The Metastatic Process of a Transplantable Lymphocytic Tumor of Chickens (RPL 16)

I. The Time of Metastasis*

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It has been suggested that the resultant pathology exhibited by an individual bird which has received an inoculation of RPL 16 tumor cells is related to factors concerning metastasis of tumor cells from the primary growth and the reaction of the host to the tumor (15). As to the phenomenon of metastasis, such factors as the time of onset, the relative number of cells freed into the circulation, the uniformity of the metastatic process once established, and the fate of the tumor cells are probably of significance.

As indicated by the review of Coman (8), the subject of the time of onset of metastasis has not been emphasized in studies dealing with metastasis. Yet, it would seem that an appreciation of the time of onset of metastasis is important in the characterization of an experimental tumor and important in the comparison of tumors or the study of a given tumor subjected to varying influences. Plenk et al. (19) demonstrated that metastatic cells from a primary growth of C-1300 (neuroblastoma) of mice could be detected in lymph nodes as early as the 4th day after inoculation in a few instances. Metastatic cells could be detected in all cases 12 days after inoculation. Time of onset of the metastatic process should be of further significance when it is considered in reference to the survival times of animals inoculated with a particular tumor.

This is a report of the results of a series of experiments designed to establish one of the characteristics of the metastatic process of RPL 16, the time at which tumor cells are freed from a primary growth into the circulation.

MATERIALS AND METHODS

Tumor.—RPL 16 is a transplantable lymphocytic tumor of chickens. It was isolated from a White Leghorn hen by Burmester and Prickett in 1945 (5). The tumor has been employed by a number of investigators (2, 3, 6, 7, 10-13). It has been under study at this laboratory since 1952. Although it is probable that the tumor arose as a virus-induced neoplasm, no tests have been performed to establish the presence of a virus. All reports dealing with RPL 16 have been confined to its characteristics as a transplantable tumor. The classical criteria which distinguish a tumor cell transplant from a virus transfer, the production of a local tumor at a site of inoculation and a short survival period of recipients, have been fulfilled by the characteristics of RPL 16 in the experiments reported below. The short survival times of birds inoculated with cellular suspensions of RPL 16 are emphasized by the fact that the tumor must be transferred each 4-6 days to assure perpetuation of the tumor. The average survival times of birds for the tumor passages utilized for the experiments of this report were 6.7-11.6 days.

Plan of experiments.—RPL 16 produces local growth upon intramuscular inoculation of a tumor cell suspension. The term “massive metastasis” has been employed to describe the manner of distribution of tumor cells throughout the host (14).
No leukemic reaction is detectable by conventional technics (9, 14). The presence of tumor cells in blood taken from veins leading from growing tumors can be demonstrated by inoculation into young chicks. Typical lesions of experimental leukemia and death are produced. Survival times are of the order associated with cell transplants of RPL 16.

The results reported below are based on five experiments designed to determine the time of onset of metastasis. For these experiments, groups of birds were utilized in which each animal had received 0.5 cc. of a tumor cell suspension inoculated into the musculature of the tibial segment of the right leg. These constituted the routine transfers by which the tumor is perpetuated in this laboratory. Three birds were chosen from each experimental group for examination on each day. The blood was diluted 1:10 with Ringer's solution, and 0.5 cc. of this material was inoculated intraperitoneally into each of ten birds. The times at which death of the remaining birds served as an indication of the growth characteristics of the tumor in the particular passage and permitted comparison of the results of the five experiments. The times of death of the remaining birds of the passage were pertinent to die of leukemia. The times at which death occurred were recorded. The average of the times of death of the remaining birds served as an indication of the growth characteristics of the tumor in the particular passage and permitted comparison of the results of the five experiments. The characteristics of the five passages are presented in Table 1.

**Criteria for presence of metastatic cells.**—The recipient chicks were kept under observation for 3–6 weeks. All which succumbed were autopsied. The production of typical lesions of experimental leukemia in some or all of the chicks given inoculations of the blood of a given donor provided direct evidence of the presence of metastatic tumor cells in the inoculum.

