New Properties of Immunotropic Preparation from Porcine Skin

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We studied new immunological and physicochemical properties of K-activin, immunotropic preparation from porcine skin isolated by the acetone method. The preparation restored the sensitivity of background rosette-forming cells in the spleen of thymectomized mice to the inhibitory effect of azathioprine *in vivo* and practically normalized serum thymic activity reduced in thymectomized mice. The molecular weight of proteins present in K-activin and previously detected by SDS-PAAG electrophoresis was determined by MALDI mass spectrometry

Key Words: *Immunotropic preparation; skin; T lymphocytes; serum thymic activity; MALDI mass-spectrometry*

The development of new immunotropic preparation is an important problem of modern immunopharmacology. V. Ya. Arion *et al*. proposed an acetone method for isolation of immunotropic preparation from porcine skin [2]. Using this method we obtained 3 preparations (P1, P2, and P3) with molecular weights of 15, 1.4-15, and 1.4 kDa, respectively. These preparations were characterized by isoelectrofocusing, SDS-PAAG electrophoresis, and electrophoresis in the presence of 7 M urea [3]. The effects of these preparations on proliferation and differentiation of human skin keratinocytes in primary culture [3], on the number of rosette-forming cells in the spleen of CBA mice at the peak of primary immune response (Erne method), and on the recovery of sensitivity of background rosette-forming cells (bRFC) of the spleen in thymectomized (TE) mice to the inhibitory effect of azathioprine *in vitro* [1] were evaluated. P1 stimulated proliferation and slightly stimulated differentiation of human keratinocytes [3]. P2 inhibited proliferation and stimulated differentiation of human keratinocytes [3]. P3 stimulated proliferation and had no effect on differentiation of human keratinocytes [3]. All three preparations were active in the Erne test, but only P3 demonstrated activity in the azathioprine test.

Since hyperproliferation of keratinocytes and their incomplete differentiation are typical of psoriasis, we hypothesized that P2 (working name K-activin) can produce a therapeutic effect in this pathology. Preclinical study of K-activin was performed at Laboratory of Experimental Hemosorption and Oxidative Methods of Detoxification, Institute of Physicochemical Medicine. Skin-resorption effect, acute toxicity, chronic toxicity [6] and allergic effects of K-activin in the reactions of systemic anaphylaxis (anaphylactic shock) and active cutaneous anaphylaxis were evaluated. Preliminary data obtained on volunteers (psoriasis patients) showed that the preparation produced a therapeutic effect.

Here we evaluated activity of K-activin in the test of recovery of sensitivity of splenic bRFC from TE mice to the inhibitory effect of azathioprine *in vivo* and its effect on the level of serum thymic

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activity (STA) in TE mice, and measured molecular weights of K-activin proteins by MALDI mass spectrometry.

MATERIALS AND METHODS

Restoration of the sensitivity of splenic bRFC from TE mice to the inhibitory effect of azathioprine *in vivo* was performed as described previously [10].

Male C57Bl/6J mice were divided into 4 groups (5 mice per group) and subjected to TE. Three weeks after TE, the mice received 3 daily subcutaneous injections of 0.1 ml physiological saline (group 1, control), or 100 μg/mouse K-activin in 0.1 ml physiological saline (group 2), or 10 μg/mouse Tactivin in 0.1 ml physiological saline (group 3), or both preparations in the same doses (group 4). The mice were sacrificed on the next day after the last injection. The spleen from each mouse was taken into a separate tube and a splenocyte suspension was prepared. The number of bRFC in the spleen of TE mice per 10^4 nucleated cells was counted. The results were expressed in percents of bRFC by the formula:

$$A = \frac{M_{Ex}}{M_C} \times 100\%,$$

where M_C and M_{Ex} are the arithmetic means of bRFC numbers in the control (without azathioprine) and experiment (with azathioprine).

The level of STA in the blood was measured by the method of restoration of the sensitivity of bRFC from the spleen of TE mice to the inhibitory effect of azathioprine [8,9]. Male C57Bl/6J mice were divided into 4 groups (5 mice per group). Group 1 mice were sham-thymectomized (the skin was cut and then sutured, but the thymus was not removed). Group 2 mice were subjected to TE. After 3 weeks, the mice of groups 1 and 2 were subcutaneously injected with 0.1 ml physiological saline for 3 days. Group 3 mice were subjected to TE and after 3 weeks received Tactivin (10 μg/mouse in 0.1 ml physiological saline). Group 4 mice were subjected to TE and after 3 weeks received K-activin (100 μg/mouse in 0.1 ml physiological saline). The mice were decapitated 1 day after the last injection and the blood was collected into tubes. After clot formation, the serum was collected, applied on a cone filter CF 25A or CF 50A (Amicon), and centrifuged at 800*g* for 30 min to remove high-molecular-weight inhibitory factors. The solution passed through the filter was used in further reactions. The serum filtrate was stored at -20-70°C for 6-12 months. The level of STA was measured. Twofold dilutions of the serum filtrate were prepared. After the reaction, the number of bRFC in the spleen of TE mice per 10^4 nucleated cells was counted.

