CHARACTERISTICS OF HETEROLOGOUS ANTISERA AGAINST STROMAL FIBROBLASTS

A. A. Ivanov-Smolenskii

UDC 612.398.017.1:576.8.097.5

Experiments with heterologous rabbit antifibroblast sera (AFS) against guinea pig stromal mechanocytes showed that culture media from subcultured stromal mechanocytes from the bone marrow, spleen, and thymus contain a specific trypsin-resistant fibroblast protein (AG-1), which is present also in normal blood serum and on the surface of the stromal fibroblasts. AG-1 is insensitive to the action of collagenase and evidently differs from the chief surface protein (CSP) of fibroblasts. AG-1 is a γ_1-globulin and is probably the hitherto unknown specific surface protein of fibroblasts. Antibodies against a nonspecific fibroblast protein (AG-2) and an α_1-globulin related to AG-1 also were found in the AFS.

KEY WORDS: antifibroblast serum; stromal mechanocytes.

Recent publications have given descriptions of the properties of specific antisera against collagen and the chief surface protein (CSP) of fibroblasts — two known specific proteins of fibroblasts [8, 11, 14, 15]. On the other hand, only isolated items of information are available on antifibroblast sera (AFS) obtained by immunization with whole cells [5].

In a previous paper [2] the writers described the properties of a rabbit AFS obtained by immunization with stromal fibroblasts from guinea pig bone marrow. This antiserum, as the results of the cytotoxic and immunofluorescence tests showed, was specific for fibroblasts (mechanocytes) and did not bind with macrophages, lymphocytes, or hematopoietic cells [2].

The object of the present investigation was to study the characteristics of a serum against stromal mechanocytes from the thymus and bone marrow and their reaction with antigen.

EXPERIMENTAL METHOD

Rabbits were immunized by the method described previously [2] with stromal mechanocytes from guinea pig bone marrow and thymus between the third and tenth passages [3], separated from the glass either by 0.25% trypsin (treatment for 30-40 min and 37°C) or by means of a rubber spatula.

The AFS was exhausted as described previously [2] with either a mixture or homogenate of guinea pig bone marrow, thymus, lymph node, spleen, and liver cells, with medium supporting cultures of stromal fibroblasts, and with adherent and nonadherent bone marrow cells.

The precipitation test in agar with AFS was carried out by the method described in [1], with normal guinea pig sera, with media supporting cultures of stromal fibroblasts from guinea pig bone marrow, thymus, spleens between the 5th and 15th passages, with water-soluble extract of the organs used for exhaustion, and also with aqueous extracts of fibroblasts (for all the water-soluble extracts, the same quantity of cells by volume was taken). Starting from the dilutions of the materials containing antigen, and allowing for the fact that the sensitivity of the precipitation test is about 4 μg antigen/ml, its approximate concentration was calculated in the culture media and in normal guinea pig blood serum. Immunoelectrophoresis was carried out by the method described in [4]. Culture media and normal guinea pig sera were subjected to electrophoresis followed by the precipitation test with AFS.

Electrophoresis in flat polyacrylamide gel [12] was carried out in 5.6% reacting gel (pH 8.8) and in 3% concentrated gel (pH 6.8) with Tris-glycine electrode buffer (pH 8.4) with

Laboratory of Immunomorphology, N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR P. A. Vershilova.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 86, No. 10, pp. 454-458, October, 1978. Original article submitted February 10, 1978.

0007-4888/78/8610-1347$07.50

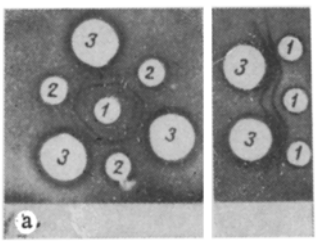

Fig. 1

Fig. 2

Fig. 1. Detection of specific fibroblast antigen (AG-1) in culture media and normal guinea pig serum by means of heterologous rabbit antifibroblast sera in agar diffusion test. a: 1) AFS (against stromal fibroblast of guinea pig bone marrow or thymus, separated by trypsin or with a rubber spatula), exhausted with guinea pig erythrocytes and NBS (see text), 2) medium concentrated 10-20 times after lyophilic drying from cultures of stromal fibroblasts of thymus, spleen, or bone marrow, 3) normal guinea pig serum (see text); b: 1) AFS exhausted with guinea pig erythrocytes, NBS and either mixture or extract of bone marrow, spleen, thymus, lymph node, peritoneal exudate, and liver cells of guinea pigs, or with bone marrow or spleen cells separately, and also simply with nonadherent population of bone marrow cells, 2) culture medium of stromal fibroblasts, 3) normal guinea pig serum. AG-1 inmedia identical with antigen in normal serum.

