Kinetics of the Acute-Phase Reaction in Rats after Tumor Transplantation

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ABSTRACT

Transplantation of Yoshida sarcoma (solid type) and Zajdela ascites hepatoma tumors in rats induces a biphasic change in the concentration of the following five acute-phase proteins: α1-acid glycoprotein; α1-antitrypsin; haptoglobin; hemo-
pepxin; and ceruloplasmin. These proteins and other plasma proteins were quantitated by two-dimensional immunoelectrophoresis relative to normal serum concentrations. The elevation of most of these acute-phase proteins was greater in the second phase, during which serum levels increased continuously as the tumor burden increased until the animals died. The increase in haptoglobin concentration during the second phase was much higher in rats bearing Yoshida sarcoma than in rats bearing Zajdela tumors. Rats receiving irradiated tumor cells showed neither tumor growth nor second-phase protein changes. Significant increases in uptake of 3H-amino acids by isolated perfused livers of tumor-bearing rats provided evidence for an increase in the hepatic synthesis rates of the acute-phase proteins. Removal of the solid tumor resulted in a gradual decrease of acute-phase protein concentrations with concomitant increase in serum albumin concentration. These alterations in serum acute-phase proteins during tumor growth and after removal of the tumor may make their use attractive as biological markers of the response of the tumor-bearing animal to its tumor.

INTRODUCTION

Acute-phase proteins are a group of glycoproteins, the concentration of which increases in plasma in response to a wide variety of stimuli, such as acute and chronic inflammation (11, 28), tissue injury (2, 8, 13), and tumor growth (10, 29). The mechanism by which these stimuli induce increased acute-phase protein synthesis by the liver is not well known and may be influenced by a variety of factors (4, 6, 14, 17). The application of 2-dimensional immunoelectrophoresis has made it possible to evaluate the complex changes of plasma acute-phase reactants evoked by subjecting experimental animals to various noxious stimuli (20, 25).

In malignant neoplasms, the plasma protein composition may be altered qualitatively and quantitatively by the effect of the tumor and the host reaction to it. Many attempts have been made to detect specificity in the pattern of plasma protein changes in order to provide additional parameters for the diagnostic and prognostic evaluation of the clinical stages of cancer. Scherer et al. (24) demonstrated different patterns of correlation between the concentration of 20 individual plasma proteins and erythrocyte sedimentation rate in patients with neoplasms and other diseases. Such changes in the concentration of the proteins studied were found to be specific to the respective diseases.

In the present study, the kinetics of alterations in plasma concentration of several acute-phase proteins were investigated after transplantation of tumors in rats.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male Wistar rats (Mus-Rattus, Brunnthal, Federal Republic of Germany) weighing 125 to 150 g were maintained on laboratory chow and water ad libitum. In each experiment, 100 rats were randomly subdivided into control and experimental groups. Each group contained at least 6 rats.

Tumor Origin. An original stock of rats bearing Yoshida sarcoma (solid type) and Zajdela ascites hepatoma was generously donated by Prof. Dr. Gercke, Laboratorium für Krebsforschung (Hoechst AG, Federal Republic of Germany). The tumors have since been routinely transplanted in our laboratories.

Transplantation of Tumor Cells. Yoshida sarcoma (solid type) tumors were removed after about 10 days when the tumor diameter reached 3 to 5 cm. The tumors were dissected in sterile 0.9% NaCl solution to remove connective and necrotic tissue. Pieces of this tumor material (2- to 3-mm diameter) were implanted s.c. into experimental rats. Solid tumor growth was detected by manual palpation of the implanted zone. Positive detection was possible at the fifth day after inoculation when the tumor size reached a diameter of about 1.5 cm. Sham operations were carried out on a control group of rats.

Zajdela ascites hepatoma were isolated by sterile syringe puncture from peritoneal fluid at 6 to 7 days after inoculation when maximal ascitic fluid had developed. The experimental rats were directly inoculated i.p. with 0.5 ml of the extracted fluid containing about 10⁶ Zajdela cells. A control group of rats was similarly inoculated i.p. with sterile pyrogen-free 0.9% NaCl solution.

