Serum Mucoid Levels in Rats Bearing Walker Carcinoma 256 and the Effect of Surgical Exirpation of the Malignancy*

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SUMMARY

In rats given implants of Walker 256 tumor a sizable increase in tumor mass has been shown to precede significant increases in the serum mucoid levels. Surgical extirpation of the tumor leads to a return of the serum mucoids to normal values. If substantial metastasis has occurred, the serum mucoid values do not return to normal. If minimal amounts of tumor remain in situ or in unsuspected metastatic areas, then serum levels may fall to normal, only to rise again when substantial growth occurs. The chromatographic behavior of serum mucoids in tumor-bearing rats is shown to differ significantly from that of normal rat serum.

Serum mucoids form a small fraction of serum proteins. They contain hexosamines and sialic acid but not hexuronic acid, phosphate, or sulfate. They are divisible into a number of fractions in accordance with their chromatographic behavior (8, 16). Their importance is exemplified by the rise in total serum mucoid level in a variety of diseases (1, 5, 7, 9, 20). This increase is particularly pronounced in malignant disease (4, 22). Although considerable work has been reported in this area, little or no information is available on the temporal relationship between the onset of malignancy and the rise in serum mucoid levels. Furthermore, it has not been sufficiently clear whether this rise reflects a proportional or a disproportional increase in the various fractions of serum mucoid. The present investigation was designed to clarify these points. The results reported herein show that the rise in serum mucoid level of rats bearing Walker 256 tumor begins after the tumor becomes demonstrably palpable. This rise is, therefore, of little value as an early indicator of tumor growth. However, there was a definite and reproducible deviation from the normal serum mucoid pattern reflected by a disproportionate change in the various serum mucoid fractions. Our results further indicate that, after complete removal of the tumor, the serum mucoid level drops accordingly and remains at normal levels. If extensive metastasis is already present, the serum level does not fall but remains elevated. If minimal amounts of tumor remain in situ or in unsuspected metastatic areas, then serum levels may fall to normal, only to rise again when substantial growth occurs.

MATERIALS AND METHODS

Male, Sprague-Dawley, adult rats weighing 150-175 gm. were used throughout this study. Walker 256 rat carcinoma mince was prepared from freshly excised tumor that was maintained by means of repeated transfer into the hind leg.

All steps in the preparation of the tissue were carried out under aseptic conditions at 6°-8° C. Freshly excised tumor was scraped free of necrotic material and trimmed of excess fat and fascia. The tissue was transferred to Hanks solution containing 300 µg of penicillin and 300 µg of streptomycin per ml. and was cut into small pieces, first with scissors, then by repeated forced ejection through an 18-gauge needle. The minced suspension was sedimented at 1500 × g for 10 minutes. The volume of the packed mince was recorded and 10 volumes of fresh Hanks solution added; 0.5 ml. of this suspension was injected intramuscularly into the hind leg (or legs) of each recipient animal. No implantation failures occurred. It is of interest that the mince remains viable when stored at −50° C. suspended in 50 per cent glycerol in Hanks buffer for periods up to 90 days.1

The procedure used for assay of serum mucoids is a modification of that reported by Winzler et al. (13). The modifications consist of using one-half the final concentration of perchloric acid and 4 times the amount of serum. Since both the acid concentration (8, 17, 21) and serum concentration (2, 8) influence the composition of the final product, the ratio of hexose to tyrosine of 3.5:1 (21) was taken as the standard. The following procedure was found to give a product with the proper ratio in 25 per cent higher yields (8). All manipulations were performed at 0° C.; 0.2 ml. of serum was mixed with a 0.2-ml. volume of 0.15 M NaCl, followed by 0.4 ml. of 0.6 M perchloric acid. The precipitated protein was centrifuged off, and the mucoids present in 0.4 ml. of the clear extract were subsequently precipitated by the addition of 0.1 ml. of 2.5 per cent solution of phosphotungstic acid in 2 N HCl. The precipitate was separated by centrifugation and

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washed with 5 ml. of 70 per cent ethanol. The washed precipitate was then dissolved in 0.1 N NaOH for protein assay. Protein was determined by the method of Lowry (11).