Fig. 2. Detection of AG-2 in normal guinea pig sera (see text). 1) Any AFS exhausted by conditioned culture medium of any stromal fibroblast; 2) conditioned medium from cultures of stromal fibroblasts on thymus, spleen, or bone marrow; 3) normal guinea pig serum.

0.1% sodium dodecyl sulfate. The gels were stained with a 0.25% solution of Coomassie R-250 in a solution containing 5 volumes of distilled water, one volume of acetic acid, and five volumes of methanol. Washing was carried out in 7% acetic acid with 5% methanol.

Highly active bacterial collagenase from *Clostridium histolyticum* was used; the quantity of the enzyme required for destruction of collagen [9] was determined empirically from disappearance of the characteristic bands of collagen during electrophoresis. The concentration of collagen in the culture medium was determined by the color reaction for hydroxyproline [6].

The indirect immunofluorescence test on living cells and exhaustion with labeled antiserum against rabbit γ-globulin (Sevac) were carried out by methods described in [2, 7]. The cytotoxic test in suspension was carried out on bone marrow and thymus cells and also on trypsinized stromal thymus and bone marrow mechanocytes with AFS exhausted with erythrocytes, normal bovine serum (NBS), and bone marrow [10].

EXPERIMENTAL RESULTS

All the AFS, i.e., those obtained by immunization with fibroblasts removed from the glass both with trypsin and with a rubber spatula, after exhaustion with guinea pig erythrocytes and NBS, gave two bands in the precipitation test in agar with normal guinea pig serum and one band with culture media of stromal mechanocytes from bone marrow, thymus, and spleen. The precipitation band with the media was completely identical with one of the bands formed by AFS with normal sera (the antigen responsible for this precipitation band will be called AG-1), and partly identical with the other band, named AG-2 (Fig. 1a). On exhaustion of the AFS with extract of organs or a mixture of cells from different organs, including spleen cells or bone marrow cells only (and also with nonadherent bone marrow cells separately), but not with thymus cells, the precipitation band with normal sera, partly identical with the band formed with the culture media, disappeared (Fig. 1b). The remaining precipitation band disappeared after exhaustion with normal guinea pig sera (1:1). After exhaustion in this manner the cytotoxic test and the immunofluorescence test for fibroblasts and their precursors alsobecame negative [2]. After exhaustion with media the AFS gave one precipitation band in the precipitation test with AG-2 or normal sera (Fig. 2). The AFS against AG-1 gave no precipitation band with any of the water-soluble extracts, including extracts of stromal fibroblasts. The precipitation reaction with AFS took place in the zone of equivalence with unconcentrated normal sera and with media concentrated 25 times. The approximate concentration of AG-1 was about 1 μg/ml culture medium and 4 μg/ml normal serum. The AG-2 concentration in normal serum was about 10 μg/ml.

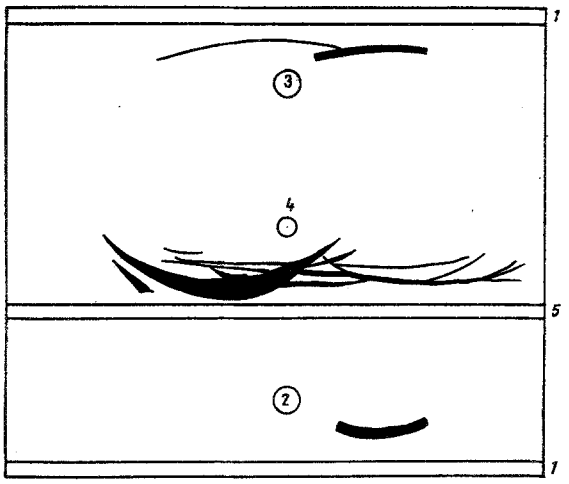

Fig. 3. Immunoelectrophoretic analysis with AFS of AG-1 in culture media of stromal fibroblast and of AG-1 and AG-2 from normal sera. Comparative electrophoretic mobility of AG-1 and AG-2. 1) AFS; 2) culture medium of stromal fibroblasts; 3) normal guinea pig serum; 4) normal mouse serum; 5) antiserum against normal mouse serum.

According to the immunoelectrophoretic data, AG-1 migrated in the α_1-globulin zone and AG-2 in the α_1-globulin zone (Fig. 3).

To discover whether AFS are antisera against collagen, the following experiment was carried out. A test preparation of type I collagen was subjected to electrophoresis in polyacrylamide gel. Three groups of bands corresponding to the α-, β-, and γ-chains of the molecule were obtained. On treatment with an excess of collagenase for 1.5 h at 37°C the bands disappeared.