All rats inoculated with either Yoshida sarcoma or Zajdela ascites hepatoma developed tumors. No weight loss was observed in the tumor-bearing rats during the duration of the experiments. Under these conditions, all of the inoculated rats died within 10 days after tumor implantation.

Tumor Growth. Tumor growth was followed daily from Days 4 to 9 by caliper measurement of 2 perpendicular diameters. Solid tumors were also excised and weighed. Ascitic tumor cells were counted. The mean values of both tumor weights and number of Zajdela ascitic cells were plotted as a function of time after transplantation. The Yoshida sarcoma showed no metastasis, whereas metastatic Zajdela cells were found in paratracheal lymph nodes.
Kinetics of Changes in Acute-Phase Protein Concentration after Tumor Transplantation in Rats. Blood was collected from the tumor-bearing animal at Days 1, 2, 3, 4, 6, and 8 after inoculation. On each of these days, one group of rats bearing either Zajdela hepatoma or Yoshida sarcoma was sacrificed, and the sera of each group was pooled. A standard pool of sera was collected from a large number of rats immediately after transplantation of solid tumor or i.p. injection of ascitic tumor cells (zero time). Blood was collected from groups of control animals at the beginning and at the end of the experiment. Serum samples were also collected at similar time intervals from groups of rats inoculated previously with irradiated control animals at the beginning and at the end of the experiment. Serum samples were also collected at similar time intervals from groups of rats inoculated previously with irradiated control animals.

Immunoelectrophoresis. The method of 2-dimensional immunoelectrophoresis, as described by Laurell (15) and modified by Clarke and Freeman (5), was applied for the quantitative determination of rat serum proteins using the modified Desaga UGI apparatus (Desaga, Heidelberg, Federal Republic of Germany). Polyvalent rabbit antiserum to whole rat serum proteins was purchased from Dakopatts A/S (Copenhagen, Denmark). Quantitation of the immunoprecipitated peaks was done using digital planimeter (Digiplan AM02; Kontron GmbH, Munich, Federal Republic of Germany) after defined enlargement by projection. For quantitative comparison of various serum protein concentrations, the individual peak areas within the electropherogram of the standard pool of control rat sera and the sera of other experimental animals were measured. At least 30 immunologically distinct rat serum proteins were demonstrated within the immunoelectrophoretic pattern. Fifteen of these proteins were identified on the basis of their electrophoretic mobility, specific staining methods, and other physicochemical parameters or biochemical functions (1). For example, prealbumin and albumin were identified according to their electrophoretic mobility. Further proof for the identity of albumin was obtained by reacting the rat serum with monospecific anti-rat albumin (Nordic Immunological Laboratories, Tilburg, The Netherlands). In addition to electrophoretic mobility, α and β lipoproteins were stained with Oil Red O. Haptoglobin and hemopexin were stained with benzidine. Ceruloplasmin was identified by staining with p-phenylene diamine, and cholinesterase was identified by indoxyl acetate staining. Tranferrin was identified by autoradiography utilizing its iron-binding capacity. C3 complement component was detected by partial conversion of β,C globulin to β,A globulin after repeated freezing and thawing of rat serum as well as after prolonged storage of the serum at 4°C and by reacting against specific antiserum to rat C3 (Nordic Immunological Laboratories). α1AT2 was identified according to its affinity to bind trypsin. Antiprotease enzym complexes with the subsequent cathodal shift of the precipitated peak were identified by their immunoelectrophoretic pattern. α1AT identity was further confirmed using anti-rat α1AT provided by Dr. A. Koj (Institute of Molecular Biology, Krakow, Poland). The identity of rat AAG was characterized according to its electrophoretic mobility and glycoprotein staining. The microheterogeneity of the protein, indicated by the asymmetrical peak shape, is in agreement with the molecular heterogeneity of AAG, as isolated by Gordon and Dykes (9). AAG identity was further confirmed using anti-rat AAG provided by Dr. M. Billingham (I.C.I., Cheshire, England). For detailed procedures of identifications, see Ref. 1. A major advantage of 2-dimensional immunoelectrophoresis is its ability to resolve, identify, and quantify (relative to control values) many serum proteins simultaneously. Also, the technique demonstrates clearly the physiological and pathological changes in the complex mixture of serum proteins. It should be noted that rat plasma proteins are less well characterized than are human proteins and that monospecific antisera for most of individual rat plasma proteins are not available.

