Changes in red blood cell osmotic fragility induced by total plasma and plasma fractions obtained from rats bearing progressive and regressive variants of the Walker 256 tumor

Abstract

Two variants (A and B) of the widely employed Walker 256 rat tumor cells are known. When inoculated sc, the A variant produces solid, invasive, highly metastasizing tumors that cause severe systemic effects and death. We have obtained a regressive variant (AR) whose sc growth is slower, resulting in 70-80% regression followed by development of immunity against A and AR variants. Simultaneously with the beginning of tumor regression, a temporary anemia developed (~8 days duration), accompanied by marked splenomegaly (~300%) and changes in red blood cell osmotic fragility, with mean corpuscular fragility increasing from 4.1 to 6.5 g/l NaCl. The possibility was raised that plasma factors associated with the immune response induced these changes. In the present study, we identify and compare the osmotic fragility increasing activity of plasma fractions obtained from A and AR tumor bearers at different stages of tumor development. The results showed that by day 4 compounds precipitating in 60% (NH₄)₂SO₄ and able to increase red blood cell osmotic fragility appeared in the plasma of A and AR tumor bearers. Later, these compounds disappeared from the plasma of A tumor bearers but slightly increased in the plasma of AR tumor bearers. Furthermore, by day 10, compounds precipitating between 60 and 80% (NH₄)₂SO₄ and with similar effects appeared only in plasma of AR tumor bearers. The salt solubility, production kinetics and hemolytic activity of these compounds resemble those of the immunoglobulins. This, together with their preferential increase in rats bearing the AR variant, suggest their association with an immune response against this tumor.

Key words
• Plasma fractions
• Temporary anemia
• RBC osmotic fragility
• Tumor regressive variant
• Cancer immunity
• Walker 256 tumor

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Research supported by CAPES and CAISM/UNICAMP. C.C. Gregorini was the recipient of a CAPES/CNPq fellowship. Publication supported by FAPESP.

Received August 21, 2002
Accepted April 22, 2003
Introduction

The Walker 256 (W256) tumor cells have been extensively used in studies of cancer pathophysiology (1-4). Two variants of this tumor have been reported, namely A and B (3). The A variant has been widely employed. It is known that tumor variants can be obtained by repeated passages through different media, a phenomenon that can be reversed by successive passages through the original medium (5). In our laboratory, after 60 successive intraperitoneal (ip) passages, a regressive variant (AR) of the W256 A tumor was obtained. The subcutaneous (sc) unifocal growth of the AR tumor variant is slower when compared to the A variant, and in 70-80% of the individuals tumor regression occurs followed by development of immunity against both the A and AR variants (Rettori O, Vieira-Matos AN, Guimarães F and Cavalcanti TC, unpublished data). Similar examples of the development of immunogenic variants of several tumors can be found in the literature (6).

Previous studies of the host’s response to the AR variant have shown that 8 to 12 days after unifocal inoculation and preceding overt tumor regression, a temporary anemia always develops, followed by an increase in red blood cell (RBC) osmotic fragility, a marked hypertrophy of the spleen (7) and presence in plasma of a substance able to increase the osmotic fragility of healthy RBC (Rettori O, Vieira-Matos AN, Guimarães F and Cavalcanti TC, unpublished data). Spleenectomy always greatly augmented the levels of anemia, with a further increase in RBC osmotic fragility. The hemolytic mechanism of the anemia and its early and temporary manifestation (both suggesting the involvement of polyclonal immunoglobulins), the apparent role of the spleen in modulating its evolution and the fact that it appeared only in the rats bearing the AR tumor variant raised the possibility that factors appearing in the plasma, associated with the immune response, could be responsible for the increase in RBC osmotic fragility observed in rats bearing the AR tumor variant, and that splenectomized tumor bearers could be a convenient source of these factors.

