PREPARATION AND CHARACTERISTICS OF INTERLEUKIN-6-DEPENDENT HYBRIDOMA

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UDC 616-006-092.4:578.245]-07

KEY WORDS: interleukin-6; hybridoma growth factor; factor-dependent hybridoma

B-cell hybridomas of mice in the early stages of culture require the presence of a growth factor, secreted by macrophages and some other types of cells [1, 2]. Molecular cloning of cDNA has shown that hybridoma growth factor is identical with the T-cell factor which stimulates differentiation of B cells into immunoglobulin-secreting cells, β_2-interferon, and hepatocyte stimulating factor [6], after which it was called interleukin-6 (IL-6).

To study the relationship between IL-6 production and various pathological processes, it is necessary to create highly sensitive and specific methods of testing this cytokine. Hybridomas whose survival and growth in culture require the obligatory presence of IL-6 have now been obtained [1-3, 7]. Dependence of hybridomas on IL-6 is evidently specific, for several biological factors such as interleukin-1, -2, -3, and -4, and β- and γ-interferon, as well as others, have been shown not to influence their growth [3, 5].

The aim of this investigation was to obtain a similar factor-dependent hybridoma and, on its basis, to develop a method of detecting hybridoma/IL-6 growth factor.

EXPERIMENTAL METHOD

Preparation of factor-dependent hybridomas. August rats were given an intraperitoneal injection of 400 μg lipopolysaccharide (LPS) from *E. coli* 0111:B4 ("Difco") [7]. Splenocytes were fused with myeloma X65. AG8-653 with the aid of polyethylene-glycol ("Merck-4000") 3 days after immunization. The hybridomas were cultured in the presence of feeder cells (peritoneal macrophages) in medium DMEM ("Flow Laboratories"), containing hypoxanthine, thymidine, and aminopterine (2% HAT medium, "Flow"), 50 μg/ml gentamicin, 4 mM glutamine, and 10% fetal calf serum (FCS, from "Gibco," "Myoclone plus"). As the source of growth factor we used medium conditioned by activated macrophages (MCM, see below). The hybridomas thus obtained were cloned by the limiting dilutions method, and later in semisolid agar in the presence of 12.5% MCM.

Preparation of Conditioned Media. To prepare MCM, BALB/c mice were given an intraperitoneal injection of 50 μg LPS. Peritoneal cells, extracted next day, were cultured in a concentration of 10^6/ml for 5 days in medium DMEM containing 1% FCS at 37°C and in 5% CO_2 [5]. Human mononuclears were isolated from heparinized blood from healthy donors on lymphocyte separation medium ("Flow") and incubated in plastic Petri dishes in a concentration of $2 \cdot 10^6$/ml for 2 h, after which nonadherent cells were removed and the monocytes were cultured for 5 days in medium RPMI 1640, containing 1% FCS and 10 μg/ml of LPS [1].

Cells of mouse monocytic line P388D1 and human promonocytic line U937 were cultured in medium RPMI 1640 with 10% FCS. To obtain conditioned media, P388.D1 cells (10^6/ml) were induced by LPS (10 μg/ml) for 5 days [1], and U937 cells ($5 \cdot 10^5$/ml) were induced with phorbol myristate acetate (PMA, 20 ng/ml) for 3 days [4] in medium RPMI 1640, containing 1% FCS. All supernatants were dialyzed against 50 volumes of medium DMEM.

Research Center for Molecular Diagnosis, Ministry of Health of Russia, Moscow. (Presented by Academician of the Russian Academy of Medical Sciences A. P. Avtsyn.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 114, No. 8, pp. 179-181, August, 1992. Original article submitted November 28, 1991.

