Inhibition of Hepatic Phosphoenolpyruvate Carboxykinase by Avian Reticuloendotheliosis Viruses

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ABSTRACT

Severe weight loss is associated with many malignant diseases of humans and animals. Avian reticuloendotheliosis viruses (REV viruses) induce runting in experimentally infected chickens. Chickens infected with a replication-competent REV virus, reticuloendotheliosis-associated virus, weighed 30-50% less than control birds at the time of death. Chickens infected with reticuloendotheliosis virus, a replication-defective acute leukemia virus, weighed 30% less than the controls. The runting induced by REV viruses does not occur because of reduced food intake. Activities of phosphoenolpyruvate carboxykinase, a key gluconeogenic enzyme in the liver, were reduced approximately 40 and 50%, respectively, by infection with reticuloendotheliosis-associated virus and reticuloendotheliosis virus. REV virus infection, however, did not affect the hepatic pyruvate carboxylase activity, indicating that inhibition of phosphoenolpyruvate carboxykinase is not due to a general inhibition of all liver enzymes. Birds given injections of UV-inactivated REV viruses or reticuloendotheliosis virus-transformed, non-virus-producing tumor cells also exhibited a reduction in phosphoenolpyruvate carboxykinase activity.

INTRODUCTION

Neoplastic diseases of humans are frequently complicated by a variety of symptoms, including weight loss, immunosuppression, and anemia, which are collectively termed cachexia (1-3). Retroviruses have provided excellent animal models to study neoplastic diseases, and many retroviruses have been shown to induce host responses which are similar to responses in human cachexia (4-18). Several retroviruses have been shown to induce immunosuppression in experimentally infected animals (4-12). Weight loss or runting has been demonstrated in chickens infected with avian myelocytomatosis virus (MAV-O; 13-14) or Rous-associated virus-7 (15) and in mice infected with Abelson leukemia virus, a murine acute leukemia virus (16). Certain avian leukemia viruses have also been shown to induce anemia (17, 18).

Avian REV-T, an acute leukemia virus, is capable of inducing a fatal lymphoid leukemia in nearly 100% of infected animals (19-24). REV-T transforms very immature lymphoid cells both in vivo (20) and in vitro (25-27). Unlike hematopoietic cells transformed by other avian acute leukemia viruses, REV-T-transformed lymphoid cells have infinite growth potential in vitro and are capable of proliferation and metastasis when injected into histocompatible animals (8, 25, 26). The disease induced by REV-T was originally described as a running syndrome because of the failure of the infected animals to gain weight (19). REV-A, a replication-competent REV group virus which serves as helper virus for REV-T, is capable of inducing a chronic running disease (28). REV group viruses also induce a well-characterized severe suppression of the cellular immune response [4, 5, 8; reviewed by Bose (11)]. The objective of this study is to establish the REV virus system as a model to study metabolic defects during retrovirus-induced disease. In this publication we demonstrate that the activity of a key hepatic gluconeogenic enzyme, PEPCK (EC 4.1.1.32), is inhibited in animals infected with REV group viruses. This metabolic aberration may contribute to the running syndromes induced by REV viruses. We also examined the role of the helper virus and of tumor cells in inducing the inhibition of PEPCK activity.

MATERIALS AND METHODS

Viruses. REV-T phenotypically mixed with the helper virus REV-A was obtained from the culture fluid of RECC-UT1, a REV-T-transformed bone marrow cell line (25). REV-A was cloned from these mixed stocks by endpoint dilution and propagation in leukosis-free chick embryo cell cultures (SPAFAS Corp., Norfolk, CT) as described previously (29). Sucrose gradient-purified REV-A was inactivated, when indicated, by exposure to a General Electric germicidal UV lamp at a distance of 10 cm (31). A standard endogenous reverse transcriptase assay (32) was used to determine biological activity of the virus preparations after UV exposure.

Non-Virus-producing Cells. Derivation and biological characterization of REV-T-transformed non-virus-producing cell (NP) lines have been described previously (8, 26). RECC-UT310, a NP cell line isolated by in vitro infection of spleen cells from an SC chick (BbBb homozygous at the major histocompatibility locus of chickens), has high tumorigenic potential and is capable of inducing lethal reticuloendotheliosis when injected into SC birds (26).

