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Inhibition of Tumor Growth by Recognition Factors (37800)

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Recognition factors, which reside in the a_2-globulin fraction of plasma (1–3), have been demonstrated to enhance the phagocytosis of a variety of particulates (1–4) and are, therefore, essential for optimal expression of macrophage function. In the absence or depletion of these recognition factors (5,6), impairment of phagocytosis results due to the inability of macrophages to detect the presence of the injected foreign particulates. These findings denote that recognition factors are essential for macrophage surveillance and the initiation of the phagocytic event.

Previous studies from this laboratory have demonstrated that plasma from patients with carcinomas was depleted of recognition factors (7–9). The rapid recovery of recognition factor activity following surgical treatment of patients with primary tumors (7) was suggestive of a possible relationship between malignant tumors and recognition factor activity.

Studies conducted in rats employing Shay chloroleukemic tumor cells indicated that the proliferation of leukemic cells in the circulation was similarly associated with depletion of recognition factors (10). Additionally, plasma recognition factor activity was decreased immediately following the intravenous injection of leukemic leukocytes (11). The loss of circulating recognition factors upon tumor cell administration denoted that tumor cells were perceived as foreign particulates. This finding suggested that one of the immediate events which occurs upon the entrance of neoplastic cells into the circulation is the formation of a tumor cell-recognition factor complex. The formation of such a complex would be anticipated to assist the macrophage in the recognition and possible destruction of the foreign cell. This conclusion is based upon previous observations that macrophages possess the capability of not only phagocytizing tumor cells (12,13) but also to induce contact lysis (14). These composite findings suggest that by increasing recognition mechanisms of macrophages at the tumor site, it may be possible to effectively inhibit tumor development. The present study was, therefore, conducted to evaluate the influence of recognition factor administration on tumor growth.

Materials and Methods. Long–Evans Hooded rats, 3–4 wk old, were employed as hosts for chloroleukemic tumor cells (15). The Shay chloroleukemic tumor was maintained by the subcutaneous injection of 20×10^8 tumor cells in Long–Evans rats.

The recognition factor fraction was isolated from normal human plasma by the addition of 20,000 units of heparin per 100 ml of plasma. The plasma was maintained at 0° for 3–6 hr to precipitate the recognition factor fraction. Plasma samples were then centrifuged and the supernatant was removed. The precipitate was then washed with distilled water (0°), lyophilized and stored at —20°. The yield of the lyophilized recognition factor fraction approximated 1.0 mg of protein per ml of plasma. The lyophilized fraction was dissolved in sterile saline to a protein concentration of 20 mg/ml immediately prior to injection. Immunoelectrophoresis denoted a heterogenous plasma protein fraction. In agreement with previous studies (1–3), further purification with Sephadex G-200 revealed the phagocytic promoting activity to reside in the a_2-globulin fraction.

In the evaluation of the ability of the recognition factor fraction to modify tumor growth, 20×10^6 tumor cells were injected

subcutaneously with either (a) saline, (b) recognition fraction (7.5 mg), or (c) human serum albumin. The animals were killed 10 days following injection, tumor excised, and weighed.

The assay of phagocytic promoting ability of normal human plasma, as well as the supernatant and heparin-precipate fraction was conducted according to previously described procedures (5–11). Liver slices weighing approximately 200 mg were prepared with a Stadie–Riggs tissue slicer and placed in Erlenmeyer flasks which contained either 3 ml of Krebs–Ringer phosphate buffer or 1.5 ml of buffer and 1.5 ml of the various plasma fractions. The recognition fraction was added in the amount of 15 mg of protein to the buffer medium. The test particle was a gelatinized reticuloendothelial (RE) test lipid emulsion labeled with ^{131}I-triolein 2,5,6). The RE test lipid emulsion was added to the incubation flasks in a concentration of 2 mg. Heparin was added in the amount of 100 units to each flask. The flasks were then gassed with 95% O_2 and 5% CO_2 and incubated for 30 min at 37° in a Dubnoff metabolic shaker. After incubation, the liver slices were washed in saline to remove loosely absorbed lipid particulates. The amount of ^{131}I-triolein labeled lipid emulsion phagocytized by Kupffer cells of liver slices was determined with a Nuclear Chicago-Auto-Gamma scintillation counter. Results are expressed as the percentage of the lipid emulsion phagocytized per 100 mg of liver tissue (%ID/100 mg). The expression of recognition factor activity is denoted by the degree of phagocytosis manifested.