The doses of the preparation were considered active, if the percent of bRFC was ≤50%. Activity was expressed as negative logarithm of plasma dilution to the base 2 ($-\log_2 2^{-N}$).

MALDI mass-spectrometry of K-activin was performed on a Reflex IV spectrometer (Bruker Daltonix) [11]. MALDI is characterized by high sensitivity, requires no complicated processing of samples, and allows using heterogeneous samples. The method allows measuring the molecular weight of a protein of hundred thousand Daltons with an accuracy of 0.50-0.01%. It is characterized by protonated molecular ions MH^+ and adducts with cations of alkaline metals $[M+Na]^+$ and $[M+K]^+$ [4]. Samples were prepared as described previously [12]. Sinapinic acid and 2,5-dihydroxybenzoic acid were used as the matrix.

RESULTS

K-activin exhibited no activity in the test of restoration of sensitivity of bRFC from the spleen of TE mice to the inhibitory effect of azathioprine *in vitro*, but had no effect on humoral immune response to T-dependent antigen sheep erythrocytes [1]. Since K-activin had no effect on T cell maturation, we hypothesized that it can inhibit the effect of preparations inducing T cell maturation, similarly as it inhibits keratinocytes proliferation. We studied combined and separate effects of K-activin and Tactivin in the test of restoration of sensitivity of bRFC from the spleen of TE mice to the inhi-

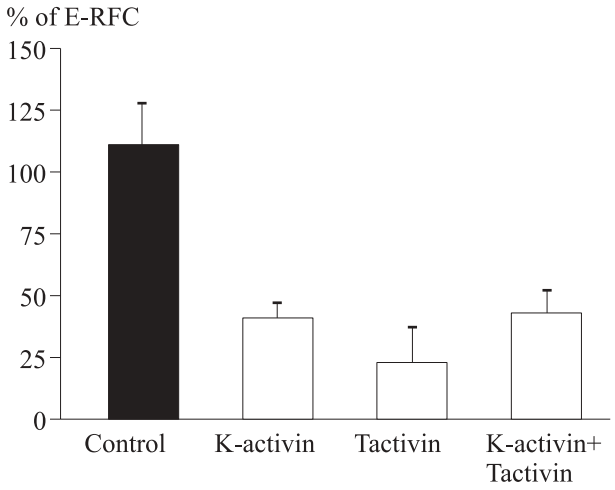

Fig. 1. Effect of K-activin and Tactivin on restoration of sensitivity of bRFC from the spleen of TE mice to the inhibitory effect of azathioprine *in vivo*.

bitory effect of azathioprine *in vivo* and found that K-activin was active in this test (Fig. 1), although it was ineffective *in vitro*. K-activin in a dose of 100 μg/mouse restored sensitivity to azothioprine *in vivo*. The percent of E-RFC was 43%.Tactivin in a far lower dose (10 μg/mouse) exhibited higher activity and the percent of E-RFC decreased to 23%. After combined use of K-activin and Tactivin, the percent of E-RFC was the same as after treatment with K-activin alone.

Activity of K-activin in the test of restoration of azathioprine sensitivity attests to an increase in relative content of mature T cells among splenic cells and to its effect on T cell maturation. The preparation exhibited activity similar to that of thymic factors. After combined use of K-activin and Tactivin, activity corresponded to that of K-activin.

The level of STA in sham-thymectomized mice was 3 (Fig. 2). After TE the level of STA decreased to 1.5. Treatment with K-activin increased STA to 2.7, *i.e.* to almost control level. After treatment with Tactivin, STA increased to 3.5, *i.e.* surpassed the control level. Thus, K-activin restored the level of STA in TE mice to almost control level, although its activity was lower than that of Tactivin.

Thus, K-activin, similarly to Tactivin isolated from the thymus, increased STA and stimulated maturation of T cells.

The level of STA depends on age and reflects the state of the thymus. The decrease in STA indicates immunodeficiency. Thymic hormones (α_1-thymosin, thymopoietin, and thymulin) contribute to STA. Humans and animals have certain normal concentration of thymic factors. The concentration of these factors decreases in T-immunodeficiency, which, in turn, leads to disturbances in T-mediated immunity.

