PURIFICATION OF EPIDERMAL G_2-CHALONE BY IMMUNOAFFINITY CHROMATOGRAPHY

S. A. Ketlinskii and A. S. Simbirtsev

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In the early 1960s a theory was put forward that tissue homeostasis is maintained by the negative feedback principle on account of synthesis of endogenous tissue-specific inhibitors of proliferation, called chalones, by tissue cells [5]. By now tissue-specific antiproliferative activity has been found in extracts of most tissues [1]. However, since the time of the first attempt to isolate the active principle of tissue extracts, many workers have met with difficulties in obtaining purified chalones, due to the complex process of biological testing of active material in different stages of purification, and the absence of an accurate, rapid, and reliable method of identifying them. One possible approach to the solution of this problem is by the use of antibodies against chalones [3].

This paper describes an attempt to isolate a biologically active inhibitor, with the aid of monospecific antibodies, by the method of immunoaffinity chromatography.

EXPERIMENTAL METHOD

The monospecificity of rabbit immune serum against rat epidermal G_2-chalone [2] was verified by crossed immunoelectrophoresis [12]. A 55-81% alcoholic extract of rat skin prepared by the method in [6] was subjected to electrophoretic fractionation. Electrophoresis was then carried out in the perpendicular direction in gel containing immune serum (0.5 ml to 15 ml of 1% agarose gel in 0.02 M Tris-glycine buffer, pH 8.6). The plate with gel was washed, dried, and stained with Amido black. Immunodiffusion analysis by the method of Ouchterlony and Mancini was carried out in 1% agarose gel made up in buffered physiological saline, pH 7.4.

Isolation of purified G_2-chalone from the 55-81% alcoholic extract of rat skin was carried out by immunoaffinity chromatography. For this purpose the immunoglobulin fraction of the immune serum, obtained by salting out with ammonium sulfate at 37.5% saturation, was bound with CNBr-activated Sepharose 4B according to the manufacturer's recommendations (Pharmacia Fine Chemicals, Sweden). Nonreacting active groups were blocked with 0.1 M monoethanolamine. The immunosorbent was then transferred to a chromatographic column with cooling. All subsequent procedures were carried out at 4-8°C. The alcoholic extract was dissolved in 0.1 M $NaHCO_3$ with 0.5 M NaCl, centrifuged for 20 min at 6000 rpm, and applied to a column equilibrated with the same solution. The yield of protein fractions was estimated by their extinction at 280 nm. The column was washed with the equilibrating solution and then with 1 M NaCl to reduce nonspecific binding. Desorption was carried out with 0.1 M glycine-HCl buffer, pH 2.8. Fractions containing desorbed material were at once neutralized with 0.1 N NaOH, concentrated, and dialyzed against physiological saline at 4°C for 24 h.

Disc electrophoresis was carried out in 7.5% polyacrylamide gel [8], stained with Amido black. The molecular weight was determined by disc electrophoresis in 10% polyacrylamide gel in the presence of 0.1% sodium dodecylsulfate [11]. A standard kit for molecular weight determination (from Pharmacia Fine Chemicals) was used as reference substances.

Biological activity of the alcoholic extract and of the purified G_2-chalone was investigated in C57B1 mice. Physiological saline was injected into the group of control animals. Simultaneously with the test material, all animals were injected with colcemid in a dose of 0.1 mg/10 g body weight. All injections were intraperitoneal. The animals were killed 4 h

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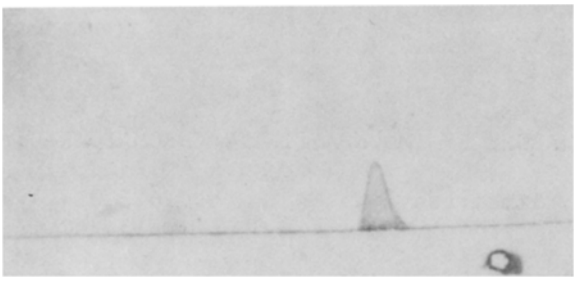

Fig. 1. Crossed immunoelectrophoresis of alcoholic extract of rat skin with antiserum against epidermal G_2-chalone. Stained with Amido black.