Liver Perfusion. Livers were removed from control animals or from tumor-bearing animals on the sixth day after tumor transplantation. The perfusing medium was L-15 containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (0.05 m) which was oxygenated for at least 30 min before the operation. The liver was perfused by recirculation of 100 ml of medium. The portal flow rate was maintained at 10 ml/min at 37°C. During the entire period of perfusion, the pH of the perfusion medium was kept constant at 7.4. The oxygen flow was maintained to achieve a constant gaseous O2 and CO2 content in the medium of about 95 and 5%, respectively. Fifteen min after starting the perfusion, 10 μCi 3H-radioactive amino acid mixture (high specific tritiated radioactive amino acid mixture; American, England) was added to the perfusion medium. Samples (2 ml) were taken at zero time and hourly thereafter for 4 hr for determination of total radioactivity. Another 4 ml of the perfusate were removed and dialyzed for 4 days in 0.9% NaCl solution at 4°C, followed by radioactive counting. After the withdrawal of the 6-ml sample of the perfusate, another 6 ml of L-15 medium containing 10 μCi 3H-amino acid mixture were added at each time interval. The perfusion lasted for 4 hr, and 40 μCi of 3H-amino acid mixture were added to the perfusion medium during the entire period of perfusion. The samples were mixed with Triton X-100 (5 ml) and toluene/PPO solution (0.6%, 10 ml) and counted in an SL 30 liquid scintillation spectrometer (Intertechnique, Paris, France).

RESULTS

Serum Proteins of Tumor-bearing Rats. By means of 2-dimensional immunoelectrophoresis, we were able to determine quantitatively and simultaneously 30 rat serum proteins with high degree of resolution. Fifteen of these proteins were identified by specific staining methods and other physicochemical parameters or biochemical functions (Fig. 1). The technique is highly reproducible for normal control rat sera. Distinct changes in the plasma protein pattern were precisely measured upon examination of the sera of tumor-bearing rats.

Kinetics of Changes in the Concentrations of the Acute-Phase Proteins. From Charts 1 and 2, it is clear that there is a biphasic change in the concentration of most of the plasma proteins determined in the sera of both groups of rats bearing Zajdela ascites hepatoma and Yoshida sarcoma. One day after transplantation, the concentrations of the following acute-phase reactants, AAG, haptoglobin, α1AT, hemopexin, ceruloplasmin, and Peak X, were substantially elevated. A maximum

1 The abbreviations used are: α1AT, α-1-antitrypsin; AAG, α-1-acid glycoprotein.
was obtained on the second day for most of these proteins (Figs. 2A and 3A). Their levels then declined gradually to a level slightly higher than normal. A concomitant decrease was observed in the levels of prealbumin, albumin, cholinesterase, and transferrin during the first 48 hr, after which their concentrations began to increase again.

The second phase of response started 4 days after transplantation of both tumors and was characterized by a second pronounced increase in the concentration of most of the acute-phase proteins at the sixth day (Figs. 2B and 3B). The rise in their levels continued at different rates as the tumor load increased until the rats died.

In both groups of experimental rats bearing the different types of tumors, the concentration of ceruloplasmin reached its maximum during the first 2 days. However, its level did not change significantly during the second phase of response. Interestingly, the concentration of haptoglobin decreased sharply at the fourth day in Zajdela tumor-bearing rats. Thereafter, its serum level increased again, but the percentage of increase was much lower than the very substantial rise demonstrated in Yoshida tumor-bearing rats during the same time intervals (Charts 1 and 2). The concentration of prealbumin, albumin, cholinesterase, and transferrin showed further decreases at different rates in the sera of experimental rats as compared to the control groups. The percentage of decrease was more pronounced in this second phase, especially for albumin in rats bearing Yoshida sarcoma. Analysis of the sera from rats inoculated with irradiated tumor cells showed a similar increase in the concentration of the estimated acute-phase