Considerable difficulty in experimental cancer studies, which usually employ unifocal tumor inoculations, is related to the fact that the initiation of the systemic effects of cancer in individuals of the same group is a random phenomenon. It may start any time between 6 and 47 days or more after tumor inoculation, with deaths occurring between days 14 and 58 or more (7). By employing multifocal simultaneous sc inoculations (2, 4 or more inoculations/rat), the beginning of the systemic effects of cancer is rapid and highly synchronized among individuals of the same group, beginning 3-5 days after tumor cell inoculation and leading to death within 10-13 days (7-9). Therefore, studies employing multifocal inoculations yield rapid and highly reproducible results.

The purpose of the present study was to partially characterize the putative plasma factors responsible for the increase in RBC osmotic fragility in AR tumor-bearing rats. For this purpose, we monitored the time course of appearance of increasing RBC osmotic fragility activity in fractions obtained from plasma of splenectomized rats receiving multifocal inoculations of the A and AR variants of W256 tumor cells.

Material and Methods

Experimental design

After splenectomy under ether anesthesia, the rats were randomly divided into three groups of 6 rats each: control, A tumor bearers, and AR tumor bearers. Twenty-four to 48 h later (day zero), all rats were inoculated sc under slight ether anesthesia. Control rats received 250 µl Ringer-lactate solution employed to dilute the tumor cells, and rats in the experimental groups (A and AR) re-
received the same amount of Ringer-lactate containing the appropriate tumor cells. Before tumor inoculation, 0.7 ml blood was collected from the suborbital plexus into a syringe containing 0.05 ml heparin (5000 IU/ml). A similar blood sample was also obtained on day 4 after the inoculations. On day 10, all animals were sacrificed under anesthesia and a large blood sample was collected following thoracotomy, iv heparin injection and heart section. Hemoglobin levels and the osmotic fragility of fresh RBC were also determined in all blood samples. On days 0, 4 and 10 after the inoculations, pools of plasma from 6 animals were obtained. One aliquot of each pool was frozen (-18°C) for further testing of its effect on healthy RBC osmotic fragility, and the remainder was used in ammonium sulfate precipitation studies. Finally, the effect of the plasma and its fractions on RBC osmotic fragility was tested employing fresh RBC from normal donors as described below.

Tumor and animals

The W256 A tumor line was provided by Dr. Maria C. Cintra Gomes of the Departamento de Fisiologia, Instituto de Biologia, UNICAMP. The line originally came from the National Cancer Institute Bank, Cambridge, MA, USA. This tumor and its AR variant are stored under liquid N2, and maintained by ip or sc passage in rats.

Eight-week-old male Wistar rats were used in this study. The animals were housed (5 per cage) at controlled room temperature (21º ± 2ºC) on a 12-h light-dark cycle. Animals were allowed free access to standard rat chow (Labina/Purina, Campinas, SP, Brazil) and water. The rats were splenectomized and then randomly divided into 3 groups: a) controls (N = 6), b) W256 A variant tumor bearers (A, N = 6), and c) W256 AR variant tumor bearers (AR, N = 6). Each tumor-bearing rat received multifocal simultaneous sc inoculations at four dorsal sites, spaced at least 1 cm apart, with 5 × 10⁶ tumor cells (A or AR) suspended in 250 µl Ringer-lactate, in each site. The controls received identical inoculations of Ringer-lactate (placebo). Plasma pools (6 animals/pool) were used in the ammonium sulfate fractionation studies. Tumor cells with 98% viability assessed by Trypan blue were obtained from the ascitic fluid of donor rats. General United Kingdom Coordinating Committee on Cancer Research guidelines for animal welfare were followed (10).

Ammonium sulfate fractionation of plasma proteins

Ammonium sulfate was slowly added to the pool of total plasma (2.0-5.0 ml, diluted 1/10 in PBS) immediately after collection from control and tumor-bearing rats under constant stirring to a final concentration of 60%, w/v (11). Stirring was maintained for another 60 min and the solution was then centrifuged at 375 g for 30 min. The supernatant and precipitates were named S60 and P60, respectively. The ammonium sulfate concentration of the S60 fractions was increased to 80% to obtain a P80 fraction by centrifugation as described earlier. The P60 and P80 fractions were resuspended in 2.0 to 5.0 ml of 10 mM PBS, pH 7.4. Finally, all fractions were extensively dialyzed against PBS for 48 h with six buffer changes. Samples were diluted in PBS or concentrated with a 10-kDa molecular membrane (Spectrum, type C, POR®, Houston, TX, USA) to reconstitute the original volumes. All operations were carried out on ice at ± 4ºC. The protein content of the different fractions was measured by the method of Lowry et al. (12).

