by the CFT (27). However, with the use of the monoclonal antibody DAS-ELISA with an increased sensitivity it would seem that the minimum number of albumen samples required to be tested can be reduced (33,34).

No differences were observed in proportions of gsa-positive and gsa-negative eggs when batches of eggs collected from the same hen, either early or late in the laying period, were examined. However, the timing of egg collection appeared to have some influence. Statistical evaluation of large numbers of test results showed that two-week intervals between collection periods yielded higher scores of congenitally ALV-shedding chickens than uninterrupted collection of the same number of eggs (De Vries, personal communication).

Failure to obtain complete eradication may also be due to a poor association between gsa-positive test samples and virus transmission to offspring.

As already indicated in section 2.3, vaginal/cloacal swab or meconium testing are less efficient procedures for identification of hens which congenitally transmit ALV. It is not clear whether the predictive value of vaginal/cloacal swab testing can be improved by testing more swabs per individual laying hen.

4.2. Horizontal transmission of ALV during the early rearing period

As described before, a great proportion, perhaps the majority, of ALV infections are established via horizontal exposure, originating from congenitally infected hatch-mates. Short-term, small-group rearing can significantly reduce the horizontal spread of infection (50,61), but this is difficult to apply in LL control programmes for large breeding flocks.

Genetic differences between the various lines of chickens may influence susceptibility to horizontally transmitted ALV (62), and with that the outcome of LL control efforts. In addition, genes for genetic resistance at the cellular level are more prevalent in meat-type chickens than among layers (63), which is reflected in the field by lower incidences of infection in heavy breeds (15). As discussed previously, horizontal spread is less effective in the presence of maternal antibodies (48,64).

Susceptibility for ALV infection following horizontal ALV exposure is further influenced by immunological stresses which can be imposed by interaction with viral agents such as Marek's disease virus (MDV), reticuloendotheliosis virus (REV) and infectious bursal disease virus (IBDV). These and further unknown environmental conditions are detrimental to eradication programmes, as demonstrated by rearing of "hard lines" under laboratory conditions (65).

4.3. Gsa of endogenous origin

Generally the various ev genes express endogenous gsa at relatively low levels (28,37). Differentiation between gsa of endogenous or exogenous origin can therefore be obtained by testing diluted samples (34,35). However, a recent study by Ignjatovic (66) has reported replication-competent endogenous ALV to be prevalent in the majority of Australian lines of meat-type chickens. Nine commercial lines of meat-type chickens appeared refractory to LL control measures because of "false-positive" reactions in DAS-ELISA caused by endogenous gsa. Hens which transmitted complete endogenous ALV and shed endogenous gsa in albumen, consistently produced gsa-positive albumens, although no endogenous gsa could be detected in vaginal/cloacal swabs from the hens nor in meconium samples from their newly-hatched chickens. Thus far, these aberrant

properties of meat-type lines have not been observed in other parts of the world, e.g. in the Netherlands for which a total of 16 meat-type flocks are included in Table 3. Nevertheless, the possible presence of endogenous gsa in detectable quantities should be investigated if "hard lines" are encountered.

4.4. Vertical transmission of ALV by roosters

As will be described in section 4.5, elimination of ALV shedding can be obtained in a single generation employing an alternative LL control procedure, by mating roosters from an ALV-infected flock with ALV-free hens. This approach, however, remained without success in one White Leghorn breeding flock demonstrating an extremely high ALV shedding rate (80%). No reduction of ALV shedding was obtained by mating three generations of roosters of this flock with ALV-negative hens of another related flock. The shedding rates of these three CD flocks (see Table 2) were 61%, 63% and 74%! In this particular line, the LL control efforts were extended by selecting ALV-free roosters on the basis of absence of infection or low levels of gsa in both blood and semen. In a group of 137 roosters of the first generation that was examined, only two individuals were shown to be non-viremic by examination of white blood cell preparations (43) and only twenty-five plasma samples were free of ALV. Infectious ALV was recovered from 127 out of 137 semen samples. In addition to the roosters with ALV-negative semen, those with the lowest gsa levels in their semen were employed for reproduction. With this selection procedure, additional to albumen testing of eggs, a significant reduction of ALV shedding was obtained in the following generation. Similar circumstantial evidence relating to vertical ALV transmission by roosters, which is at variance with the existing literature (4,5,57, Chapter 3), have also recently been observed elsewhere (D. von dem Hagen, W.B. Chase and L. Morrisroe, personal communication). These observations demonstrate that occasionally a flock may be encountered with vertical ALV transmission via semen, and accordingly such a chicken flock will be designated as a "hard line" with regard to LL control.