Chart 1. Time course of increase and decrease in rat serum protein levels after i.p. injection of Zajdela ascites hepatoma. AG, AAG; Alb, albumin; AT, a1AT; PA, prealbumin; HG, haptoglobin; Tf, transferrin; Hpx, hemopexin; CE, cholinesterase; Cep, ceruloplasmin; pX, unidentified peak. The mean standard deviation of the percentage of increase or decrease for each protein is: AG, 24; AT, 2.3; HG, 12.1; Hpx, 3.8; Cep, 9.5; Alb, 2.0; PA, 5.2; Tf, 2.2; CE, 2.9; pX, 7.3.
Acute-Phase Protein Changes in Tumor-bearing Rats

DISCUSSION

Numerous investigators are involved in studying the systemic action of the tumor on the organism. Several studies documented serum protein changes in tumor-bearing animals and different serum acute-phase protein patterns in cancer patients (12, 21, 30–32). In the present study, we found that both tumors, Yoshida sarcoma and Zajdela ascites hepatoma, induced a biphasic response after transplantation which can be followed quantitatively by measuring the changes evoked in the plasma proteins, especially the acute-phase proteins. The high resolving power of the 2-dimensional immunoelectrophoresis aided these studies. The primary phase following tumor implantation is most likely associated with the initial stress of injury together with the tissue necrosis which takes place following tumor transplantation, since the serum levels of most of the acute-phase proteins returned to a level only slightly higher than normal on the third day. The duration of the acute-phase protein changes during this phase is in agreement with previous results obtained after local inflammatory stimulation with carrageenan (23).

In the second phase of the response, the concentration of the acute-phase proteins increased continuously but at different rates as the tumor burden increased. The pronounced rise in the blood levels of the major acute-phase proteins, AAG, AlAT, haptoglobin, ceruloplasmin, and hemopexin, could be the result of de novo synthesis by the liver in response to the proteins reaching a maximum on the second day after inoculation, decreasing gradually to normal levels on the third day, and remaining unchanged until the end of the experiment.

Correlation of Tumor Growth with Elevation of Acute-Phase Proteins. Chart 3 shows the increase in both the number of Zajdela hepatoma cells and tumor weights of Yoshida sarcoma (solid type), respectively, after tumor transplantation. Tumor growth was least on the fourth day, after which both tumors grew fast and continuously until the death of experimental animals within 10 days after transplantation. Basically, a linear correlation could be demonstrated when the linear regression of the number of ascitic tumor cells (Chart 4A) or tumor weights of Yoshida sarcoma (Chart 4B) was plotted versus elevation of each of the acute-phase reactants during the second phase.

Release of $^{3}$H-Amino Acid-Labeled Proteins by Perfused Livers. As shown in Chart 5, the perfused livers from control rats and rats bearing Zajdela hepatoma and Yoshida sarcoma were active in plasma protein synthesis over a 4-hr period of perfusion. Also, the livers from tumor-bearing rats incorporated comparatively more of the $^{3}$H-amino acid mixture into the protein fraction of their perfusates.

Total Serum Proteins. Slight hypoproteinemia was observed in the experimental rats during the different time intervals of the experiments as compared to the control rats. The mean values of the total protein concentration of the pooled sera from control, and Yoshida and Zajdela tumor-bearing rats were 6.10 ± 0.15 (S.D.), 5.78 ± 0.22, and 5.71 ± 0.31 g/100 ml, respectively. These differences were insignificant in case of the rats bearing Yoshida tumors and slightly significant ($p < 0.02$) in the group bearing Zajdela tumors.

Chart 2. Time course of increase and decrease in rat serum protein levels after s.c. implantation of Yoshida sarcoma (solid type). Abbreviations are as in Chart 1. The mean standard deviation of the percentage of increase or decrease for each protein is: AG, 22.4; AT, 9.7; HG, 26.0; Hpx, 6.1; Cep, 6.6; Alb, 1.3; PA, 2.5; TF, 3.1; CE, 7.8; pX, 6.5.