The maximal collagen concentration in the culture medium, showed by the test for hydroxyproline, was 20 µg/ml. The culture media were treated with sufficient collagenase to cause complete disappearance of the bands characteristic of collagen in the gel (allowing for the concentration of collagen in the medium). The precipitation test with AFS was then carried out on the collagenase-treated media. The reaction was not weakened, and, consequently, AG-1 (the test was set up with AFS exhausted with bone marrow also) was insensitive to the action of collagenase.

In the indirect immunofluorescence test with AFS against AG-1 on living trypsinized stromal fibroblasts from bone marrow at the seventh passage (on spherical cells, not spreading on the surface) specific fluorescence of circular type was observed.

In the complement-dependent cytotoxic test with AFS against AG-1, trypsin (2.5%; Bethesda, Maryland), diluted tenfold, and a 0.25% solution of trypsin from the Institute of Poliomyelitis and Virus Encephalitis, Academy of Medical Sciences of the USSR, when the cells were treated under ordinary conditions (30-40 min at 37°C), did not remove this antigen from the stromal fibroblasts. This is clear from the fact that AFS (in dilutions of up to 1:1000) showed a cytotoxic action comparable with that during the reaction with adherent fibroblasts [2]. The reaction with thymus and bone marrow cells under these circumstances, however, was negative (Table 1).

Judging from the results of the immunofluorescence test, the cytotoxic test, and the precipitation test, AG-1 was specific for fibroblasts (AG-1 is absent in hematopoietic cells, lymphocytes, and macrophages). AG-1 was synthesized by stromal fibroblasts both *in vivo* and *in vitro*. AG-2, partly identical with AG-1, according to the results of the precipitation test was present in fibroblasts, in bone marrow and spleen cells, but not, for example, in thymus cells or erythrocytes (results of exhaustion). AG-2, judging from the results of exhaustion, is a surface water-soluble protein of hematopoietic cells and lymphocytes. AG-2 is evidently synthesized *in vivo* by some bone marrow and spleen cells (and, probably, by other cells), but not by thymus cells. It is not yet known whether AG-2 is synthesized by fibroblasts *in vivo*. It is likewise unknown whether any of the known cell antigens, such as the protein described previously [5], or the α_1-protein present in lymphocytes and fibroblasts, corresponds to AG-2.

TABLE 1. Results of Cytotoxic Test of AFS with Guinea Pig Cells (in suspensions)

Dilution of AFS[1]	Cytotoxic index (CI)[2]				
	stromal bone marrow fibroblasts		stromal thymus fibroblasts	bone marrow	thymus
	I*	II†	II		
1:10	0,60	0,81	0,91	0,00	0,00
1:100	0,59	0,55	0,57	—	—
1:1000	0,26	0,20	0,19	—	—

[1]AFS against trypsinized stromal bone marrow fibroblasts exhausted with erythrocytes, NBS, and guinea pig bone marrow.
[2]CI = % of cells killed during experiment − % of cells killed with normal serum multiplied by 100, and divided by percent of cells killed with normal serum.
Legend: *) trypsinization of cells with trypsin (2.5% trypsin, Bethesda, Maryland) in a concentration of 0.25% for 30 min at 37°C; †) trypsinization of cells with 0.25% trypsin solution from Institute of Poliomyelitis and Virus Encephalitis, Academy of Medical Sciences of the USSR, for 30 min at 37°C.

According to the results of the cytotoxic test and the immunofluorescence test of living fibroblasts, AG-1 is a surface protein of stromal mechanocytes. According to the results of the precipitation test, AG-1 is not a component of the "internal" proteins of fibroblasts. AG-1 is not collagen, for after treatment of the culture media with sufficient collagenase to destroy the collagen present in them, the AG-1 still remains. On the basis of the results of previous investigations and the writer's own observations it can be postulated that AG-1 is present in different amounts on the surface of different stromal fibroblasts, and this could play an important role in the recognition of each other and of cells interacting with them (hematopoietic cells, lymphocytes). AG-1 is a trypsin-resistant protein (as shown by the results of the cytotoxic and immunofluorescence test on freshly trypsinized cells with AFS obtained by immunization with trypsinized fibroblasts), which suggests that it is not CSP, which is an extremely trypsin-sensitive protein of fibroblasts [11, 13]. According to preliminary data, AFS do not react in the precipitation test with a urea extract of fibroblasts containing CSP (by contrast with anti-CSP-sera). AG-1 is thus a γ-globulin (unlike CSP, which an α_2-globulin), it is neither CSP nor collagen, and is evidently a hitherto unknown specific surface protein of fibroblasts.

The author is grateful to Professor A. Ya. Fridenshtein for a critical discussion of the results of this investigation, to A. G. Grosheva for much assistance of different sorts, A. K. Yazova for valuable advice and for discussion of the results of the precipitation test, T. V. Zamaraeva for providing the collagen and for help in the work with it, and to A. E. Berman for providing the purified collagenase.