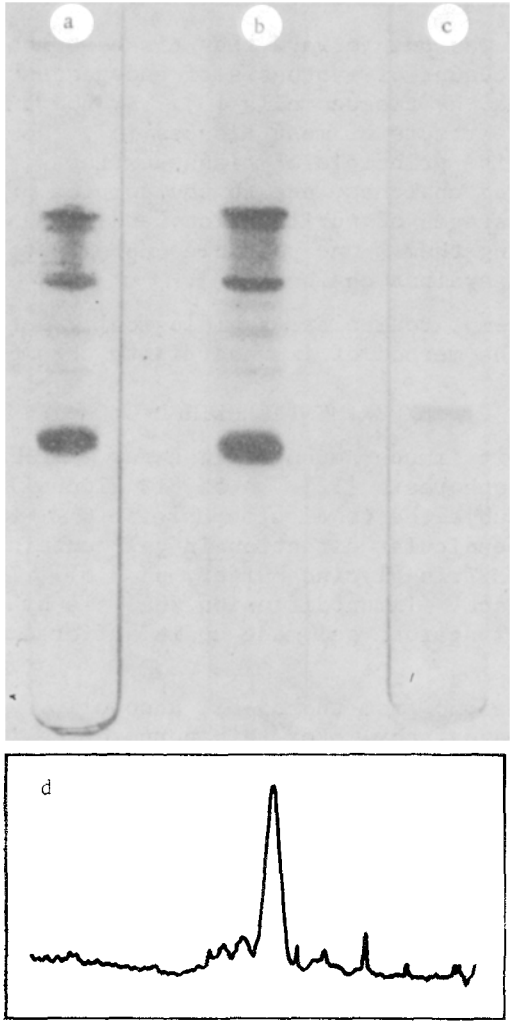

Fig. 2. Polyacrylamide gel disc electrophoresis of alcoholic extract of skin and of its fractions obtained after immunoaffinity chromatography. a) Whole extract, b) material not bound with antibodies, c) material bound with antibodies and eluted at pH 2.8, d) scannogram of stained gel (c).

later by dislocation of the cervical spine. The external ear was taken to prepare histological sections. The number of mitoses arrested by colcemid was counted in 4000 cells of the basal layer of the epidermis and in 100 sebaceous glands [13].

EXPERIMENTAL RESULTS

It was shown by the crossed immunoelectrophoresis method that the immune serum used to isolate the G_2-chalone possessed monospecificity (Fig. 1). This was confirmed also by analysis of fractions obtained after immunoaffinity chromatography. For instance, according to data of disc electrophoresis, the desorbed antigenic material consisted of one component (Fig. 2).

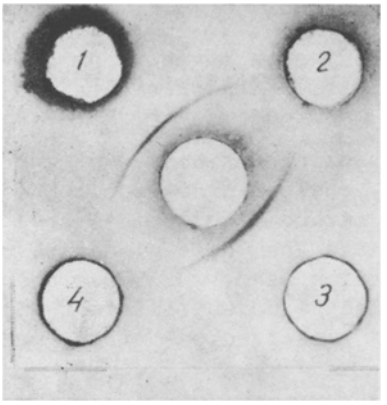

Fig. 3. Ouchterlony's double immunodiffusion test with monospecific immune serum against epidermal G_2-chalone (in central wall). 1) Alcoholic extract of rat skin, 2) material not bound with antibodies, 3) purified G_2-chalone, 4) rat blood serum.

TABLE 1. Number of Mitoses Blocked by Colcemid (MC_{col}) in Epidermocytes and Sebaceous Gland Cells of Externalar of Mice 4 h after Injection of Alcoholic Extract of Skin and of Epidermal G_2-Chalone Isolated by Immunoaffinity Chromatography

Material tested	Number of animals	MC_{col}, %		% of depression		P relative to control	
		1	2	1	2	1	2
Control	10	6,68±0,86	5,67±0,33	—	—	—	—
Alcoholic extract	6	2,25±0,84	1,25±0,25	66,3	78,0	≤0,004	<0,001
Purified G_2-chalone	6	2,55±0,74	5,85±0,68	61,8	—	≤ 0,004	—

Legend. 1) Epidermis, 2) sebaceous glands.

