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Immunoregulatory Characteristics of Human Recombinant Angiogenin

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The possibility of directed regulation of immunogenesis attract the attention of both scientists and to an even greater extent, physicians. The ever increasing array of bioactive substances modulating one or another immune process provides a rich resource for the optimal choice of a method with due consideration for the individual characteristics of the chosen agent and of the object of immunocorrection. For obvious reasons, substances of endogenous origin are most desirable here; the present paper is devoted to one such substance, the recently discovered bioactive agent angiogenin (AG). It was found on the plasma membranes of human adenocarcinoma cells [5]; like other solid tumors, this tumor constantly induces neovascularization. Since this phenomenon occurs in other diseases, as well as in some physiological states, for instance, in diabetic retinopathy, rheumatiod diseases, wound healing, cyclic physiological processes, and embryonal development, the detection of AG in mammalian blood serum [8] and cells [7] should be considered natural, in the same way as the detection of AG gene expression not only in tumors, but in normal human and animal tissues [9, 10] as well. Angiogenin represents a single-chain polypeptide with a molecular weight of 14000 D consisting of 123 amino acid and characterized by primary structures similarity to ribonucleases, by a restriction ribonuclease activity, and by an extremely high capacity for stimulating the growth of the vascular network. This unique property of AG suggest its use to enhance the healing of wounds, ulcers, and burns [6], the recovery of the myocardium after infarction, etc.

Since the above processes are associated with activation of the immune system function, we investigated its response to an increase of the AG level in the environment.

MATERIALS AND METHODS

A total of 197 CBA mice aged 3 to 4.5 months were used, at least 7 per group. Sheep erythrocytes (SE) were used as the antigen. Immune system activation induced by the antigen was assessed by three tests. For the rosette formation test (RFT) the mice were intraperitoneally injected with sheep erythrocytes or SE plus AG, 5×10^6/mouse. Then at various periods

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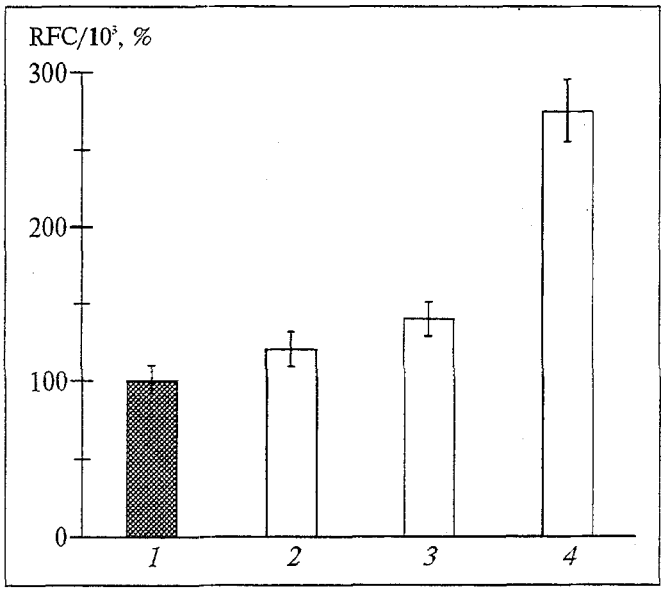

Fig. 1. Dose–dependent immunostimulating effect of angiogenin. Abscissa: *1*) mice injected SE (control, 100%); *2–4*) mice injected SE+AG in doses: *2*) 10^{-9} g/kg; *3*) 10^{-7} g/kg; *4*) 10^{-5} g/kg.

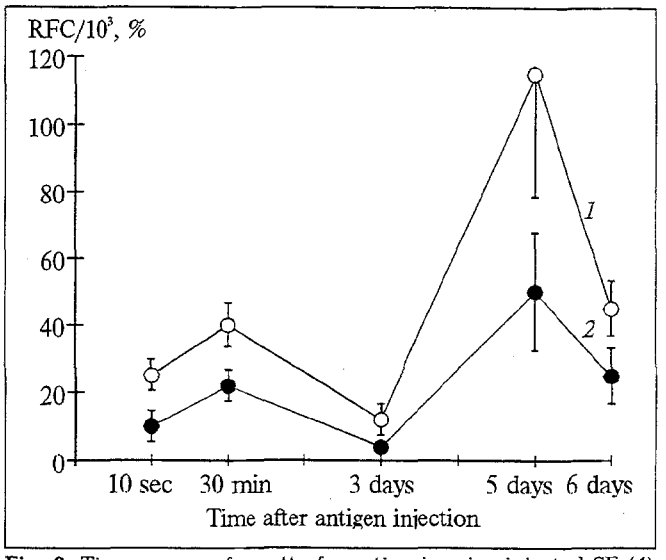

Fig. 2. Time course of rosette formation in mice injected SE (*1*) or SE+AG in a dose of 10^{-5} g/kg (*2*).

postimmunization splenocyte suspension were incubated with equal volumes of 3% SE suspension for 15 min at 37°C and a mobile preparation was made in which the slide rested on a mineral oil ring encircling the test suspension [4]. This was followed by estimation of the number of rosette-forming (antigen-binding) cells (RFC/10^3 cells) by a phase-contrast device, magnification ×1000.