Chemotactic activity of the normal human plasma, the heparin-treated supernatant plasma, and the recognition factor fraction was assessed employing modified Boyden chambers (16) to ascertain whether the isolated recognition factor fraction possessed chemotactic activity as well as phagocytosis-stimulating activity. Peritoneal macrophages of rats induced by sterile sodium thioglycolate were obtained and washed twice with saline. The macrophages (1.0×10^6 cells) were placed in the upper chamber and normal human plasma or heparin-treated supernatant fraction were added in the amount of 0.2 ml to lower chamber. The isolated recognition fraction was added in the amount of 1.34 mg chamber. Sodium caseinate in the amount of 1 mg was also added to certain chambers to serve as a chemotactic agent. The chambers were maintained at 37° for 5 hr. Following incubation the filters were removed, stained with Bullard's hematoxylin stain, and the number of cells on the lower portion of the membrane counted. Ten high power fields were counted on each filter. Duplicate determinations were performed in each instance. Statistical analyses were performed on the basis of paired observations.

Results. The recognition factor fraction isolated from normal human plasma clearly enhanced the phagocytosis of the gelatinized RE test lipid emulsion by rat Kupffer cells (Table I). Normal human plasma induced a 24-fold enhancement in the uptake of the test particulates as compared to that manifested in buffer medium. The supernatant fraction from heparin treated plasma showed a mean 87% depletion in its ability to support phagocytosis. In contrast, the addition of the heparin precipitable fraction to buffer media produced mean 69-fold elevation in phagocytic uptake. Enhanced phagocytosis was also observed when the recognition factor fraction was administered intravenously to rats injected with the RE test lipid emulsion.

The role of recognition factors as chemotactic agents was also evaluated in modified Boyden chambers. It was observed that in the presence of sodium caseinate a 2.9-fold increase occurred in the number of macrophages that had migrated across the membrane by the fifth hour (Table I). Plasma depleted of its recognition factor activity did not support chemotaxis by peritioneal macrophages. No significant increase in macrophage migration occurred when sodium caseinate was added to plasma which was depleted of its recognition factor activity. In contrast, the addition of the isolated recognition factor fraction to buffer medium produced a significant increase in cell migration over that observed with normal human plasma. Likewise, migration was increased when sodium caseinate was added in the presence of the isolated recognition factor fraction. These studies denote the recognition

TABLE I. Phagocytic and Chemotactic Promoting Ability of a Heparin Precipitable Fraction of Normal Plasma.

Sample	Number	Phagocytic uptake	Chemotaxis	
			sodium caseinate	
		% ID/100 mg	−	+
Buffer	18	0.36 ± 0.01		
Normal plasma	18	8.88 ± 2.1	1.00	2.94 ± 0.84
Heparin-treated plasma, supernatant	18	1.12 ± 0.18	1.06 ± 0.49	1.31 ± 0.41
Heparin-treated plasma precipitate	18	24.9 ± 3.79	3.72 ± 0.87	10.9 ± 4.7

Values relative to the chemotaxis studies are derived from five to seven experiments conducted in duplicate. The − and + designations denote the absence or presence of the chemotactic substance, sodium caseinate in the lower chamber. The number of macrophages (mean 21.1 ± 7.4) per microscopic field (x450) which migrated across the membrane in the presence of normal plasma was considered as 1.0 in the chemotaxis experiments.

factor fraction, which has the capability of enhancing phagocytosis, also has chemotactic properties.

In saline injected rats that received tumor cells, the mean tumor weight approximated 10 g on the tenth day (Table II). In contrast, the administration of the recognition factor fraction with tumor cells resulted in an 86% decrease in tumor weight. The administration of albumin did not modify tumor development demonstrating the selective action of the recognition factor fraction in the inhibition of tumor growth. Since the inhibitory effect of the recognition fraction could be due to the direct cytotoxicity of the fraction on the transplanted cells, studies were conducted *in vitro* with tumor cells and the recognition factor fraction employing trypan blue as a vital stain. A 30-min incubation of cells and the recognition factor fraction indicated no direct cytotoxicity.