Vertical ALV transmission through roosters, however, remains an exception. Artificial insemination of three different strains of chickens with virus-positive semen from roosters of the above mentioned White Leghorn flock did not result in the production of ALV-positive embryos and gsa-positive albumens. This experiment confirmed earlier observations along these lines (67). Either the virus dose or the genetic make-up of the bird seems to play a decisive role.

4.5. Alternative LL control procedure in heavily ALV-infected flocks

Consequent elimination of all gsa-positive birds of a heavily ALV-infected flock is usually not welcomed by geneticists. They prefer a gradual reduction during a number of generations, in order to save valuable genes, or otherwise to reject the entire flock from the breeding programme. Complete eradication of ALV shedding in such flocks within one generation, however, can be achieved by the application of an alternative approach of LL control. This procedure, like the one described in sections 4 and 5.2, is again based on the principle that roosters generally do not contribute to congenital ALV transmission, even if infectious virus is present in semen (41,67, Chapter 3). The eggs are collected irrespective of the ALV shedding status of the hens, but in the next parental generation only the roosters are used for reproduction by mating them with hens of an ALV-free flock. If desirable, valuable genes of the ALV-positive stock can be retrieved via back-crossing.

We have applied this alternative procedure in two flocks with ALV shedding rates in the order of 35%. The chickens of these flocks were mated with those of a closely related ALV-negative line. All four possible combinations of Table 2 were examined for ALV shedding when the hens came into lay. Both combinations employing females of the ALV-positive flocks demonstrated shedding percentages at a similar level as the parent flocks. ALV shedding, however, was not observed in the offspring of both combinations utilizing females of the ALV-negative parent flocks. Therefore, employing the approach of "outbreeding", complete eradication of ALV shedding was obtained in a single generation by using only the roosters of such an ALV shedding flock.

5. MANIPULATION OF THE IMMUNE RESPONSE

5.1. Vaccination studies with RSV preparations

Relatively few vaccination studies with avian retroviruses have been undertaken. Initially, RSV preparations were mainly utilized because of the common envelope glycoproteins provoking similar neutralizing antibodies and because of the availability of a challenge model. At that time it was not yet recognized that the immune response to ASV tumors differs from the immune response to lymphomas induced by ALV. Cellular immunity is most important in controlling the growth of ASV tumors while humoral immunity is dominant in the prevention of ALV infection and, probably, also ALV lymphoma induction (see Chapters 6 and 7). Protection against tumor induction by RSV prior to infection could not be

obtained with inactivated virus preparations (68,69) or administration of neutralizing antibodies directed to RSV. Passive immunization of chickens with larger doses of RSV immune serum resulted only in a transient delay in tumor formation but did not affect the rate of tumor growth, once sarcomas were palpable (70).

However, immunization with live virus preparations of RSV(RAV-1), or the Rous-associated virus alone (RAV-1), elicited a strong resistance to establishment of sarcomas after challenge exposure with RSV. A marked cross protection was observed after vaccination with RAV of either subgroup A, B or C against tumor induction by RSV of any of the three subgroups (69). Chickens and quails immunized with extracts of RSV-transformed fibroblasts demonstrated group-specific immunity. Since gp85-defective RSV also induced immunity, the investigators involved suggested that in addition to subgroup- and group-specific antigenic determinants of the major glycoproteins a non-virion structure, called tumor-specific surface antigen (TSSA), might play a role in the induction of immunity (71). However, experiments in other laboratories failed to confirm the involvement of TSSA in anti-ASV tumor immunity (see Chapter 7). Protection is thought to be elicited by the immune response to the envelope glycoproteins of RSV and cross protection is explained by the shared subgroup-common antigenic determinants in the envelope glycoproteins of the various virus strains.