Determination of hemoglobin and RBC osmotic fragility

Hemoglobin and RBC osmotic fragility were determined by the method of Dacie et al. (13). For practical purposes, mean cor-
Puscular fragility was employed for data analysis of RBC osmotic fragility defined as the NaCl concentration (g/l) causing 50% RBC lysis (13).

RBC from normal rats were used to test the osmotic fragility changes induced by the putative factors present in the plasma and its fractions from tumor bearers. After the centrifugation of blood, 150 µl of normal packed RBC was resuspended (final hematocrit: ~20%) and incubated at 37ºC for 30, 120 and 240 min in 0.6 ml of the following solutions: a) plasma from splenectomized controls; b) plasma from A tumor bearers; c) plasma from AR tumor bearers; d) fractions S60 control, A or AR; e) fractions P60 control, A or AR; f) fractions P80 control, A or AR; g) autologous plasma from RBC donors and last change dialysis buffer as controls. Before incubation, in order to achieve a glucose concentration of ~150 mg/100 ml, 3.5 µl of 25% glucose was added to the solutions lacking glucose (dialysis buffer and dialyzed fractions).

Autopsy

Autopsies were performed in all tumor-bearing rats. Special attention was paid to the eventual presence of metastases, significant tissue invasion and bleeding.

Statistical analysis

The results are reported as means ± SEM and means$_{\text{diff}}$ ± SEM$_{\text{diff}}$ when analyzing paired data. Statistical significance was determined by ANOVA followed by the post hoc Dunnett test (P < 0.05), and by the paired Student t-test.

Results

Development of anemia

Although there were no changes on day 4, ten days after tumor inoculation (11-12 days after splenectomy), hemoglobin levels dropped markedly in tumor bearers, from 15.2 ± 0.5 to
5.2 ± 0.4 g/100 ml in A tumor bearers and from 15.2 ± 0.5 to 6.7 ± 0.8 g/100 ml in AR tumor bearers. In control splenectomized rats, no significant changes were observed in hemoglobin levels (Figure 1).

Changes in RBC osmotic fragility induced by tumor growth

There was no significant change in the fresh RBC osmotic fragility of A tumor bearers in spite of the pronounced degree of anemia, while in AR tumor bearers a marked 58% rise in RBC osmotic fragility was observed on day 10 after inoculation (Figure 2). The increase in RBC osmotic fragility in these splenectomized animals usually started at about day 8 and reached a maximum (with intravascular hemolysis) around day 10, when the animals were sacrificed.

Changes in RBC osmotic fragility induced by unfractioned plasma and plasma fractions from A and AR tumor bearers

Only plasma samples obtained on day 10 from AR tumor bearers were able, after incubation at 37°C, to induce a significant increase in osmotic fragility of fresh RBC from normal donor rats (Figure 3). A full effect of increase in osmotic fragility was already observed after 30 min of incubation at 37°C and maintained from then on up to 240 min (data not shown).

Regarding the plasma fractions, four days after inoculation, P60 obtained from A and AR plasmas induced an increase in RBC osmotic fragility of fresh RBC from normal donor rats. On day 10, this activity disappeared in the plasma of the A tumor bearers, but remained in the plasma of the AR tumor-bearing rats (Figure 4). In addition, an important activity increasing RBC osmotic fragility appeared on day 10 in the S60 fraction from AR tumor bearers. This new activity precipitated completely at 80% ammonium sulfate saturation; no RBC osmotic fragility increasing activity could be detected in the S80 fraction (Figure 4). Full effects were already detectable after a 30-min incubation and therefore the results obtained after 120- and 240-min incubation have not been included.