The results of scanning of the stained gels on an ISCO (USA) instrument showed that the degree of purification of the substance obtained was close to 90% (Fig. 2d). The impurities discovered were possibly connected with nonspecific sorption during affinity chromatography. According to the results of immunodiffusion, the test antigen could not be found in material not bound with antibodies, i.e., it was completely removed from the original alcoholic extract (Fig. 3).

Biological testing was carried out next (Table 1). The concentration of the test substance in solution after purification by immunoaffinity chromatography was estimated by Mancini's quantitative immunodiffusion method, and was brought to the same value as its concentration in the dose of alcoholic extract which, on intraperitoneal injection, inhibited by 50-60% mitotic activity of the epidermocytes of the external ear of the mice (150 µg/g body weight). On biological testing the quantity of test material injected into animals in the composition of the alcoholic extract and in the purified form was thus equal. The protein concentration in the latter also was determined by Lowry's method. The dose of the purified substance, expressed as protein, corresponded to 2 µg/g body weight.

The results of biological testing (Table 1) showed that material bound with antibodies had a marked antimitotic action on epidermocytes of the external ear, similar to the action of the whole alcoholic extract. To test the tissue specificity of the isolated inhibitor, its effect was studied on mitotic activity of sebaceous gland cells, which have their own chalone regulation [7] and which are an adequate test system for evaluating tissue-specificity of the action of epidermal chalones [13]. The substance thus obtained was found not to have any antimitotic action on sebocytes, whereas the whole alcoholic extract of skin induced such an effect. The resulting inhibitor evidently had a true tissue-specific antimitotic action on cells of the epidermis, i.e., it was epidermal G_2-chalone.

Investigation of purified G_2-chalone by SDS-PAG electrophoresis showed that it is a single component with molecular weight of 13,000 daltons. The results are a little at variance with the results of investigations by other workers, who found that the molecular weight of epidermal

G_2-chalone was 20, 25, and 35 kilodaltons [4, 9, 10]. It should be pointed out that molecular weight was determined in the studies cited above by gel-filtration and ultracentrifugation; moreover, only partially purified chalones obtained from different species of animals were studied.

By immunoaffinity chromatography using antibodies of a monospecific immune serume it was thus possible to isolate epidermal G_2-chalone from an alcoholic extract of skin in one stage. It can evidently be obtained by this method also from an aqueous extract of skin without preliminary fractionation with alcohol, and the whole procedure of purification can be reduced to a minimum. The method of purification used, which has been successfully applied for obtaining various substances, proved also to be suitable for epidermal G_2-chalone. The purified inhibitor was biologically active and preserved one of the main properties of chalones, namely tissue specificity of action.

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STIMULATION OF DNA SYNTHESIS IN SPLENIC LYMPHOCYTES OF GUINEA PIGS BY STAPHLYOCOCCAL PEPTIDOGLYCAN

V. V. Khorobrykh and V. K. Pozur

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The study of the role of staphylococcal antigens in immunologic reactions is of both theoretical and practical importance, for it can deepen our knowledge of interaction between individual structures of bacteria and lymphocytes of the host. Several investigators have shown that the peptidoglycan in the cell wall of Gram-positive and Gram-negative bacteria has mitogenic activity against lymphocytes of man, rats, and mice [7-10]. Formation of the immune response and the ability of the lymphocytes to respond to mitogens are linked with the degree of differentiation of the lymphocytes and the organization of their receptor apparatus [1, 6, 11].

With these considerations in mind it was decided to study DNA synthesis in splenic lymphocytes of intact guinea pigs in response to peptidoglycan and to compare it with that observed in the presence of nonspecific B and T cell mitogens, and to identify the lymphocyte subpopulation on which peptidoglycan acts.

N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow.

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