5.2. Vaccination studies with ALV preparations

Burmester et al. (72) performed a series of vaccination studies using multiple injections of experimental vaccines prepared of filtrates of livers infected with the RPL12 strain of ALV. Preparations were administered either as a live virus preparation or inactivated, and with or without adjuvant. The extent of immunity was determined by the relative responses to challenge infection of progeny obtained from eggs collected before or after vaccination. Whilst all vaccines employed resulted in a significant passive immunity in offspring, the best protection was obtained with the live virus preparations.

During a sabbatical year in Burmester's laboratory, the late B.H. Rispens was greatly encouraged by the above results. Back in the Netherlands, he initiated, in collaboration with H.J.L. Maas, a research project of long continuance aimed at the control of LL under field conditions. A controlled exposure with high doses of the ALV subgroups A and B, which prevail in the field (15,16), was introduced in order to prevent congenital transmission following horizontal ALV exposure. The three elements of the Dutch LL eradication programme are depicted in Figure 1:

I. Identification of hens which congenitally transmit ALV infection to their progeny. Such hens are eliminated from the stock and only eggs from non-shedders are employed for the production of the next generation.

II. Rearing in isolation during the first 8 weeks of life.

III. Active immunization by intramuscular inoculation of 10^5 $TCID_{50}$ ALV of subgroups A and B, to further prevent infection by horizontal spread and to induce maternal antibody in the offspring.

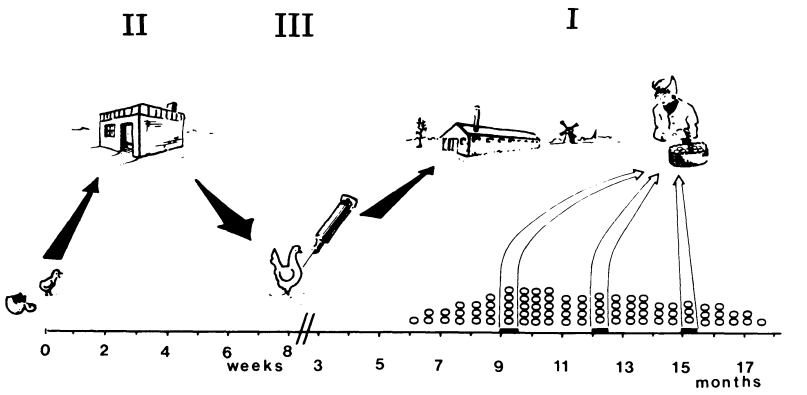

Figure 1. Schematic design of the Dutch LL eradication programme. I. Selection of hens producing ALV-free eggs. II. Rearing in isolation during 8 weeks. III. Inoculation with ALV of subgroups A and B, and subsequent transfer to the conventional chicken house.

The three elements of the Dutch LL eradication programme are presented in the order of priority (prevention of ALV exposure from outside is of little use if virus shedding chickens are already present in the incubator). Immunization alone as a control measure is inadequate, and is only effective if applied in virus-free chickens. The three elements support each other towards successful LL eradication. In the first series of experiments, the age was determined at which resistance to lymphoma development had fully developed and also the age at which vaccination did not result in congenital virus transmission to offspring. In these trials, and in many that followed, it was shown that controlled ALV exposure could safely be performed at the age of 8 weeks (16,17,43, 58,73,74,75,76). Immunization was only obtained if ALV-free chickens were used. The controlled virus exposure was followed by a low-grade persistent viremia, demonstrable as a latent infection associated with white blood cells. Although the

mode of antigen presentation is not understood, the persistent ALV infection elicited a strong immune response so that vaccinated birds became refractory to horizontal ALV exposure (16,17,48). Slightly elevated levels of gsa were recorded in serum samples from ALV-vaccinated hens. Eggs with elevated levels of maternal antibody were produced (77). The titers of neutralizing antibodies to subgroup A were generally higher than those induced against subgroup B (73), suggesting differences in immunizing capacity between ALV of different subgroups. Since some field strains of ALV have been shown to be less immunogenic (78), it was perhaps fortunate that laboratory strains were incorporated in the vaccine.