K-activin was inactive in the suspension of splenocytes isolated from TE mice probably because this

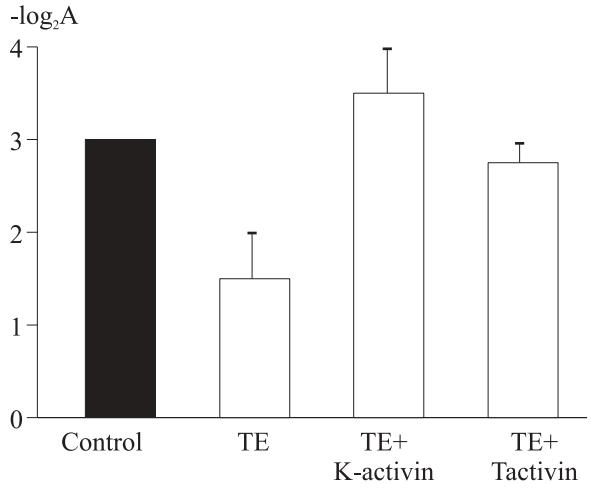

Fig. 2. Effect of K-activin and Tactivin on STA in C57Bl/6J mice.

suspension contains no target cells for the preparation. At the same time these cells were detected in the organism of TE mice. They can present in the bone marrow (pre-thymocytes or earlier precursors). Since K-activin modulates not only on T-, but also on humoral β-dependent immune response, we can hypothesize that it affects common precursors of T and B cells. K-activin probably contains not one, but several factors modulating lymphocytes differentiation. It can be also hypothesized that K-activin undergoes changes in the body of TE mice leading to the appearance of activity. Modified K-activin acts on immature T cells in the spleen and promotes their differentiation. It cannot also be excluded that K-activin indirectly modulates T cell differentiation by modulating target cells producing differentiation factors for T lymphocytes. The first hypothesis seems to be more reasonable. We believe that K-activin after subcutaneous injection enters the circulation (which manifests in increased STA) and then is transported to the bone marrow, where it stimulates T cell differentiation from the corresponding precursors. Differentiated T cells migrate into the corresponding zones of the spleen, where they are detected by the azathioprine test.

STA in mice did not decrease to 0, but was 1.5, probably because this level of STA is maintained by skin factors, which perform immunoregulatory functions. However, the skin, though produced factors increasing STA, cannot maintain normal level of STA (STA is low in TE mice). In adult humans, the T system works properly despite thymus involution. A certain role can be played by the skin, which regulates differentiation of T cells under these conditions. Immunity impairment is observed in old humans, in whom immune functions of the skin probably decrease.

T cell differentiation is still observed in the absence of the thymus [7]. In nude mice Thy-1$^+$ lymphocytes were found in thymus-dependent zones of the spleen and lymph nodes. The number of these cells increases with age. T cells of nude mice also express Lyt-1 and Lyt-2 antigens. The expression of α- and β- genes of T cell receptor in the total population of peripheral lymphocytes decreases in nude mice, but expression of γ-genes does not differ from the normal. In nude mice, activity of cytotoxic T lymphocytes was detected, but the level of T helpers considerably decreased. The development of T cells in nude mice is similar to normal differentiation of T cells, but proceeds more slowly. And probably animal life is insufficient for the realization of some stages of this process [7].

We believe that skin factors detected by us modulate differentiation of T lymphocytes in the absence of the thymus.