Discussion. Wright and Douglas (17) initially proposed the concept that plasma contained agents, classified as opsonins, which promoted the phagocytosis of a variety of agents by leukocytes. Hektoen (18), Vaughan (19), Boyden (20, Rowley (21), and Saba *et al.* (2) have demonstrated significant specificity of recognition factors in the phagocytosis of a variety of particulate agents. These investigators demonstrated that selective absorption of one recognition factor could be associated with little or no loss of recognition factor activity to another particulate agent. Additionally, Stuart (22) demonstrated that recognition factor existed in an invertebrate population, namely the *Eledone cirrosa.* He also reported that in contrast to the opsonic specificity which is manifested in the vertebrate population (2, 17–21), the octopus was characterized by a single plasma protein fraction which was polyreactive to a variety of particulate antigens.

TABLE II. Influence of Recognition Factor Administration on Growth of Shay Chloroleukemia Tumor Cells in Normal Rats.[a]

Group and treatment	No. of animals	Tumor weight g
Control, saline	42	10.0 ± 1.12
Recognition factor fraction	21	1.4 + 0.34
Human/Albumin	31	8.1 ± 1.41

[a]Normal Long–Evans rats received 20×10^6 tumor cells subcutaneously. A single injection of either saline, humoral recognition factor, or human albumin was made at the tumor injection site. Values are expressed as means ± standard error.

Studies in this laboratory have demonstrated that patients with carcinoma manifest a profound depletion in the activity of recognition factors (7,8,23). The degrees of loss was associated with the extension of the tumor as patients with primary tumors did not show the degree of depletion of recognition factor activity which was manifested in those individuals with metastatic states. Recognition factors were shown to be profoundly depleted in plasma of cancer patients when gelatinized RE test lipid emulsion, colloidal gold, or technetium sulfate (8,23) colloids were employed. However, when *S. albus* was employed as a test particle, normal activity was manifested

in patients with carcinoma (23). These findings may denote a specific deficiency in recognition factor which is required for the phagocytosis of gelatin stabilized particulates.

Additionally, it was demonstrated that following radiotherapy or surgical removal of primary tumors, patients showed a rapid elevation in plasma recognition factor activity (7). These findings suggested the possibility that tumor cells were acting as an absorbent and depleting plasma of recognition factors. In support of this concept that tumor cells might complex with recognition factors, it was demonstrated that normal rats which received an injection of leukemic cells showed an immediate loss of recognition factor activity (9,10) an event not observed when normal leukocytes were administered. The depletion of recognition factors, which also occurs upon the introduction of certain inert particles (5,6,11), suggested that tumor cells are perceived as foreign by the host and one of the initial reactions is the complexing of the tumor cells with recognition factors. This event would not only serve to enhance phagocytosis, but also to enhance chemotaxis of macrophages.

The present observation that the administration of a recognition factor fraction with tumor cells exerted profound inhibitory effect on tumor growth and development supports the concept of an important role of recognition factor activity in tumorgenesis.

Since macrophage inhibition of tumor growth is associated with an activation of macrophages as well as the induction of cellular immune responses (24–28), the activation as well as the mobilization of macrophages by the recognition factor fraction may be the mechanism of its antitumor action. It remains to be established whether administration of macrophages activating agents, such as BCG, *Mycobacterium bovis,* or glucan in conjunction with recognition factors will exert a synergistic action on tumor cell destruction.

The present findings that a water soluble protein component, with demonstrated ability to enhance macrophage phagocytosis and exert chemotatic activity, possesses the capability of modifying tumor growth further accents the concept that recognition factors may be important regulatory factors in macrophage mediated mechanisms in the detection or recognition of tumor cells. The recognition of tumor cells and possible assistance in destruction may be a regulatory role which recognition factors possess for maintaining internal environment as related not only to certain colloidal and particulate materials but also to malignant cells.

Summary. Recognition factors have been demonstrated to constitute one component of macrophage surveillance. Since depletion of plasma recognition factors occurs in experimental and clinical neoplasia, studies were undertaken to evaluate the contribution of macrophages and recognition factors to neoplasia. Employing Shay chloroleukemic cells as the transplant, the administration of recognition factors at the tumor cell transplantation site induced an 86% decrease in tumor weight. Specificity of this inhibitory response on tumor growth was demonstrated by the observation that administration of human serum albumin resulted in no modification in tumor growth. The isolated recognition factor fraction enhanced phagocytosis *in vitro* and also exerted a pronounced chemotactic effect. Macrophages and recognition factors may be of significant importance in tumor cell detection and rejection mechanisms.

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