A second criterion for the presence of circulating tumor cells was employed. All chicks which survived the original inoculation were challenged with 0.5–1.0 cc. of tumor cell suspension inoculated into the pectoral musculature. The challenge procedure was a test for resistance to the transplanted tumor, resulting from previous experience with tumor cells, in birds which had received numbers of metastatic cells insufficient to initiate fatal tumor growth. The rationale of this procedure is based on the fact that birds which survive a cellular inoculation of this, or similar, tumors are resistant to a second inoculation (5, 6, 17). It has been established that a cytotoxic effect can be demonstrated in the serum of birds which survive a cellular implant (1) and that cells of the reticulo-endothelial system are involved in the reaction of the host to the second implant (16). It has been concluded that viable tumor cells are required to produce resistance (5, 4, 17) and that cell products are not capable of eliciting the response (18). These investigations indicate that for many transplantable lymphocytic tumors of chickens (including RPL 16) two dose-dependent responses to the inoculation of tumor cells are to be expected. One level of tumor cells results in death of the bird; another, lower, dose level fails to kill but elicits immunity which is demonstrable upon challenge of the bird by a subsequent inoculation of tumor cells. Results of challenge of surviving chicks were compared with the response of a group of control chicks of the same age. Resistance was exhibited by survival of test chicks or by prolonged survival times as compared with the control group. It might be argued that the challenge test employed in these experiments did not test for previous contact with viable cells but merely for "natural resistance" of the chicks. Although results of inoculation of cellular suspensions of RPL 16 are variable as to the times of death of recipients, experience with over 4,000 young birds used in routine maintenance passages indicated that very few survive. The inocula used in the challenge of surviving chicks of the experiments reported below were comparable to those used in perpetuating the tumor strain; and, therefore, it would seem unlikely that the degree of resistance usually encountered in a challenged group could be due to "natural" causes. In some instances the test for experience with tumor cells by challenge was more sensitive than the original inoculation. In one case (Experiment 4), surviving chicks from a group, some of which had died showing typical lesions, died as a result of challenge. This might be explained on the basis of nonuniform distribution of cells in the original inoculum which resulted in no experience which tumor cells for some of the chicks and thus left them incapable of resisting the challenge inoculation. A second explanation might lie in the variable factors concerned with the inoculum used in the challenge. At the time of these experiments no effort was made to quantify the materials used for challenge.

**RESULTS**

**Experiment 1.**—One group of three birds was tested at 120 hours after inoculation. Some of the chicks inoculated with blood from all three birds died of typical leukemia.

**Experiment 2.**—Three groups were tested.
Birds tested at 48 hours after inoculation showed no evidence of metastasis either by original inoculation or subsequent challenge of the recipient chicks. By 72 hours metastasis had occurred. None of the chicks died, owing to the original inoculation; however, resistance was demonstrated in all three groups upon challenge. At 96 hours, blood from two of the three birds produced typical lesions upon inoculation into young chicks; and the third was determined to be positive on the basis of results of challenge of the recipient chicks.

Experiment 3.—Five groups were tested. Blood from one of the three birds tested at 53 hours after inoculation produced typical lesions (local tumor and enlarged liver, spleen, etc.) in one chick. Challenge of the surviving chicks given inoculations of blood from the three test birds showed no evidence of resistance. The one positive case resulting from the original inoculation was considered sufficient to designate the test bird as exhibiting metastasis.

Experiment 4.—Two groups were tested. At 28 hours, metastasis was not detectable by either original inoculation or subsequent challenge. At 51 hours, metastasis had occurred in one of three birds tested. Blood from this bird produced typical lesions (local tumor and enlarged liver, spleen, etc.) in one chick. Challenge of the surviving chicks given inoculations of blood from the three test birds showed no evidence of resistance. The results of the five experiments are summarized in Table 2. It will be noted that there was no evidence of metastasis by the end of the 1st day (23–28 hours): however, one-half of the birds tested on or soon after the end of the 2d day (24–53 hours) exhibited evidence of metastasis. At 3 days and thereafter, all birds tested revealed that metastasis had occurred.

Table 2 presents a number of apparent discrepancies which are resolved by correlating the results with the average survival times of the particular passages. In both Experiments 2 and 5, groups were tested at 48 and 72 hours after inoculation. None of the birds tested at 48 hours in Experiment 2 revealed the presence of metastatic inoculation produced typical lesions in a young chick. Surviving chicks and chicks inoculated with blood from another test bird showed evidence of resistance to challenge. The third bird showed no evidence of metastasis either by original inoculation or subsequent challenge of the young chicks. At 76 hours, metastasis had occurred in all three birds tested; two were positive by initial inoculation of chicks, and the third proved positive by challenge. At 102, 123, and 148 hours after inoculation, blood of all birds produced typical cases of experimental leukosis upon inoculation into young chicks.

Experiment 5.—In this experiment, three groups were tested. At 23 hours after inoculation, metastasis had not occurred. Chicks showed no evidence of having had previous experience with tumor cells either by original inoculation or by
cells in their circulations; however, metastasis had occurred in the cases of all three birds of Experiment 5 tested at 48 hours. It will be noted in Table 1 that the average survival time of birds of passage 81, employed in Experiment 2, was long (239 hours) as compared with the average survival time of birds of passage 102 employed in the fifth experiment. In the latter case, 48 hours represented the 30th percentile of the average survival time of the passage, whereas that time represented but the 20th percentile of the estimated survival time of the birds used for Experiment 2. The same tendency is evident from the results of the tests at 72 hours. In this case all three birds of each group showed evidence that metastasis had occurred. However, in the experiment dealing with a passage of a longer survival time, the levels of tumor cells in the circulations of the birds tested were not sufficiently high to produce typical lesions and death upon inoculation into recipient chicks but were sufficient to elicit resistance as exhibited by challenge. The three birds tested from passage 102 (Experiment 5) all revealed levels of tumor cells sufficient to cause death in the recipient chicks upon original inoculation. Seventy-two hours was estimated to represent the 30th and 44th percentiles of the survival times of the passages from which these two experiments were derived. Since it is possible to relate variation in the time of onset of metastasis of individual experiments to the growth characteristics of the tumor at the times of the experiments (as expressed by the average survival times of the passages), it is of value to consider all the data from the standpoint of the per cent of the survival time represented by each test. In this manner, it is possible to describe the onset of metastasis in relative terms, which might apply to any group of birds inoculated with RPL 16. In addition, this method of presentation is of value in formulating further questions concerning features of metastasis.