Animal Studies. Approximately 10⁵-10⁷ PFU of REV-A were injected intravenously into 2-week-old SPF AS chickens. Mock-infected animals were given injections of culture supernatants from uninfected chick embryo cell cultures. Virus-infected animals and age-matched control animals were housed in cages modified to prevent the loss of feed. Birds were fed measured amounts of Purina chick starter and given water ad libitum. Body weights and food consumption were determined by weighing on a Metier balance. UV-inactivated REV-A was injected intravenously (10⁶ PFU equivalents twice daily) into 2-week-old SPF chickens. Cells (10⁴) from RECC-UT310 were injected intravenously into 2-week-old SC chickens.

Enzyme Assays. Livers from decapitated chickens were homoge-
nized in 9 volumes of 10 mM imidazole-HCl buffer (pH 6.9) containing 0.25 M sucrose at 0°. Samples were subjected to three freeze-thaw cycles using a dry ice/acetone bath before centrifugation at 100,000 × g for 45 min at 4°. The supernatant fraction was used for the determination of PEPCK activity by the method of Ballard and Hanson (29) as modified previously (30; 33–38). Reaction mixtures for determination of PEPCK activity contained 100 mM imidazole-HCl (pH 6.6), 2 mM MgCl₂, 1 mM glutathione, 1.25 mM inosine diphosphate, 2.5 mM NADH, 1.5 mM phosphoenolpyruvate, 2 units malate dehydrogenase/ml, and 2 μCi 14C-bicarbonate/ml. PC (EC 6.4.1.1) activity was determined on the same liver supernatant fluids by the method of Ballard et al. (39). Reaction mixtures for determination of PC activity contained 2.5 mM Tris-HCl (pH 7.4), 10 mM sodium pyruvate, 2.5 mM sodium ATP, 0.75 mM acetyl CoA, 5 mM MgCl₂, and 2 μCi 14C-bicarbonate/ml.

Mitogen Stimulation of Lymphocytes. Procedures used by this laboratory for chicken lymphoid cell culture and mitogen stimulation have been described previously (4, 5, 8). Spleens were removed aseptically and macerated through a fine wire mesh screen into RPMI1640 medium, and lymphocytes were separated from erythroid cells by centrifugation over Ficoll-Hypaque. Single cell suspensions were cultured at 5 × 10⁸ cells/ml in 12- x 75-mm Falcon culture tubes with or without the mitogen PHA. [3H]Thymidine (1 μCi/ml) was added to each culture at 48 hr incubation. After an 18-hr labeling period, incorporation of radiolabel into trichloroacetic acid-precipitable form was determined by scintillation spectroscopy.

Statistical Evaluation. Statistical significance was determined by the Student’s t-test (40).

RESULTS

Food Consumption and Runtng in Birds Infected by RE Group Viruses. RE group viruses induce a number of manifestations which are similar to certain symptoms of human cachexia, including runting (4, 5, 8, 11, 19–28). Injection of 2-week-old chickens with REV-A (10⁶ PFU) resulted in severe runting by 10 weeks after injection (Fig. 1). In this experiment, REV-A infected birds weighed only one-half as much on average as the control birds. The REV-A-infected birds also showed evidence of abnormal feathering. Animals infected with RE group viruses may be deficient in a metabolic function or functions which affect the ability to gain weight. Another possibility is that the infected animals are impaired in their ability to eat. To distinguish between these alternatives, 2-week-old chickens (SPAFAS Corp.) were infected with REV-T or REV-A or were mock-infected and were housed in modified cages to monitor their food consumption. The mean body weight of chickens in all groups was approximately 60 g at the beginning of the experiment. Body weights of mock-infected birds did not differ significantly from those of uninjected animals. Differences in body weight between the REV-A-infected and mock-infected group in this experiment were statistically significant (P < 0.001) at 3 weeks (Chart 1). Chickens infected with REV-A consumed amounts of food comparable to the mock-infected group throughout the course of the experiment. At 12 weeks the mean body weight of the birds infected with REV-A was approximately 40% less than that of the control group, yet at this time food consumption per kilogram of body weight was unchanged. The results suggest that the runting induced by REV-A is not due to anorexia. Four out of the 10 birds infected with REV-A also developed neurological dysfunctions after 12 weeks and became unable to stand. Two of the birds that did not develop neurological symptoms died (14–15 weeks post-infection) of a disease characterized by large nodular lesions composed largely of mononuclear cells in the liver and spleen. Both the cells and the lesions were distinct from those characteristic of reticuloendotheliosis induced by REV-T. The rapidity of onset and severity of the runting induced by REV-A was variable (Fig. 1 and Chart 1). REV-A-infected animals...
weighed between approximately 30 and 50% less than control animals at the time of death in other experiments.