Another type of cellular reaction was tested by delayed type hypersensitivity (DTH). For this porpose the animals were intraperitoneally injected with SE or SE plus AG, 5×10^5/mouse, then on day 4 the antigen was injected repeatedly subcutaneously in a dose of 10^8 into one of the hind paws, 0.9% NaCl being injected into the other paw. Twenty-four, 48, and 72 h; later edema size was determined by the difference between the volumes of expelled liquid in the right and left paws. The the other mice the humoral response was assessed from the blood serum hemagglutinin level (total per group) on days 4, 8, 11, and 14 after the primary immunization (5×10^6 SE per mouse) and on days 3 and 4 after the secondary immunization. Angiogenin was injected intraperitoneally in a single dose, except in one experiment described below, together with SE. The mixture was prepared individually for each animal directly before injection. Such a mode of using the preparation provided, on the one hand, a physiologically possible situation with an AG concentration increase at the site where and at the moment when the host organism was first exposed to the antigen and, on the other hand, a single injection minimized the organism's exposure on the whole. The results were statistically processed using the parametric Student *t* test. The figures show the arithmetic mean values with 95% confidence intervals.

RESULTS

Angiogenin of Russian manufacture was used in the study. Its creation is the result of a remarkable investigation carried out by a group of scientists headed by Associate Member of the Russian Academy of Natural Sciences N. P. Mertvetsov [3]. Using genetic engineering technology, chemico-enzymatic synthesis and molecular cloning of the human AG gene were carried out and expression of synthetic AG gene was

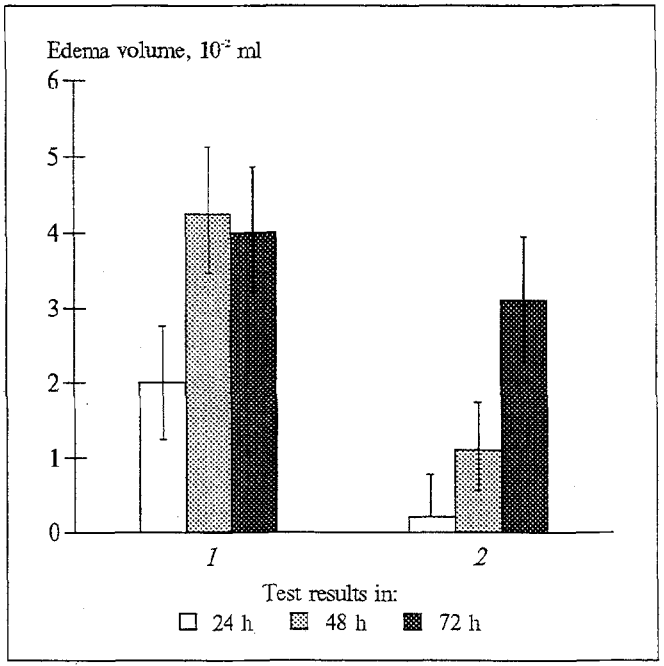

Fig. 3. Delayed type hypersensitivity in mice injected SE (*1*) and SE+AG in a dose of 10^{-5} g/kg (*2*).

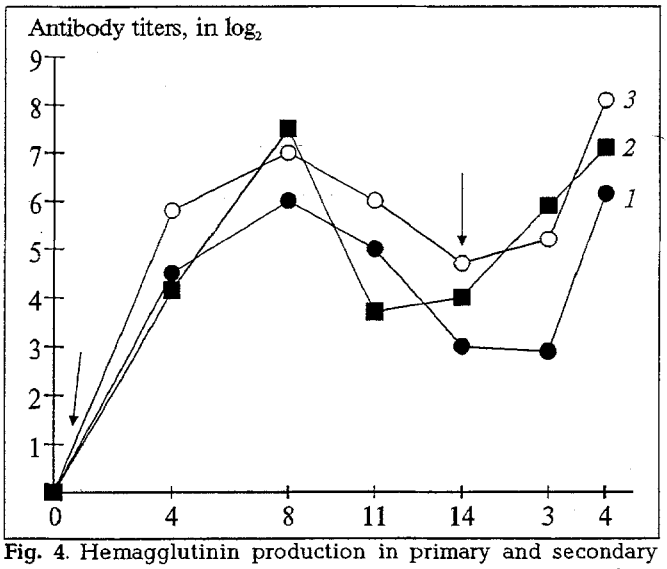

Fig. 4. Hemagglutinin production in primary and secondary immune response. Abscissa: days postimmunization (arrows show the first and second immunizations) in mice injected SE (*1*) and SE + AG in a dose of 10^{-5} g/kg once (*2*) or daily for the first 4 days (*3*).

demonstrated in *E.coli* cells, resulting in the isolation of pure genetically engineered, biologically active AG [2]; this work was carried out at the Novosibirsk Institute of Bioorganic Chemistry of the Siberian Branch of the Russian Academy of Sciences, with a contribution fron the Institute of Therapy of the Siberian Branch of the Russian Academy of Medical Sciences. The resultant AG producer strains were sent to the Department for Experimental Production of the FERMENT Research and Production Amalgamation (Vilnius), and medium-scale cultivation has been carried out there since. Preclinical trials of experimental batches of the agent are in progress, our studies being among them.