 0007-4888/92/0008-1156$12.50

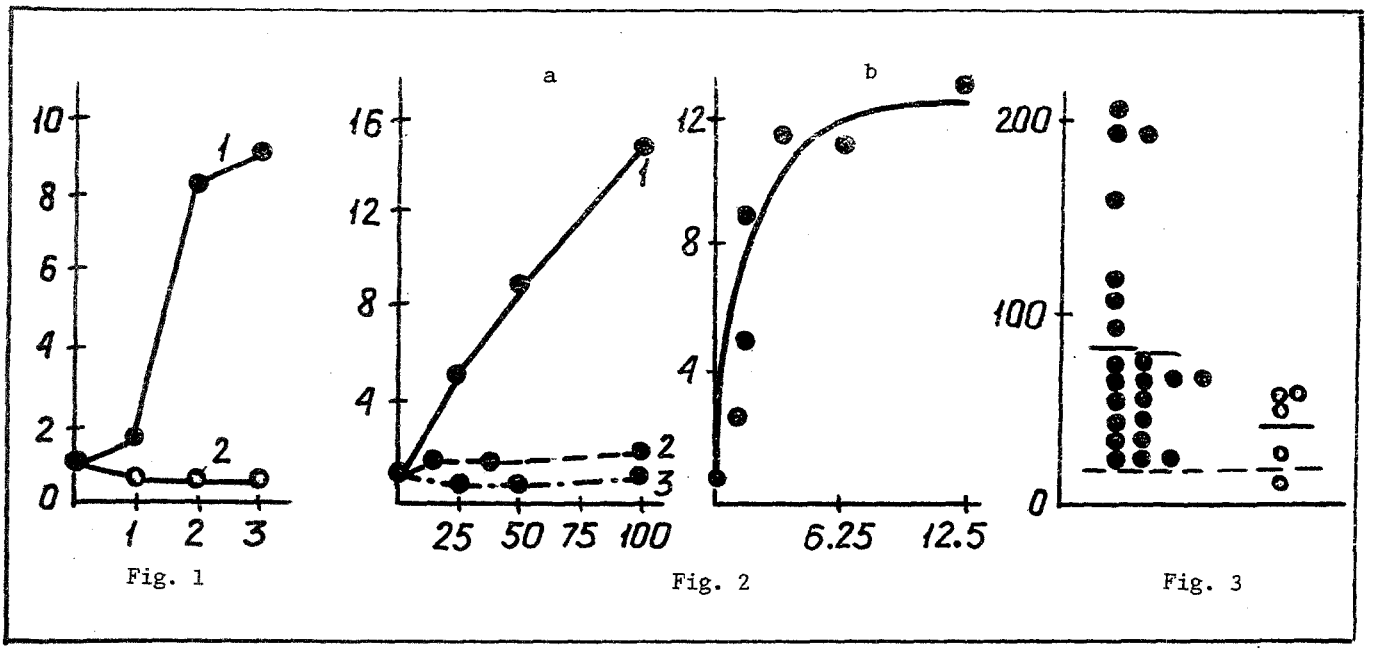

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Kinetics of action of MCM on DNA synthesis by D6C8 cells: 1) 12.5% MCM, 2) medium without MCM. Abscissa, duration of culture, days; ordinate, incorporation of ^{3}H-thymidine, cpm $\cdot 10^{-4}$.

Fig. 2. Action of IL-6 on proliferation of clone D6C8: a) effect of recombinant cytokines; b) effect of MCM. Abscissa, concentration: a) U/ml, b) %; ordinate, incorporation of ^{3}H-thymidine, cpm $\cdot 10^{-4}$; 1) IL-6, 2) TNF, 3) IL-1β.

Fig. 3. Serum IL-6 level in rheumatoid arthritis patients and healthy blood donors. Ordinate, content of IL-6, U/ml; filled circles – patients with RA, empty circles – healthy donors; broken line indicates before determination, continuous line denotes means values.

Clinical Material. Blood sera from 21 patients with rheumatoid arthritis (RA) were obtained from the Research Institute of Rheumatology, Russian Academy of Medical Sciences. To abolish nonspecific toxicity the samples were inactivated at 56°C for 30 min. The minimal dilution of human serum not inhibiting proliferation of D6C8 cells was determined in preliminary experiments.