Infection with REV-T also resulted in runting, as described previously (19). At day 3 the mean body weight of the chickens in the REV-T-infected group was significantly less ($P < 0.01$) than that of the mock-infected controls (Chart 2A). By 7 days the weight of the REV-T-infected birds was approximately 30% less ($P < 0.001$) than that of control birds. REV-T and mock-infected chickens consumed approximately equal amounts of food until day 5 of the experiment (Chart 2B). Therefore, the REV-T-infected birds did not lose weight because they failed to eat. On day 7 the consumption of food by the REV-T group had dropped below that of the control group, but all of the birds in the group died of reticuloendotheliosis by the next day.

Inhibition of Hepatic PEPCK by Infection with RE Group Viruses. Birds infected with RE group viruses consumed approximately the same amounts of food as control animals. Therefore, it is likely that RE viruses induce a metabolic defect(s) in infected animals. The liver is a key organ in carbohydrate metabolism. To define whether birds infected with RE viruses have a defect in carbohydrate metabolism, we monitored the activity of PEPCK and PC. PEPCK and PC are the key regulatory hepatic gluconeogenic enzymes (33-38). PEPCK is under regulation by corticosteroid hormones, while PC is not under stringent hormonal control. We were also interested in the effects of RE virus infection on the activity of PEPCK, because prior results have indicated that infection of mice with Sindbis virus, an α-virus, inhibited the hormonal induction of this enzyme (33, 34, 41).

Two-week-old SPAFAS chickens were infected with REV-T or REV-A or were mock-infected and were sacrificed at various times, and liver samples were taken to determine PEPCK and PC activity. The activity of PEPCK began to decline in REV-A-infected birds (Chart 3) relative to control animals at 3 weeks ($P = 0.05$) and in REV-T-infected birds (Chart 4) at 3 days ($P < 0.01$). Blood glucose and liver glycogen levels were also significantly lower in chickens infected with RE group viruses (data not shown). Efforts to demonstrate corticosteroid induction of PEPCK activity in uninfected and infected chickens gave inconsistent results. In comparison with mammals, birds appear to be insensitive to parentally administered corticosteroids. Levels of liver PC activity, however, did not differ significantly between either the RE virus-infected group or their mock-infected age-matched controls. The decrease in hepatic PEPCK activity, therefore, cannot be explained by the general depression of all liver enzymes.

Inhibition of Hepatic PEPCK by Injection of UV-inactivated REV-A and by REV-T-transformed Non-Virus-producing Cells. Immunosuppression and weight loss are common features of cachexia. RE group viruses induce a severe suppression of the cellular immune response. The induction of immunosuppression by RE viruses requires active virus replication, and therefore UV-inactivated virus fails to induce immunosuppression (5). To
determine whether the immune-suppressive properties of REV-A contribute to the acute runting phenomena, birds were given injections of UV-inactivated virus, and the levels of their PEPCK and PC were monitored. Gradient-purified preparations of REV-A were UV-irradiated as described in "Materials and Methods." This procedure results in the formation of uracil dimers in the viral RNA (31). As shown in Chart 5, UV irradiation at a dose of greater than 960 ergs/mm² (exposure for 1 min) resulted in the loss of detectable endogenous reverse transcriptase activity. UV inactivation under these conditions prevents the transcription of the retroviral RNA into DNA, a necessary event in the life cycle of the virus. Furthermore, infectious virus could not be rescued by passage of the UV-inactivated stocks in chick embryo fibroblasts. An amount of UV-inactivated virus (1920 ergs/mm² irradiation) equivalent to 10⁶ PFU of uninactive virus was injected twice daily into 2-week-old chickens for 4 consecutive days (8 injections). This level of inactivated virus was chosen to compensate for the fact that animals receiving uninactive virus would be exposed to increased levels of viral components as a result of virus replication. Two-week-old birds infected with REV-T were also included in this experiment. On day 5 of the experiment, livers were taken from the animals for determination of the activities of PEPCK and PC, and spleens from the same animals were taken for assay to determine the mitogen responsiveness of the lymphoid cells. UV-inactivated REV-A was capable of inhibiting the activity of hepatic PEPCK but not the activity of PC (Table 1). The rapid decline in PEPCK activity in birds given injections of UV-inactivated REV-A may be explained by the large dose of virus injected. The observation that injection of UV-inactivated RE virus can inhibit the activity of PEPCK suggests a role for virion components in inducing this effect. PEPCK levels returned to control levels within 2 weeks post-injection of the UV-inactivated virus. Birds given injections of UV-inactivated REV-A which inhibited hepatic PEPCK were monitored for up to 4 months. None of these animals developed the chronic runting syndrome or other overt symptoms associated with REV-A infection. These results imply that virus replication is necessary to maintain the inhibition of hepatic PEPCK activity and to develop the runting associated with REV-A infection. In contrast to the effects on liver PEPCK activity, the PHA responsiveness of spleen cells from birds given injections of UV-inactivated REV-A was not significantly different from that of mock-infected animals (Table 2). These results indicate that birds infected with these UV-inactivated virus preparations were not immunosuppressed. Spleen cells from birds given injections of non-inactivated REV-T were unresponsive to PHA, indicating a severe suppression of the cellular immune response. These results suggest that immunosuppression and inhibition of PEPCK activity induced by RE viruses involve independent mechanisms.