Figure 4. Changes in osmotic fragility induced in normal donor RBC by incubation with total plasma and fractions from controls and from Walker 256 (W256) A and W256 AR tumor bearers obtained at different times after tumor inoculation. Data are reported as means ± SEM. The number of experiments is given in parentheses. P60: precipitate from 60% ammonium sulfate saturation; S60: supernatant from 60% ammonium sulfate saturation; P60-80: precipitate from 60-80% ammonium sulfate saturation. MCF, mean corpuscular fragility. *P < 0.05 compared to total plasma (ANOVA followed by the post hoc Dunnett test).
Autopsy

No evidence of metastasis was found in any of the experimental animals, nor significant tissue invasion or bleeding that could have masked the present observations.

Discussion

Cancer anemia

Cancer anemia is classified as an anemia of chronic diseases, whose pathogenic mechanism has been difficult to establish. Bone marrow production of RBC is usually slightly increased but insufficient to compensate for a moderately increased RBC destruction (14-17). At present, there are no clear explanations for either phenomenon, i.e., bone marrow relative insufficiency and increased RBC destruction. It has been suggested that the activation of the immune and inflammatory systems through the increasing levels of pro-inflammatory cytokines such as interleukins 1 and 6, TNF-α and INF-δ would induce iron retention by the reticuloendothelial system, gastrointestinal tract and liver, therefore exerting an inhibitory effect on erythroid precursors (18-20). The erythropoietin production and bone marrow response to it are decreased in cancer anemia (4,18,20,21) and treatment with recombinant erythropoietin has been reported to be only of temporary help (14,22). Macro- and/or microscopic alterations of bone marrow have long been ruled out as factors involved in cancer anemia in humans (23) and rats (4). In the terminal stage of cancer, RBC are rapidly destroyed, even transfused ones, at a rate of up to five times the physiological rate, without an increase in RBC osmotic fragility (8). This terminal anemia is not temporary but continues to increase with a very high correlation (r² = 0.86) with the other severe systemic effects involved in terminal general failure (8). Development of hemolytic anemia associated with increased RBC osmotic fragility is not frequent in cancer and has been associated with a) autoimmune antibodies, b) microangiopathic alterations, and c) chemotherapy (24,25).

The present study emphasizes the complexity of the mechanisms involved in anemia induced by cancer. We had previously reported that in intact rats bearing the progressive A variant of the W256 cancer the rapid terminal destruction of RBC was associated with a decrease or no change in RBC osmotic fragility (8). In contrast, rats inoculated with the AR tumor variant systematically developed a temporary anemia associated with a marked increase in RBC osmotic fragility at the beginning of tumor regression. This anemia progressed and rapidly led to lethal intravascular hemolysis only in splenectomized rats, a model used in order to amplify the effect we wanted to study in the present investigation. Chemotherapy was not employed in the present study and the characteristic fragments of RBC present in microangiopathic anemia were not found in the morphological studies performed on blood. The hemolytic nature of the anemia, its early and temporary manifestation in intact AR tumor bearers and the apparent role of the spleen in modulating it raise the possibility that immunoglobulins associated with an initial polyclonal immune response to the tumor could be involved in its mechanism, perhaps signaling the elaboration of a further step toward the development of an adaptive host immune response, whose success, however, is still not guaranteed.

Development of anemia and changes in RBC osmotic fragility induced by tumor growth

The development of anemia among A and AR tumor-bearing rats was highly synchronized. It started about day 4 and was marked at day 10 (see Figure 1). These results agree with the usual kinetics of the development of anemia induced by multifocal inoculation of W256 tumor cells in intact
rats, whose RBC are destroyed at about five times the physiological rate around the 10th day, as previously reported (8). Since splenectomized rats were used in the present experiments, this marked degree of anemia indicates that the spleen may not be the only site of RBC destruction in rats bearing the W256 tumor.

