IN VITRO INDUCTION OF MIF-PRODUCING CELLS

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The initial and essential stage of formation of delayed-type hypersensitivity (DTH) to soluble protein antigens is processing of the antigen by accessory cells and its presentation to T cells along with Ia antigens. To obtain effector cells of DTH *in vitro* the principle of culture of normal lymphocytes with macrophages pretreated with antigen is used [2, 5, 6, 8, 10]. Effector cells, forming macrophage migration inhibition factor (MIF) are one of the cell subpopulations participating in the effector stage of DTH. The principles governing formation of MIF-producing cells *in vitro* have not yet been studied.

The aims of the present investigation were: to obtain MIF-producing cells *in vitro* specific for egg albumin (EA) and methylated bovine serum albumin (MBSA), to compare the activity of MIF-producing cells obtained *in vitro* and *in vivo*, and to study the effect of the doses of antigen used for processing macrophages on the formation of MIF-producing cells.

METHODS

Experiments were carried out on female CBA mice weighing 20-22 g. MBSA and EA (from Serva, West Germany) were used as antigenic material. Adherent spleen cells of intact mice were treated with MBSA in doses of between 0.2 and 5 μg/ml and with EA in doses from 0.2 to 250 μg/ml. To stimulate sensitized lymphocytes in the macrophage migration inhibition test, MBSA was used in a dose of 25 μg/ml and EA in a dose of 50 μg/ml.

Lymphocytes sensitized *in vitro* were obtained by culturing nonadherent splenocytes with adherent spleen cells, treated beforehand with antigen by the method described in [8] in the writers' modification [2]. The cells were cultured in medium of the following composition: medium RPMI-1640 (from Serva, West Germany) with the addition of 10% embryonic calf serum, 2 mM L-glutamine, 1 mM HEPES, $5 \cdot 10^{-5}$ M 2-mercaptoethanol, essential amino acids, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. After 5 days the nonadherent cells were separated, their viability determined, and their ability to produce MIF in response to stimulation by specific antigen was tested. Splenocytes sensitized *in vivo* were obtained on the 5th day after immunization of the mice by subcutaneous injection of MBSA or EA in a dose of 250 μg/mouse, mixed with Freund's complete adjuvant.

In the experiments of series I MIF production was studied in response to stimulation of DTH effector cells, obtained *in vitro* and *in vivo*, by specific antigen in the direct capillary test [7, 9] in the modification in [1]. In the experiments of series II activity of the supernatants obtained on activation of sensitized lymphocytes by the corresponding antigen was investigated, by determining their ability to induce inhibition of macrophage migration in the indirect capillary test [4].

Peritoneal exudate cells (PEC) of intact mice served as the target cells in both the direct and the indirect macrophage migration inhibition test. When the direct capillary test was set up, capillary tubes (Pyrex glass, internal diameter 0.4 mm) were filled with a cell population consisting of PEC and 15% of lymphocytes, sensitized *in vitro* and *in vivo*. When the indirect test was set up, glass capillary tubes were filled with PEC and placed in wells with MIF-containing supernatants. The latter were obtained by culturing cells sensitized *in vitro* or *in vivo* in medium containing antigen (in the control, in medium without antigen). The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 18 h.

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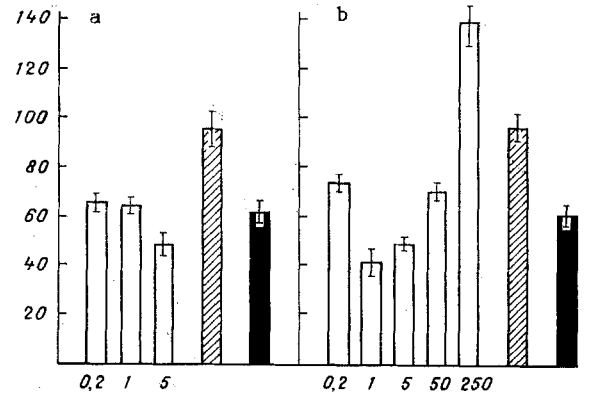

Fig. 1. Determination of MIF-producing cells induced by MBSA or EA *in vitro* or *in vivo*, by the direct method of inhibition of migration of peritoneal exudate cells. A) results of experiments in which MBSA was used as antigen, B) results of experiments in which EA was used as antigen. Unshaded columns, migration index of PEC with addition of 15% of lymphocytes sensitized *in vitro* by EA or MBSA, during interaction with specific antigen (cells adherent to the plastic were treated with the corresponding antigen in doses of 0.2, 1, 5, 50, and 250 μg/ml); obliquely shaded columns, the same, for interaction with nonspecific antigen (purified preparation of human γ-globulin in a dose of 50 μg/ml); black columns, migration index of PEC with addition of 15% of splenocytes, sensitized *in vivo* with EA or MBSA, on interaction with specific antigen. Here and in Fig. 2: ordinate, MI (in percent).

The zone of migration was determined by measuring two diameters on a Diavert-Leitz microscope (West Germany) by means of an optical micrometer. The results were assessed as the migration index (MI), determined by the formula:

$$MI = \frac{\text{Mean area of migration zone in experimental}}{\text{Mean area of migration zone in control}} \cdot 100\%.$$

The numerical results were subjected to statistical analysis by Student's t test.