In birds infected by REV-T, the acute transforming virus, PEPCK levels decline significantly earlier than in those birds infected by the non-transforming REV-A. In addition to the virion components, animals infected with REV-T, phenotypically mixed with REV-A, are exposed to antigens expressed by tumor cells which are accumulated in the liver and spleen. To establish a possible role for the tumor cells in the runting induced by REV-T, SC chickens were given injections of cells from a line of REV-T-transformed hematopoietic cells (UT-310) which do not produce virus particles. The NP cell line was isolated by in vitro infection of spleen cells from SC chickens which are homozogous for the B locus (B₂B₂), the major histocompatibility complex in chicken. These REV-T-transformed non-virus-producing cells are tumorigenic in histocompatibly matched birds. These cells multiply in the visceral tissue of infected birds, leading to the development of lethal reticuloendotheliosis (25). REV-T-transformed NP cells were also capable of inhibiting the activity of PEPCK in the absence of a productive virus infection (Table 3).
The activity of PC was not significantly affected by the injection of NP cells.

DISCUSSION

Birds infected by members of the avian RE virus group fail to gain weight at the same rate as that of uninfected animals. However, the runting induced by RE group viruses is not due to an impairment in the ability of the infected animals to eat. We have demonstrated that hepatic PEPCK activity is decreased during infection by RE viruses, relative to control birds. This enzyme is of major importance in the regulation of gluconeogenesis, and the decrease in activity could be responsible for depletion of carbohydrate reserves in the infected animal. Mobilization of these reserves is presumed to be important for normal energy-requiring metabolic processes and for growth. Thus, the decrease in the activity of PEPCK could be involved in the runting induced by RE viruses. Another predictable physiological consequence of decreased PEPCK activity is that animals would be more susceptible to stressful conditions than control animals (34). Indeed, we have observed that RE virus-infected animals, unlike control animals, are unable to make the metabolic adaptations necessary for extended survival in a cold environment (34). Other enzymes or metabolic processes could also be affected by RE viruses; however, the runting induced in chickens by RE group viruses is not due to a general depression of all hepatic enzymes, since the activity of hepatic PC was not measurably affected.

In an effort to define whether inhibition of PEPCK activity requires active virus replication, birds were given injections of UV-inactivated virus preparations. UV-inactivated REV-A preparations were able to decrease the levels of hepatic PEPCK activity. These results indicate that virion components may be responsible, at least in part, for inducing runting. The development of the runting syndrome, however, did require ongoing virus replication, which suggests that extended exposure to virus-specified components is necessary for development of this symptom. Runting and immunosuppression are general features of cachexia. In addition to runting, birds infected by RE viruses also develop a severe cellular immune suppression. The immunosuppression induced by RE group viruses is mediated by a population of splenic suppressor cells (4, 5, 8). The inhibition of PEPCK activity induced by RE viruses is not, however, due to this immunosuppression. UV-inactivated RE viruses fail to induce the suppressor cell population or immunosuppression (Ref. 4; Table 2). Therefore, unlike inhibition of PEPCK activity, the induction of immunosuppression by RE viruses requires active virus replication. Perhaps, release of virus or the presence of virus structural protein precursors in infected cells activates the suppressor cells. Because retrovirus-specified proteins undergo extensive processing before, during, and after packaging into virions, the possibility that different domains or regions of the same virus-specified molecule is involved in both immunosuppression and inhibition of PEPCK activity cannot be ruled out.

Replication-competent REV-A, the helper virus for the replication-defective acute leukemia virus REV-T, is capable of inducing a runting syndrome and inhibiting the activity of PEPCK. Other replication-competent retroviruses have been shown to induce runting syndromes, as well as other aspects of cachexia (6–9, 12–18). In this regard it is noteworthy that human T-cell leukemia (lymphotrophic) virus-III, a replication-competent retrovirus, has been strongly associated with acquired immune deficiency syndrome, which is characterized by immunosuppression and severe weight loss (42–46).