tumor. These changes occurred primarily in the liver, as in cases of various forms of inflammation (18, 19, 22). Furthermore, the rats inoculated with irradiated tumor cells showed no changes in the concentration of plasma proteins during the second phase and no tumor growth. This demonstrates clearly that the second-phase response in tumor-bearing rats is primarily associated with tumor growth. The level of albumin, prealbumin, transferrin, and cholinesterase was depressed during the 2 phases of the inflammatory reaction induced by both types of tumors. Hypoalbuminemia is a characteristic aspect of the acute-phase reaction which invariably accompanies the rise in the acute-phase proteins without producing an osmotic imbalance (3). It is likely that the greater haptoglobin concentrations in animals with the Yoshida solid tumor are due to breakdown of hemorrhagic necroses which are unlikely to accompany the ascites tumor. Such a difference in haptoglobin concentration during the second phase of response could serve as a parameter for differentiation between these 2 types of tumors. It should be mentioned here that ceruloplasmin was the acute-phase protein which responded least to both types of tumors, while Ungar-Waron et al. (27) recommended its use as a reliable biochemical marker for neoplastic activity in rabbits bearing carcinoma VX-2.

The correlation between the increase in both the concentration of individual acute-phase reactants and tumor growth during the second phase is linear, as demonstrated in Chart 4, for animals bearing Zajdela hepatoma and Yoshida sarcoma, respectively.

The pathophysiological mechanism of increased synthesis of individual acute-phase proteins is not fully understood, but several evidences indicate that many humoral factors or products from the proliferating tumor cells themselves could stimulate the host liver for the de novo synthesis (7, 26, 27). The increased incorporation of 3H-radioactive amino acid mixture by the isolated perfused livers from rats bearing Yoshida sarcoma and Zajdela hepatoma during the entire period of perfusion is consistent with both increased synthesis and more rapid protein turnover rates since the total serum protein concentrations are virtually identical. The results of Shiraska and Fujii (26) also demonstrated the induced high activity of livers from...
rats bearing Yoshida sarcoma (solid type) or AH 130 (solid type). They observed increased thymidine incorporation into DNA and an approximately 100-fold increase in de novo synthesis of DNA-synthesizing enzymes.

The slight reduction of total serum protein concentration, despite increased protein synthesis, may be due to the depletion from the blood and accumulation into the ascitic fluid exudate (21). Analysis of the ascitic fluid developed in rats bearing Zajdelas ascites hepatoma at the sixth day after inoculation, by 2-dimensional immunoelectrophoresis, revealed the presence of albumin in a relatively higher concentration than the other individual plasma proteins. Interestingly, removal of the Yoshida sarcoma solid tumor at the fifth day after transplantation under aseptic conditions resulted in a gradual decrease in the concentration of most of the acute-phase proteins. Three days after removal of the solid tumor, the serum albumin concentration increased appreciably, whereas the level of the acute-phase proteins declined continuously. These alterations in serum acute-phase proteins during tumor growth and after removal of the tumor may possibly make their use attractive as biological markers of tumor-host responsiveness.

The biological activity of some of these acute-phase proteins is well defined (12, 25), such as hemoglobin binding by haptoglobin, transport of copper by ceruloplasmin, antiprotease activity of α1AT. Hemopexin scavenges circulating heme from circulation. As yet, no definite major biological function of AAG has been provided. However, this acute-phase protein may be involved in the healing of wounds and tissue repair and may also represent a natural inhibitor of lysosomal enzymes (25).

In conclusion, our present study shows that the levels of the following acute-phase proteins, namely, AAG, haptoglobin, α1AT, ceruloplasmin, hemopexin, and Peak X, increased in the blood of rats bearing tumors of different types and at different sites. Similarly, their blood concentrations increased continuously as the tumor load increased. Accordingly, the determination of the blood levels of these acute-phase proteins could furnish a useful biochemical parameter of clinical value in monitoring tumor growth.

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Fig. 2. Crossed 2-dimensional immunoelectrophoresis of serum from rats bearing Zajdel ascites hepatoma at 2 days (a) and 6 days (b) after tumor inoculation. Proteins are identified as in Fig. 1.
Fig. 3. Crossed 2-dimensional immunoelectrophoresis of serum from rats bearing Yoshida sarcoma (solid tumor) at 2 days (a) and 6 days (b) after tumor transplantation. Proteins are identified as in Fig. 1.
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