Determination of Hybridoma Growth Factor. Cells of clone D6C8 were grown on medium DMEM containing 10% FCS and 12% MCM; aliquots of cells were frozen, kept in liquid nitrogen, and thawed a few days before the experiment. To determine the IL-6 content serial dilutions of the test specimens were incubated with hybridoma D6C8 cells ($5 \cdot 10^3$ cells/well) in 96-well tissue culture planchets ("Costar") in 200 μl for 48 h at 37°C and in 5% CO_2). The degree of proliferation of the cells was estimated from incorporation of ^{3}H-thymidine, which was added 5 h before the end of the experiment (17.5 Ci/mmole, 1 μCi/well). Cells labeled with the isotope were harvested on filters and the radioactivity of the samples was assessed by means of a scintillation β-counter.

Recombinant Lymphokines. We used recombinant IL-1 β (10^7 U/mg) and human tumor necrosis factor-α (10^7 U/mg, "Boehringer Mannheim"), and human IL-6 ($2 \cdot 10^7$ U/mg, "Genzyme").

EXPERIMENTAL RESULTS

During preparation of factor-dependent hybridomas, as a rule the cells after fusion are cultured in the presence of exogenous growth factor, the source of which could be medium conditioned by macrophages, endothelial cells, etc. [1, 2]. Van Snick and co-workers [7] showed that during long-term culture of hybridomas, only a very small proportion of mouse × mouse hybrids requires the mandatory presence of exogenous growth factor, whereas the frequency of formation of factor-dependent clones for rat × mouse hybrids may reach 96%. As partners for fusion we

TABLE 1. IL-6 Content in Culture Fluids of Different Types of Cells

Cells	Indicator	Activity of factor, U/ml
Mouse		
Peritoneal macrophages	LPS in vivo	715
P388D1	LPS, 10 μg/ml	115
Human		
Peripheral blood monocytes		1430
U937	PMA, 20 ng/ml	80

therefore chose spleen cells of rats immunized with LPS and mouse myeloma ×653 cells. After fusion the cells were cultured in the presence of MCM, and dependence of DNA synthesis on the supernatant concentration was estimated by measuring incorporation of ^{3}H-thymidine. As a result, clone D6C8, characterized by the highest sensitivity to growth factor, was selected. Dependence of DNA synthesis by D6C8 cells on duration of culture is shown in Fig. 1. In the presence of growth factor, stimulation of DNA synthesis was observed as early as after 24 h, and after 48 h incorporation of ^{3}H-thymidine was 10-15 times higher than initially, and 25-30 times higher than in the absence of MCM. The action of recombinant lymphokines on cell growth and also correlation between DNA synthesis and the quantity of MCM added to the culture medium are shown in Fig. 2. IL-1β and tumor TNF-α did not affect proliferation of the D6C8 line, whereas IL-6 considerably intensified DNA synthesis by these cells (Fig. 2a), i.e., hybridoma D6C8 is IL-6-dependent. When MCM was used (Fig. 2b) stimulation of growth was observed when its final concentration was 0.16%, and a near linear dependence was found at low concentrations, the curve reaching a plateau at 2.5% MCM. The content of IL-2 in biological fluids is usually expressed in conventional units. One factor unit corresponds to the volume of supernatant at which 50% maximal stimulation of DNA synthesis is observed under standard conditions [1, 2]. Table 1 gives data on the IL-6 content in supernatants obtained by cultivating activated cells of monocytic origin. According to estimates made by different workers, culture fluids of cells of this type may contain from 700 to 4000 U/ml IL-6, equivalent to about 3-20 ng/ml [1, 2, 4]. The content of the factor in supernatants of primary cultures of monocytes and macrophages, determined with the aid of hybridoma D6C8 is comparable with data in the literature, whereas in the case of cell lines it was lower, possibly due to differences between the sublines used in the experiments. It can be postulated on the basis of these estimates, although with some degree of caution, that half maximal incorporation of ^{3}H-thymidine by D6C8 cells was observed with IL-6 in a concentration of not more than 10-20 pg/ml. Incidentally, PMA and LPS in concentrations used for induction had no effect on growth of D6C8 cells, although with higher concentrations of LPS (20-40 μg/ml) incorporation of ^{3}H-thymidine by the cells was increased three-fivefold.