Chromatography on DEAE.—The procedure used was essentially similar to that previously reported (8). The serum was precipitated with perchloric acid as above, and the supernatant was adjusted to pH 5.0 with 6 N KOH. The potassium perchlorate precipitate was allowed to settle and the mother liquor separated by decanting. The latter, containing the serum mucoids, was freed of dialyzable material by dialysis overnight against ice-cold water. At this point the final yield of serum mucoid obtained by this technic compares favorably with that obtained by the phosphotungstic acid method (8). The dialyzed solution was then adjusted to pH 7.2 with NaOH and applied to a DEAE column 2.5 × 5 cm., which was previously adjusted to pH 7.2 and equilibrated with 0.05 NaCl. The mucoids were then fractionated by stepwise elution with increasing concentrations of NaCl. The following molar concentrations were used: 0.03, 0.06, 0.11, 0.16, and 0.35. Eight lbs. p.s.i. of pressure was applied to the column and yielded a flow rate of approximately 0.5-1.0 ml/min. Based on 280-μm absorbancies, the recoveries in these experiments were approximately 80 per cent, and rechromatography of the individual peaks showed that about 85 per cent of the material fell into a corresponding homogeneous peak.

RESULTS

The natural history of the progress of implanted Walker 256 carcinoma in this laboratory corresponds to those recorded by Weimer et al. (19) and by Macbeth et al. (14), with the exception that the course was more rapid. Lung lesions were apparent by the 9th day instead of the 12th, and death occurred in 2-3 rather than 3-4 weeks. These differences are reflected in the inoculum size.1

Chart 1 is illustrative of a number of experiments and represents a comparison of the average tumor growth in the hind legs of six rats, with the average rise in total serum mucoids. The mean initial serum mucoid level and standard deviation was 118 ± 16 mg. per cent and was essentially maintained for the first 8 days. It then rose sharply to a maximum of 375 ± 45 mg. per cent on the 15th day. The tumor mass was 0.21 ± 0.07 sq. cm. on the 4th day, increasing to 1.54 ± 0.7 sq. cm. by the 8th day, and reaching 14.25 ± 3.3 sq. cm. on the 15th day. These data show that a significant amount of tumor growth can be detected by palpation before a rise in serum level can be observed.

The usefulness of the serum mucoid level in assessing the occurrence of metastatic growth is shown in the following experiment: Rats given injections in the hind leg of 0.5 ml. of fresh tumor mince were randomly divided into five groups. In Group I only one hind leg of each animal was given an injection of tumor.

CHART 1.—Comparison of tumor growth and serum mucoid level. Six rats were given inoculations in the left thigh of 0.5 ml. of a 10 per cent suspension of tumor mince in Hank's solution. Periodic 0.6-ml. blood samples were taken by lancing the tip of the tail and collecting drops of blood into a 12-ml. conical centrifuge tube. The serum was removed, and the serum mucoids were measured as described in Methods. Tumor growth is expressed in square centimeters and is the product of the major times the minor axis of the tumor, as estimated by measuring the dimensions with a transparent plastic rule.
In Group II both hind legs were given injections of tumor. This group contrasts with Group I and serves to evaluate the effect of a double dose and a larger tumor mass on the mucoid level.

In Groups III and IV one hind leg was given an injection, and the tumor was subsequently removed by amputation of the leg at the pelvic joint.

In Group III amputation was performed on the 5th day when the tumor mass was small, palpable, and before a substantial rise in serum mucoid had occurred. Amputation of the leg was performed on the 9th day in Group IV, when the tumor was large and the serum mucoid level uniformly high.

Group V consisted of rats given injections of 0.5 ml of 1 per cent bovine serum albumin solution and served as control.

The data are summarized in Chart 2. The numbers in parentheses refer to the number of animals initially in each group. Mucoid levels in the control (BSA) group fall within the normal variation, with an over-all mean of 123 ± 17 mg. per cent, and show no significant alteration as a result of repeated bleedings and injections.

A comparison of Groups I and II reveals little or no significant difference in the rise of serum mucoid. For example, the means ± S.D. in the respective groups for day 3 were 117 ± 21 mg. per cent and 114 ± 19 mg. per cent; day 9, 180 ± 37 mg. per cent and 208 ± 40 mg. per cent; day 17, 312 ± 25 mg. per cent and 358 ± 71 mg. per cent. The corresponding probability values were 0.15 for day 3, 0.10 for day 9, and 0.61 for day 17. All members in both groups died by the 20th day and, at autopsy, showed massive metastasis in the lungs.

A comparison of Groups III and IV in which amputation of the hind leg was performed on the 5th and 9th day, respectively, shows that both groups experienced the expected transient post-surgical rise in serum mucoid. Recovery from the surgical stress was apparent in the fall of mucoid level by the 4th or 5th day after surgery. All animals in Group IV showed a rising level that was maintained at about 270 mg. per cent until death and upon autopsy showed extensive metastatic growth in the lungs. It is significant, on the other hand, that Group III did not develop a rise in serum mucoid level, had no fatalities, and showed no evidence of metastasis at autopsy. However, two animals in this group, not included in Chart 2, showed a rise in serum mucoid level on the 24th day. Both of these animals had small but definite metastatic lung lesions. The livers were not grossly involved. Serum mucoid determinations may therefore be of significant value in evaluating the occurrence of metastasis following surgical removal of the primary tumor.