The vaccination experiments in the laboratory were performed under conditions known to produce congenital ALV transmission after contact exposure at the age of 8 weeks (16). In various trials the severity of the contact exposure was increased by adding to the flock hatch-mates that had been injected in ovo with RAV-1, at the age of 9 weeks. This severe ALV exposure did not interrupt the production of ALV-negative eggs, but occasionally the establishment of ALV infection in the external mucosae was noticed as evidenced by examination of swabs from cloaca and throat. A similar observation was made by Okazaki et al. (79) who performed the challenge infection immediately after vaccination. This early challenge resulted also in gsa-positive albumen samples.

The results obtained in thirty-two breeding lines are presented in Table 3. The generations to which all three elements of the Dutch eradication programme were applied are described in bold characters. The impression was obtained that eradication was obtained more efficiently if vaccination was included in the programme. However, detailed comparable data did not become available for a statistical evaluation. By autumn 1985, over 40,000 chickens had been inoculated with high doses of ALV of subgroups A and B. In the treated flocks no indication was obtained for lymphoma formation, congenital transmission of vaccine virus or negative influences on egg production (80,81).

5.3. Vaccines prepared by recombinant DNA technology

5.3.1. Live virus vaccines. With the aim of developing a vaccine to protect fowl against ALV infection and LL, recombinant DNA technology has recently been employed by H.L. Robinson et al. (82) to construct a live virus vaccine. Sequences of both endogenous and exogenous ALV were utilized. As depicted in Figure 2, gene segments encoding for gag and pol of endogenous ALV were used since congenital transmission of ALV has been demonstrated to be restricted in

line K28 chickens by the main gag gene product, $p27^o$, of endogenous ALV (83). The recombinant viruses contained long terminal repeats (LTR) of both 3' (U3) and 5' (U5) termini, as well as the env gene derived from exogenous ALV subgroup A. This vaccine-induced neutralizing antibodies at levels equivalent to those produced by exogenous ALV infection (82). Application of live virus vaccines containing the endogenous gag gene should have some advantage over the Dutch vaccination scheme in not requiring a fully developed age-related resistance in order to guarantee safety with respect to congenital transmission of vaccine virus. Hence vaccination at a younger age might become feasible. However, viral strain and host factors would seem to be crucial; endogenous ALV and recombinant viruses containing endogenous $p27^o$ were excreted into eggs of subgroup E susceptible chickens of the RPRL at East Lansing (see Chapter 5, section 9.3). In addition, congenital transmission of endogenous ALV has been shown to be a common event in several lines of Australian meat-type birds (13,66). Further research will be required to determine whether the $p27^o$ restriction of congenital ALV transmission is unique for K28 chickens or applies to other lines of chickens as well.

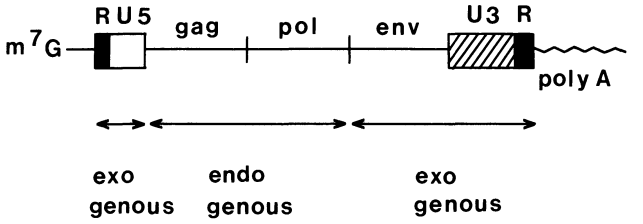

Figure 2. Experimental recombinant live virus vaccines based on gag and pol genes of endogenous ALV and the env gene and both LTRs derived from exogenous ALV. From (82), by kind permission.

The construction of viruses which replicate well, induce a persistent virus infection but do not cause disease and are not egg-transmitted, certainly will initiate research activities aimed at the incorporation of other "immunogenes", i.e. genes that encode for immunizing (glyco)proteins of other chicken pathogens, into these vectors. In addition, insertion into viral vectors of genes encoding lymphokines, such as interleukin-2 (IL-2), might be considered for immunomodulating purposes. Before application of such vaccines it should be determined whether such live virus vaccines could become lymphomagenic via genetic

recombination with the ALV of subgroup B, present in some recipients. This of course is a disadvantage not encountered with inactivated vaccines.