Chart 1 presents the results of the five experiments in terms of per cent of the average survival times of the passages from which the experiments were derived. It demonstrates that metastasis can occur as early as the 20th percentile. One of six cases tested at the 19th and 20th percentiles exhibited metastasis. At or near the 30th percentile, metastasis occurred in eight of nine birds tested. Metastasis was occurring at a sufficient level in four of these to produce typical lesions and death in young chicks. Samples from four other birds induced resistance in the inoculated chicks which was demonstrable when they were subsequently challenged. All samples tested at the 40th and 45th percentiles were positive; seven produced death in young chicks, and two elicited resistance. At the 60th percentile and thereafter, tumors were produced by all blood samples tested. It appears that metastasis occurs in all cases at, or before, the midpoint of the survival time.

DISCUSSION

As previously suggested, the resultant pathology exhibited by an individual bird which has received a transplant of RPL 16 tumor cells is related to factors concerning the growth of the tumor and the reaction of the host (13). The results of inoculation of a cellular suspension of RPL 16, or of any tumor with similar growth characteristics, into the musculature of a susceptible chicken may be responsible to the following conditioning...
phases: a period of local growth, the onset of metastasis, the distribution and deposition of metastatic cells, the fate of the deposited cells, and a period of secondary growth which results in death of the animal. Any of these phases can be conditioned by the susceptibility of the host, either reduced by a reaction resulting from previous contact with tumor cells of the same strain or altered by intrinsic characteristics such as age, size, etc.

As to the metastatic process, the time of onset, the number of cells freed from the primary growth at the time of onset, and the uniformity of the cell level in the blood once metastasis has been established are potentially variable features. Information concerning one of these, variation of the time of onset of metastasis of RPL 16, can be derived from the data produced by the experiments reported above. Metastasis may be demonstrable at 48 hours; in other cases it is not demonstrable until 72 hours after inoculation. Potential factors responsible for each variation, age and size of the host and the site of the primary tumor (and its dependency upon dose of cells), merit investigation. It is of interest to note that in Experiments 3 and 5, as compared above, the birds which exhibited relatively delayed metastasis were older (43 days of age) than those showing an earlier onset of metastasis (20 days of age).

Little can be said concerning variations in the number of cells freed from primary growths of RPL 16 at the onset of metastasis or concerning changes in the number of cells freed from a given tumor once metastasis has been established. There is evidence in the above data that metastatic cells increase in the blood stream as the disease progresses. Whether this is owing to increased shedding of cells from the primary tumor or to the gradual accumulation of cells due to incomplete clearance of tumor cells from the circulation is not known. That the numbers of tumor cells increased is suggested by the change of the proportion of birds in which metastasis was demonstrated by original inoculation as the survival time of the donors advanced. This feature is demonstrated in Chart 1. It will be noted that four of eight positive cases at or near the 30th percentile of the survival period produced lesions in chicks upon original inoculation. Between the 40th and 45th percentiles seven of nine cases tested produced death in young chicks. This situation suggests an increasing level of metastatic cells in the circulation of the donors tested at the later period. Further evidence for such an increase can be derived from a consideration of the survival times of chicks given inoculations of blood samples taken at progressively later periods in the survival times of the donor groups. Survival times of 106 chicks which succumbed owing to the initial inoculation of metastatic cell-bearing blood were available for this comparison. The decrease in survival times was demonstrated in Experiments 3 and 5, in which two or more groups were available for comparison. The lower limit of the ranges of times of death of the groups decreased slightly. In Experiment 3, the first chick of the 76-hour test group died at 301 hours. At 102 hours, the first assay chick died at 268 hours. At 123 and 148 hours, the first chicks succumbed at 216 and 217 hours, respectively.

A feature of technical value has been derived from the above experiments. Since metastasis occurred in all birds after the midpoint of the survival period, it might be judged safe to make tumor transfers for the maintenance of RPL 16 any time after the midpoint of the anticipated survival time.

**SUMMARY**

Five experiments were conducted to determine the time of onset of metastasis from primary intramuscular growths of RPL 16. Blood samples were drawn from the vein leading from the tumor, diluted, and inoculated into susceptible chicks.

Metastasis may occur as early as the 9th day after intramuscular inoculation. Metastasis was detected, either by initial inoculation or by subsequent challenge of the recipient chicks, in all birds tested after 72 hours.

The onset of metastasis may vary by 24 hours among birds of the same passage. It is suggested that this is one of the factors which influence the outcome of a given case.

Evidence is presented which suggests a progressive increase in the level of metastatic cells in the circulation during the survival period.

**REFERENCES**

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