The chromatographic distribution on DEAE cellulose columns of the various fractions of serum mucoid from pooled normal rat serum showed a striking difference in pattern from that obtained with pooled serum from tumor-bearing rats. Six pools of normal rat serum and five pools of serum from tumor-bearing rats were tested. A typical example from each category is presented in Chart 3. All chromatographic fractions appear in sera of both normal and tumor-bearing animals. However, the quantitative relationship is markedly different. The major fraction of the tumor-bearing animals is eluted with 0.11 M NaCl, whereas the corresponding peak of normal serum is relatively insignificant. In contrast, the predominant fraction of normal serum is eluted with 0.06 M NaCl, at which concentration only a minor fraction appears in tumor serum. In one experiment similar to Group III above, in which both serum levels and chromatographic characteristics of the serum mucoids were determined, the pattern which was initially like that of normal serum became tumor serum-like on the day before surgery and gave an intermediate pattern (0.06 M fraction equal to 0.11 M fraction) on the 8th day after surgery. The total serum mucoid levels at the corresponding times were 112 mg. per cent, 205 mg. per cent, and 158 mg. per cent.

The basic relationship between serum mucoid levels and tumor growth remains obscure. The high level in serum is not reflected by a high level in the tumor tissue (5, 19). The fact that serum levels increase indiscriminately in a variety of disease states (7, 20) suggests a general metabolic disturbance affecting liver synthesis (3, 15) or peripheral utilization (6, 11). The liver can be affected in generalized disease states which as a consequence might exhibit a high mucoid serum level. However, it is not readily apparent how the liver can be affected by a localized and distant malignancy unless a metastatic extension occurs or a toxic factor is elaborated. In the two animals of Group III discussed above the lesion observed was in the lung and not in the liver at the time the serum

**DISCUSSION**

The basic relationship between serum mucoid levels and tumor growth remains obscure. The high level in serum is not reflected by a high level in the tumor tissue (5, 19). The fact that serum levels increase indiscriminately in a variety of disease states (7, 20) suggests a general metabolic disturbance affecting liver synthesis (3, 15) or peripheral utilization (6, 11). The liver can be affected in generalized disease states which as a consequence might exhibit a high mucoid serum level. However, it is not readily apparent how the liver can be affected by a localized and distant malignancy unless a metastatic extension occurs or a toxic factor is elaborated. In the two animals of Group III discussed above the lesion observed was in the lung and not in the liver at the time the serum

*S. Harshman, unpublished observation.*
mucoid level was high. The concept of a toxic factor is supported by two additional observations. First, a significant amount of tumor growth must occur before any change in serum mucoids is detected (Fig. 1). This finding is in agreement with the data reported by Weimer et al. (19), and, assuming that the variations in plasma carbohydrates mainly reflect variations in serum mucoids, the data of Macbeth et al. (14) and Shetlar et al. (18) could be interpreted as supporting this view. Second, an apparent maximum value exists in the rate of rise in the serum mucoid levels (see Chart 2, groups I and II). Both of these observations would be expected if the metabolic regulation of serum mucoid synthesis in the liver (13, 15) were modified by a factor elaborated by the tumor. Thus, a threshold amount of factor would be required to induce an effect, but, once maximum suppression or stimulation of a given pathway was achieved, the further addition of modifier (tumor factor) would have little effect.

The data in Chart 2 indicate that the serum level returns to near normal once the tumor tissue is totally removed. It fails to do so in those animals which have gross metastatic growth, or it may rise again after a relatively long interval in animals which subsequently show metastasis. Data which may be similarly interpreted have been reported in C3H mice with MH 134 tumors (10). It is suggested that a longitudinal study of serum mucoid levels in patients following surgical extirpation of a malignancy may be of significant diagnostic value in detecting the occurrence of metastatic growth. Chromatographic characterization may enhance this value, since sera from tumor-bearing rats were readily distinguishable from sera derived from normal rats. Whether this technic can be developed into a meaningful diagnostic tool must await further experimentation. The chemical nature of the various fractions is not yet determined. The chromatographic shape of the fractions indicates heterogeneity within each fraction. Further purification and chemical analysis are needed.

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