5.3.2. Sub-unit vaccines. Products encoded by the ALV-env gene could be utilized for the production of sub-unit vaccines. Hunter et al. (84) demonstrated that ALV-gp85 comprises 14 glycosylated regions, although the two regions of the env gene which encode for host-specificity are not glycosylated (85). Since glycosylation is not obtained in prokaryotic cell systems (e.g. E. coli), expression products to be incorporated in vaccines probably will have to be produced in eukaryotic cells. For sufficient immunogenic stimulation multiple injections and presumably adjuvants will be required. Antigen presentation to the host is critical. A number of viral sub-unit vaccines, including a feline leukemia sub-unit vaccine, have been shown to be only weakly immunogenic if presented in monomeric form, but to be highly immunogenic when multimeric immuno-stimulating complexes are employed (86,87,88). Although the technology for the production of ALV sub-unit vaccines is available, there are no published reports to indicate that this type of work has yet been undertaken in the avian leukosis field.

Chapters 6 and 7 describe that prevention of ALV infection and, probably, also of lymphoma induction are mainly mediated by humoral immunity. However, the presence of subgroup-common antigenic determinants in endogenous ALV envelope glycoproteins may exert a tolerizing effect on the specific immune response against exogenous ALV infections. In chickens which express high levels of endogenous envelope glycoproteins (e.g. chickens carrying the ev3 gene), significantly decreased production of neutralizing antibodies to ALV of subgroup A has been reported (78). Although the incidence of neoplasms was not influenced by ev gene expression, the observed phenomenon of partial immunological tolerance, described in detail in Chapter 7, may have a detrimental effect on vaccines employed in LL control programmes. For the poultry breeder it is of course of greater importance to know whether impairment of immune responses to exogenous ALV, mediated by ev genes, may be anticipated in the field.

5.4. Control by elimination of target cells

Various treatments resulting in destruction of the target cells for transformation by ALV, B-cells in the bursa of Fabricius, have been investigated for application in LL control. Surgical bursectomy (57,89,90), treatment with androgens (91,92,93) and/or cyclophosphamide (62), or early infection with IBDV (94) could prevent or reduce LL development after experimental infection. However,

all these approaches to removing the target cells for ALV transformation have side-effects that preclude their application under field conditions. Cyclophosphamide is too toxic to be considered for large scale application. In addition, this drug suppresses the immune response to other chicken pathogens (95,96). IBDV infection also impairs the immune response to various pathogens (97,98). In recent experiments, co-infection of commercial chickens with various immunosuppressive viruses, IBDV, MDV and REV, resulted in increased rates of ALV viremia and shedding (99). Burmester demonstrated that treatment of eggs with the male hormone resulted in poor hatchability, increase of non-specific mortality and masculinization of the females (91,92). Chickens fed with a diet including the androgen analogue mibolerone, however, remained fully immunocompetent as judged by their ability to resist challenge after vaccination with various vaccines. However, since this compound does not affect the course of ALV infections, its application serves only to reduce LL mortality (100,101).

6. GENETIC RESISTANCE

6.1. Selection for genetic resistance

Two levels of genetic resistance to ALV infection are recognized: (a) Genetic resistance at the cellular level to virus infection, which is controlled by single dominant alleles for susceptibility (resistance is controlled by recessive genes) and (b) Genetic resistance to tumor development. The latter type of resistance is probably inherited through multiple alleles and appears to be less ALV subgroup-specific, and therefore may be of a more general nature (102,103) (see Chapters 3 and 12).