To assess the practical significance of our test, samples of biological fluids from patients with rheumatoid arthritis (RA) and healthy blood donors were used in clinical trials. The IL-6 concentration in healthy human blood serum is known to be extremely low. In pathological states, mainly associated with inflammation, the content of this cytokine in local tissue fluids and in the blood increases. For example, in patients with RA the blood level of IL-6 rises to 200-400 U/ml, and in the synovial fluid, in some cases it may rise to 22,000 U/ml, and its level correlates with activity of the process [8]. By using heterohybridoma D6C8 as indicator cells we showed that the serum IL-6 level in the patients was higher, on average, than in the healthy blood donors (Fig. 3). In nine of 21 patients the IL-6 concentration was higher than in healthy subjects, and in three patients it reached 200 U/ml.

We thus obtained a heterohybridoma D6C8 which required the presence of IL-6 for its growth and survival in culture. Cells of the human and murine monocytic series can serve as the source of this factor. The high sensitivity and reproducibility of the method of determination of IL-6 with the aid of D6C8 cells make it possible for the concentration of this factor to be estimated both in culture fluids and in human blood serum.

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EFFECT OF INTERLEUKIN-2 ON EXPERIMENTAL EMOTIONAL HYPERTENSION

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UDC 615.275.4:612.112.94.015.2].03:
[616.12-008.331.1-02:613.863].076.9

KEY WORDS: arterial hypertension; interleukin-2; immune system

The role of immune disturbances in the development of various forms of experimental hypertension has been discussed in recent years. For instance, underdevelopment of the thymus, a deficiency of T lymphocytes, and the presence of thymocytotoxic autoantibodies have been discovered in spontaneously hypertensive rats [10, 11]. The use of immunocorrective procedures (injection of thymus tissue extracts, of antithymic serum, or of immunosuppressive preparations) in this model of hypertension has been shown to have an antihypertensive action [5-7]. A hypotensive effect of immunosuppressive therapy has been found in New Zealand Black mice with spontaneous hypertension [9]. It has been shown that the development of hypertension in hypertensive Lyon rats can be prevented by neonatal thymectomy [4]. There is also evidence of changes taking place in the immune system in human subjects with essential arterial hypertension: raised blood levels of immunoglobulins, autoantibodies to vessel walls and to smooth muscle cells, and deposition of immunoglobulins in the renal arteries [3, 6]. In connection with the facts described above, and also since in experimental models of hypertension the clearest manifestation is weakening of T-cell functions, the study of the role of the T-cell growth factor interleukin-2 (IL-2), a powerful mediator of the immune system, in the development of hypertension is particularly interesting.

The aim of this investigation was to study the effect of human recombinant IL-2 on the course of experimental arterial hypertension.

EXPERIMENTAL METHOD

Chronic experiments were carried out on 20 noninbred male and female albino rats aged 5-7 months and weighing 200-260 g. A model of emotional hypertension was created in a "conflict situation" by daily (for 1 month) exposure to a physical stress factor, namely electrodermal stimulation of the limbs for 1 h by a pulsed current (10-100 V) on stochastic mode, and allowing for individual reactivity of the animals [1]. The systolic blood pressure (BP) was recorded by an electroplethysmographic method in the caudal artery of the rats, using a mercury manometer, an

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Kharkov Research Institute of Internal Medicine. V. P. Protopopov Kharkov Research Institute of Neurology and Psychiatry. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 114, No. 8, pp. 182-183, August, 1992. Original article submitted January 14, 1992.