Ten days after inoculation a marked increase in osmotic fragility was detected only in RBC obtained from the animals receiving the AR variant of the W256 tumor cells (Figure 2). The presence of this activity only in plasma from rats bearing the immunogenic AR variant is consistent with the idea discussed previously that this hemolytic activity could reflect some kind of immunoresponse, maybe immunoglobulin production, against the W256 tumor. Contrary to the just temporary increase in RBC osmotic fragility observed in the intact AR tumor bearers, the increase in RBC osmotic fragility in the splenectomized AR tumor bearers progressed and fatally evolved to intravascular hemolysis and ultimately death, had the animals not been sacrificed in advance. These observations confirmed preliminary experiments indicating an exacerbation of the increase in RBC osmotic fragility in splenectomized W256 AR tumor bearers, and supported the use of splenectomized animals in the experiments that followed this study in order to isolate the putative substance that increases RBC osmotic fragility. In non-tumor-bearing rats, as can be seen in Figures 1 and 2, splenectomy by itself induced no significant degree of anemia or alteration of RBC osmotic fragility up to day 10 postinoculation (days 11-12 postsplenectomy; see Material and Methods).

Another interesting observation shown in Figures 1 and 2 is that the anemia induced by the W256 tumor cells was independent of the changes in RBC osmotic fragility, since the degree of anemia found in the splenectomized A tumor bearers, whose RBC showed no significant changes in osmotic fragility, was even higher than that observed in the AR tumor bearers, whose RBC osmotic fragility were markedly increased. In a previous study, intact rats inoculated with the W256 A tumor cells presented a decrease in RBC osmotic fragility associated with tumor growth (8). The lack of significant changes in RBC osmotic fragility in A tumor bearers observed in these experiments may be related to the fact that splenectomized animals were used in the latter experiments. These observations indicate the complexity of the mechanisms involved in anemia induced by cancer.

**Changes in RBC osmotic fragility induced by plasma from W256 A and AR tumor bearers**

Consistent with the increase in RBC osmotic fragility observed in animals 10 days after inoculation with the AR tumor variant, only the plasma from AR tumor bearers was able to increase the RBC osmotic fragility of normal non-tumor-bearing donors under incubation at 37ºC (Figure 3). These observations confirmed our preliminary observations and indicate that factors present in AR plasma may be responsible for the increase in RBC osmotic fragility observed in the animals bearing this regressive tumor variant.

**Changes in RBC osmotic fragility induced by plasma fractions from A and AR tumor bearers**

When RBC from normal non-tumor-bearing donors were incubated with plasma fractions from A and AR tumor bearers, two kinds of activities increasing RBC osmotic fragility could be observed (Figure 4): an early activity precipitating at 60% ammonium sulfate saturation, detected on day 4 in the plasma of both A and AR tumor bearers, and a later activity precipitating at 80% ammonium sulfate saturation, detected on day 10 only in the plasma of rats bearing the AR tumor variant. Interestingly enough, the early
activity disappeared on day 10 from the plasma of the W256 A tumor bearers, while persisting in the plasma of the animals bearing the AR tumor variant.

These results suggest that, during W256 tumor growth, compounds with a high molecular weight, which are able to increase RBC osmotic fragility in A and AR tumor bearers, were produced. These molecules could be part of an initial immune response against the tumor, which would be sustained only by the animals bearing the AR tumor variant. Furthermore, only the AR bearers were able to produce other additional molecules of smaller molecular weight with similar activities, suggesting the initiation of a possible adaptive immune response against the W256 cancer. The biochemical nature of these molecules is not clear, but their production kinetics, salt solubility characteristics and hemolytic activity resemble those of the immunoglobulin superfamily involved in immune responses.

In conclusion, changes in osmotic fragility of RBC occur during growth of the W256 tumor, with early and temporary increase in osmotic fragility being characteristic of the immunogenic AR variant. Products associated with this activity can be isolated from plasma of tumor bearers, whose chemical nature and pathophysiological role deserve further studies.

Acknowledgments

The authors are indebted to Dr. Maria C. Cintra Gomes from the Departamento de Fisiologia, Instituto de Biologia, UNICAMP, for providing the original Walker 256 A tumor line and Mr. Amilton Garcia for animal care.

References