The first step was to establish the fact of immunity modulation REF was used here as the most convenient test for the purpose, for it is characterized by high sensitivity to various influences, is valid for virtually all the immunocompetent organs and tissues, and is represented by heterogeneous cell populations. RFC counts in the spleen were estimated in four groups of mice on day % postimmunization. The data (Fig. 1) indicate that AG in the dose used (in successive 100-fold dilution) showed a dose-dependent immunostimulating effect: host response to the antogen could be increased almost threefold.

For a more precise characterization of the effect of angiogenin in the second experiment we followed up the time course of rosette formation in mice challenged with the antigen under conditions of normal and increased level of AG injected in the optimal dose (10^{-5} g/kg), resulting in maximal stimulation. The first two of the observation periods selected were dictated by an extremely early antigen-induced activation of cellular rosette formation, with two reaction peaks observed in the spleen 10 sec and 30 min after antigenic stimulus [1]. The other three terms were to involve the asknowledged period of maximal development of rosette formation. An almost regular increment in the RFC count was observed over the entire follow-up (Fig. 2).

RFC analysis by adsorbed erythrocyte counts has shown this parameter to be little sensitive to AG. In just a few cases an AG-induced shift towards RFC predominance with a high (more than 3 sheep erythrocytes) antigen-binding activity was observed. For instance, in mice injected with AG the share of such RFC 10 sec after antigen challenge increased more than twofold. Such stimulation can be due to different causes: cellular function activation, changed RFC qualitative composition (in case of antigen attractability) or, not to be disregarded, changed properties of the injected SE themselves.

In the next experiment we found that DTH formation was also liable to be influenced by AG. Mice challenged with a sensitizing dose of the antigen in the presence of AG developed a depressed reaction 24 and 48 h after repeated injection of the antigen (Fig. 3): edema volume in them was much less than in mice not administered the agent. This fact is particularly interesting because of the known similarity between the DTH and transplantation immunity phenomena.

Analysis of the course of the humoral reaction showed this component of the immune system to be little sensitive to the effect of AG on the whole (Fig. 4), the agent being used in the optimal dose determined in the first experiment. Increasing the number of injections proved to be virtually useless. We may conclude from this, nevertheless, that an increase of the AG concentration in the environment at the moment of the first contact of the host organism with the antigen may favorably affect immunological memory formation because the secondary response of mice administered the drug was higher.

Hence, our results show new features of a still insufficiently studied bioactive substance of endogenous origin. The detected ambiguity of the effect of AG on various immunological; phenomena represented by functionally differing cell populations is indicative of diverse and intricate interactions between the tested substance and the immune system. The revealed immunoregulatory capacity of AG, significant by itself, necessitates a differentiated approach when prescribing this agent to patients in the event it is approved for clinical use.

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Peculiarities of Sodium Nitroprusside Biotransformation in Tumor-Bearing Animals

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Earlier we showed, using the EPR method, a phenomenon of specific biotransformation of sodium nitroprusside by tumor cells. The essence of the phenomenon lies in the formation in the intercellular space of ferric dinitrosyl complexes with paired RS groups of proteins [3], so-called 2.03 complexes [1]. The specific biotransformation of nitroprusside was revealed and confirmed in several models of mouse ascites tumors and in experiments with human leukemia cells [3].

The aim of this work was to study the expression of the phenomenon in models of experimental solid tumors *in vivo*.

MATERIALS AND METHODS

The following tumor models were used: sarcoma-180 (recipients - F_1(C57Bl/6×CBA/cal) mice, 18th day post-transplantation) and Walker sarcoma (recipients - Wistar rats, 30th day post-transplantation). The tumors were purchased from the Cancer Research Center of the Russian Academy of Medical Sciences. They were maintained by subcutaneous injection of 0.2 ml of 20% tumor cell suspension in saline. We also used outbred white mice with spontaneous adenomatosis obtained from the Central Department of Laboratory Animals of the Russian Academy of Medical Sciences. Healthy animals of the corresponding breed served as a control. Sodium nitroprusside (Sigma, USA) was admin-

TABLE 1. Prevalence of NP−1 EPR Signals and/or 2.03 Complexes in the Plasma of Healthy and Tumor−Bearing Animals (%) after Intraperitoneal Administration of Sodium Nitroprusside (25−50 mg/kg)

Experimental group	NP-1	2.03 Complex	Change
Healthy animals (n=15, rats, mice)	0	0	0
Sarcoma−180 (n=9, mice)	56	44	100
Walker sarcoma (n=5, rats)	50	50	100
Spontaneous adenomatosis (n=8, mice)	100	0	100

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