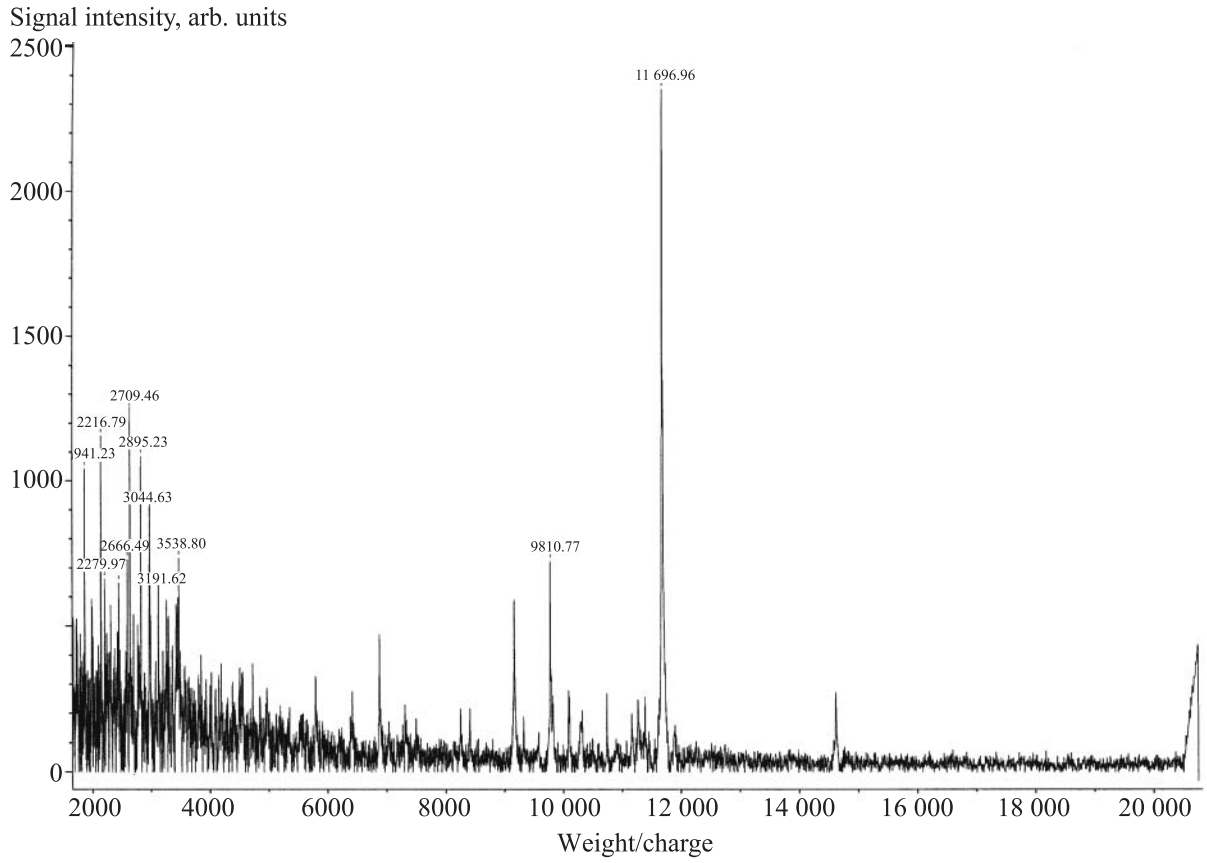

Fig. 3. MALDI mass spectrometry of K-activin in m/z range of 20,000-200,000, sinapinic acid was used as the matrix.

Mass-spectra of K-activin proteins contain major and minor peaks (Fig. 3). The peak with m/z= 11,696.96 is most intensive. We detected also major peaks with m/z 6850, 9200 and 9810.77, and 9 major low-molecular-weight peaks with m/z from 5800 to 14 600. It cannot be excluded that these peaks are presented by ions formed by some protein molecules.

When the mass-spectrum was narrowed to 4000-12,000 and 2,5 dihydroxybenzoic acid was used as the matrix, we recorded 10 major peaks with m/z from 8182.78 to 8702.09; 3 major peaks with m/z from 9807.35 to 9845.64, 1 peak with m/z 11287.51, and minor peaks with m/z 4226, 6726.20 and 7200.22. We can hypothesize that the peak with m/z 8182.78 is determined by protonated molecular ion MH^+, while the peak with m/z 8203.60 is formed by adduct with sodium cation $[M+Na]^+$, because m/z difference between the peaks is 22. Other peaks with close m/z can be determined by separate protein molecules or adducts with alkaline metal cations or other positively charged ions. The same is true for peaks with m/z 9807.35 and 9845.64. We can hypothesize that the peak with m/z 9807.35 is determined by protonated molecular ion MH^+, while the peak with m/z 9845.64 is formed by adduct with sodium cation $[M+Na]^+$, because m/z difference between the peaks is 38.

The mass-spectrum recorded in the range of 10,000 to 200,000 m/z (sinapinic acid was used as the matrix) contained a high-molecular-weight peak with m/z 22 868. No high-molecular weight peaks were found except this one. This does not mean that the sample contains no high-molecular-weight proteins. These proteins are probably present, but do not form ions during ionization. SDS-PAAG electrophoresis revealed a protein with a molecular weight of 61 kDa [3]. This protein was not detected, because was not probably ionized. Electrophoresis of K-activin in 6-20% PAAG in the presence of SDS revealed 5 major proteins with molecular weights of 6.9, 8.4, 11, 12 and 61 kDa [3]. Mass spectrometry in m/z range of 4000-12,000 revealed major and minor proteins with m/z 6850, 8452.61, 11,150 and 11,900, which corresponded to the molecular weights of proteins detected by electrophoresis.

Thus, we revealed new properties of K-activin, an immunotropic preparation from porcine skin. The preparation restored the sensitivity of bRFC from the spleen of TE mice to the inhibitory effect of azathioprine *in vivo* and practically normalized

STA reduced in TE mice. Heterogeneity of the preparation is confirmed by mass spectrometry.

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