LITERATURE CITED

1. A. I. Gusev and V. S. Tsvetkov, Lab. Delo, No. 2, 43 (1961).
2. A. A. Ivanov-Smolenskii and A. G. Grosheva, Byull. Éksp. Biol. Med., No. 4, 451 (1978).
3. A. Ya. Fridenshtein, R. K. Chailakhyan, N. V. Latsinik, et. al., Transplantation, 17, 331 (1974).
4. V. S. Tsvetkov, in: Immunochemical Analysis [in Russian], Moscow (1968), p. 120.
5. R. Bachvaroff and F. T. Rapoport, J. Immunol., 109, 1081 (1972).
6. I. Bergman and R. Loxley, Analyt. Chem., 35, 1961 (1967).
7. A. H. Coons and H. H. Kaplan, J. Exp. Med., 91, 1 (1950).
8. R. O. Hynes, Biochim. Biophys. Acta, 458, 73 (1976).
9. A. H. Kang and I. A. Piez, Biochemistry (Washington), 8, 3648 (1969).
10. A. E. Reif and M. V. Allen, J. Exp. Med., 120, 413 (1964).
11. E. A. Ruoslahti et al., Biochim. Biophys. Acta, 322, 352 (1973).
12. F. W. Studier, J. Molec. Biol., 79, 237 (1973).

13. K. M. Yamada and J. A. Weston, Proc. Nat. Acad. Sci. USA, 71, 3492 (1974).
14. K. M. Yamada and J.A. Weston, Proc. Nat. Acad. Sci. USA, 72, 3158 (1975).
15. K. M. Yamada, S. S. Yamada, and I. Pastan, Proc. Nat. Acad. Sci. USA, 73, 4 (1976).

INCREASED NUMBER OF PRECURSORS OF ROSETTE-FORMING CELLS SENSITIVE TO THYMUS HORMONE IN THE SPLEEN OF MICE VACCINATED WITH SMALLPOX VACCINE

L. P. Trubchenikova, R. M. Radzikhovskaya, V. M. Bukhman, K. L. Chimishkyan, and G. Ya. Svet-Moldavskii

UDC 612.112.94.017.1

Injection of smallpox vaccine into female C57BL/6j mice aged 2 months led on the first few days to a sharp increase in the number of precursor cells of rosette-forming lymphocytes sensitive to the differentiating effect of thymus extract. By the tenth day after vaccination the normal number of these cells was restored.

KEY WORDS: thymosine; smallpox vaccine; precursor cells; rosette-forming cells.

The immunologic response of the body to particular antigens is connected with cooperative interaction between different subpopulations of T- and B-leucocytes and macrophages. In order to understand the mechanism of the immunologic cooperative response it is essential to know the quantitative content of the various populations of immunocompetent cells reflecting the response of the host to injection of antigen.

In the investigation described below the number of precursor cells of lymphocytes capable of forming spontaneous rosettes with sheep's red blood cells (so-called E-rosettes) *in vitro* was determined in the spleens of mice vaccinated with smallpox vaccine. For this purpose a method based on the ability of thymus hormone to induce differentiation of precursor cells into rosette-forming lymphocytes was used.

EXPERIMENTAL METHOD

Female C57BL/6j mice aged 2 months, bred in the writers' laboratory, were used. The mice were obtained initially from the Stolbovaya Nursery, Academy of Medical Sciences of the USSR.

Thymus extract (TE) was prepared by Goldstein's method [4] as far as fraction 3 (TE-3). The extract contained 9 mg protein/ml, determined by Lowry's method. The TE-3 was kept at −196°C, and was thawed and used on the day of the experiment. Lyophilized bovine serum albumin (BSA, from Biomed, Cracow, Poland; batch 1/04/74) was diluted to the required concentration with physiological saline. The protein content in the thymosine and BSA preparations was the same, namely 9 mg/ml.

A commercial preparation of smallpox vaccine from the L-IVI strain, produced by the Mechnikov Research Institute of Vaccines and Sera (batch No. 0444, control No. 1388), was used. The vaccine was diluted with physiological saline.

The animals were divided into three groups: control — intact or animals receiving BSA (0.2 ml), and experimental, vaccinated subcutaneously with smallpox vaccine in a dose of 10^6 pock-forming units (PFU)/ml. On the 2nd, 7th, 14th, and 30th days after vaccination four mice in each group were killed, the spleen was removed, and a cell suspension was prepared in medium 199 in the proportion of 10^7 cells/ml. The suspension (0.1 ml) was incubated with thymosine solution in a volume of 0.15 ml at 37°C for 90 min. The lymphocytes were then

Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR L. M. Shabad.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 86, No. 10, pp. 458-459, October 1978. Original article submitted January 4,1978.

0007-4888/78/8610-1351$07.50