The helper viruses associated with replication-defective transforming retroviruses may contribute to the neoplastic disease process by inducing runting and other aspects of cachexia. However, birds infected by REV-T, the acute transforming virus, develop runting and exhibit reduced levels of hepatic PEPCK much earlier than do birds infected by REV-A. REV-T-infected birds contain tumor cells which accumulate in the liver, spleen, and other visceral organs. REV-T tumor cells may contribute to and accelerate the development of the runting syndrome and inhibition of PEPCK activity in birds infected with REV-T. Birds infected with REV-T-transformed, non-virus-producing cells become runted and exhibit a reduced activity of hepatic PEPCK. In addition, REV-T-transformed non-virus-producing tumor cells are capable of inducing immunosuppression (8). These non-virus-producing tumor cells do not contain the genomes of replication-competent RE group helper viruses and fail to produce detectable levels of the precursors to the major viral structural proteins (25, 26). Therefore, the activities present in non-virus-producing cells which are capable of inducing the inhibition of PEPCK activity and of inducing immunosuppression must be encoded by the genetic material of REV-T. Despite having several large deletions, the REV-T genome does contain almost the entire open reading frame in the gag gene of REV-A, based on comparative restriction endonuclease analysis (47, 48). These helper virus-related regions of the REV-T genome, conserved during acquisition of the viral oncogene, may be involved in either immunosuppression or runting. In addition, the REV-T-specified transforming protein is predicted to contain 12 virus-related Env-specific amino acids at the amino termini (49) and may therefore be involved in inducing immunosuppression and/or inhibition of hepatic PEPCK.

The mechanism by which PEPCK is inhibited following infection by RE group viruses has not been established. Several different agents or physiological insults, including endotoxin, polyinosinic-polycytidylic acid, and several viruses, inhibit glucocorticoid induction of PEPCK levels in mice (33–38). Polyinosinic:polycytidylic acid inhibits PEPCK activity in chickens; however, the injection of other materials such as liposomes did not inhibit the enzyme. As discussed above, NP cells, which are not anticipated

<table>
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<th>Table 3</th>
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<tr>
<td><strong>Inhibition of hepatic phosphoenolpyruvate carboxykinase by injection of REV-T transformed, non-virus-producing cells</strong></td>
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<tr>
<th>Injection</th>
<th>PEPCK activity*</th>
<th>Significance vs. mock-infected</th>
<th>PC activity*</th>
<th>Significance vs. mock-infected</th>
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</thead>
<tbody>
<tr>
<td>Mock-infected</td>
<td>118.0 ± 3.0†</td>
<td>5.42 ± 0.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>RECC-UT310 NP</td>
<td>69.3 ± 6.6</td>
<td>P &lt; 0.001</td>
<td>6.54 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>REV-T</td>
<td>51.8 ± 7.3</td>
<td>P &lt; 0.001</td>
<td>5.11 ± 0.6</td>
<td>NS</td>
</tr>
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*Units enzyme/mg protein at 5 days after injection (n = 6).
†Mean ± SE.
‡2-week-old SC chicks were given injections (i.v.) with 10⁵ RECC-UT310 non-virus-producing cells.
§Not statistically significant.

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to spread viral components in the absence of a helper virus, are capable of inducing an inhibition of PEPCk activity. In view of this observation and the fact that several diverse agents may inhibit PEPCk activity in this and other animal systems, it is likely that the changes in PEPCk activity induced by RE viruses are due to a soluble mediator. Modulation of PEPCk activity to mice infected with viruses or treated with endotoxin or polyo-

insincopolytydicyclic acid appears to be mediated by a protein we have termed glucocorticoid antagonizing factor (37, 38). A possibly related factor, termed cachectin, which has been shown to be present in wasting mice bearing tumors, has been isolated from endotoxin activated-macrophages in another laboratory (50)7. Thus, it will be important to determine the role of factors related to glucocorticoid antagonizing factor or cachectin in the RE virus-induced inhibition of PEPCk activity. It will also be important to clarify what role, if any, running or the inhibition of certain gluconeogenic enzymes might play in the oncogenic process. Therefore, it would be of interest to determine if dietary alterations could influence the inhibition of PEPCk activity, the development of the running syndrome, or the course of the diseases induced by RE viruses. Animal models, such as those provided by RE group viruses, offer a variety of experimental approaches to better understand weight loss and other aspects of cachexia.

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REFERENCES


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