Both types of resistance increase with the age of the bird, but at different paces. Virus infection at various ages resulted in differences in numbers of ALV-infected white blood cells and the age of ALV exposure was decisive for the induction of congenital virus transmission (47,73,74,75,76). Pathogenesis following ALV infection differs with the age at which exposure to virus takes place (75,76). In chickens with receptors for ALV infection, therefore susceptible at the cellular level, the susceptibility for virus infection decreases with age, but the chickens do not become fully resistant to ALV infection. Resistance to tumor development, however, is complete at about 2 months of age, and is associated with the loss of transforming ability of bursal cells.

Selection for resistance at the cellular level has been pursued by intracranial inoculation of day-old chicks, chorioallantoic membrane inoculation, culturing of

feather pulp cells from individual adult birds and, indirectly, by examining antibody formation after experimental infection (102,103). Genes for cellular resistance are more prevalent among meat-type chickens than among layers (104). Differences in susceptiblity to ALV-induced lymphomas between lines of laying-type chickens have been reported (62,105,106). However, LL control via genetic selection is difficult to achieve, and sometimes counterproductive (107), because the selection has to be for recessive genes and whereas poultry production is based on crosses from several lines (see Table 2). By comparing LL mortality in reciprocal crosses of genetically resistant and susceptible lines, the greatest loss from LL was obtained when the resistant line was used as the female parent, probably because of the lack of maternal antibodies in the offspring (106,108). It seems that at the present time little attention is being given to genetic selection for LL resistance in particular. Selection is aimed more at a balanced immune response with a broad spectrum, by eliminating those chickens that demonstrate a very low or very high immune response, either humoral or cell-mediated (107).

6.2. Insertion of retroviral genes

The modern approach of genetic engineering, by which genes encoding resistance at the cellular level are inserted in the germ line of the chicken, offers exciting prospects, both for understanding the mechanisms of gene regulation in the chicken and for improving disease resistance.

Two procedures for gene transfer have been followed: (a) Direct micro-injection of cloned DNA into zygotes and (b) Infection with (recombinant) retroviruses. Stable integration via micro-injection has already been achieved in sheep, swine and cattle. Micromanipulation of early embryonic stages in the fertilized chicken egg meets with technical difficulties and therefore in chickens insertion of DNA fragments, via retroviral injection, as developed for the mouse (109), seems to offer the best prospects. Indeed, integration of avian retroviral genes at the somatic cell level has recently been achieved by ALV infection during early embryonic development. Stable inheritance, however, has yet to be demonstrated in breeding experiments (110,111). ALV infection might establish integration of fractions of the DNA sequence of the ALV genome, including the env gene (see Chapter 4), and therefore resistance to the homologous viral subgroup might be anticipated, if transformation is established and if the env gene is properly expressed (112). Retroviral vectors might in addition be used for the introduction into the chicken genome of genes which regulate the immune response. Candidate genes for genetic manipulation are those encoding for

immunoglobulins, the major histocompatibility complex, genes for T-cell receptors and lymphokines (111,113,114,115).

Application of transgenic chickens in LL eradication programmes, however, is still far away. When the methodology for the insertion of ALV env genes becomes available, it will be required that these genes are inserted in all breeding lines that contribute to the end-product, because resistance to infection is inherited recessively (see Chapter 12).

7. CONCLUDING REMARKS

LL control measures should be aimed at the interruption of ALV transmission between successive generations. The congenitally infected chicken is the major source of virus shedding into the environment. The majority of virus infections, however, are established through horizontal virus transmission.

Based on a balance between the convenience of collection and processing of test samples and the predictive value of the laboratory technique, testing of albumen samples for ALV-gsa is preferred in LL control programmes. Complete eradication in one or two generations can be achieved if sufficient samples are tested, particularly if the shedding rate of the original flock is not too high. Proper management procedures are helpful for the prevention of horizontal virus exposure. Failures to obtain gsa-negative test samples could be ascribed mainly to horizontally acquired infections, established shortly after hatching or during the early rearing period, and occasionally to "false-positive" tests caused by gsa of endogenous ALV origin or vertical virus transmission by roosters.

Vaccination by controlled virus exposure prevents horizontal virus transmission in the treated generation, and in addition reduces the horizontal spread in the following generation as mediated by maternal antibodies.

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