RESULTS

After culture for 5 days 25-30% of the original cells put into culture remained, and 95% of these were still viable. The results showed that *in vitro*, on combined culture of nonadherent spleen cells with adherent splenocytes treated previously with MBFA or EA, MIF-producing cells are formed. During contact with specific antigen these cells can produce MIF, as was shown by direct and indirect tests of inhibition of macrophage migration from capillary tubes. For instance, when the direct macrophage migration inhibition test was set up it was found that MI of these cells in experiments with MBSA was between 50.3 ± 9.8 and 66.7 ± 7.9% on treatment of the adherent splenocytes with antigen in a dose of 0.2-5 μg/ml (Fig. 1). MI of the cells in experiments with EA was 75.6 ± 5.3% when adherent splenocytes were treated with antigens in a dose of 0.3 μg/ml. This value was considerably reduced when optimal doses of 1 and 5 μg/ml was used, when it was 42.7 ± 10.6 and 49.8 ± 2.2% respectively. With a dose of 50 μg/ml, MI was 70.7 ± 7.1%. In the experiments in which this dose was used, induction of MIF-producing cells was discovered in only 57% of cases.

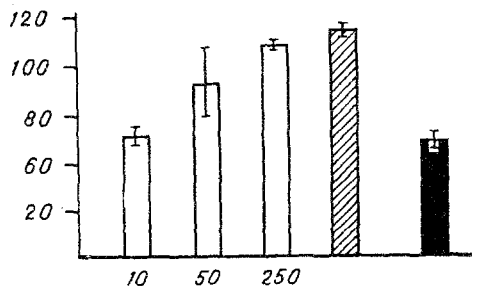

Fig. 2. Determination of MIF-producing cells induced by EA *in vitro* or *in vivo* by the indirect method of inhibition of migration of peritoneal exudate cells. Unshaded columns, migration index of PEC in supernatant obtained by interaction of lymphocytes sensitized *in vitro* with specific antigen (cells adherent to plastic were treated with EA in doses of 10, 50, and 250 μg/ml; shaded column, the same, for interaction with nonspecific antigen (purified preparation of human γ-globulin in a dose of 50 μg/ml); black column, migration index of PEC in supernatant obtained by interaction of splenocytes sensitized *in vivo* with specific antigen.

A similar picture was observed in the experiments of series II also, when the indirect macrophage migration inhibition test was set up (Fig. 2). For instance, MI of the cells, under the influence of MIF-containing supernatant obtained by contact between lymphocytes sensitized *in vitro* to EA and specific antigen, was 52.1 ± 9.8% in the case of preliminary treatment of the macrophages with antigen in a dose of 10 μg/ml. MI increased with a dose of 50 μg/ml to 75.0 ± 28.8%. The appearance of DTH effector cells was thus found to be dependent on the dose of antigen used for treating splenocytes adherent to the plastic. Combined culture of nonadherent splenocytes of intact mice with macrophages pretreated with a large dose of EA (250 μg/ml) evidently led to suppression of formation of MIF-producing cells [3]. Migration of PEC in experiments conducted in this way was virtually indistinguishable from spontaneous migration of PEC, as was shown by the direct and indirect macrophage migration inhibition tests. MIF-producing cells induced *in vitro* possessed specific activity. On contact with nonspecific antigen, MI was virtually indistinguishable from MI in the case of spontaneous migration of PEC.

To compare the activity of MIF-producing cells induced *in vitro* and *in vivo*, direct and indirect macrophage migration inhibition tests were carried out with cells obtained from donors sensitized with MBSA or EA. It will be clear from Figs. 1 and 2 that activity of DTH effector cells obtained *in vitro* and *in vivo* was virtually indistinguishable.

Thus during combined culture of nonadherent spleen cells of normal mice for 5 days with splenocytes adherent to the plastic and preincubated with optimal doses (up to 50 μg/ml) of protein antigen, DTH effector cells possessing specific activity and ability to produce MIF were formed. On treatment of macrophages with a large dose of protein antigen, followed by contact between them and spleen cells not adherent to the plastic, the formation of DTH effector cells, including specific producers of MIF, was evidently suppressed.

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POWERS OF DIFFERENTIATION OF CLONAL STRAINS OF BONE MARROW FIBROBLASTS

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KEY WORDS: stromal colony-forming cells; monoclonal strains of bone marrow fibroblasts; diffusion chambers.

Diploid strains of bone marrow fibroblasts, obtained in cultures involving passage of progenies of stromal colony-forming cells (CFU-f) have been shown to possess marked osteogenic activity, which is exhibited on back transplantation into the donor [2-5, 8, 10].

The writers showed previously [7] that the number of osteogenic cells in the composition of the strains increases during passage.

In the investigation described below the powers of differentiation of monoclonal strains of bone marrow fibroblasts, derived from single CFU-f, were studied.

METHODS

Part of the wing of the ilium was resected from Californian rabbits aged 1.5-2.5 months under pentobarbital anesthesia. Cells contained in its medullary cavity were flushed out into culture medium, after which a suspension of disaggregated cells was obtained by repeated pipeting. The cell suspension was filtered through 4 layers of Kapron. Completeness of disaggregation was tested by introducing 10^5 cells into plastic flasks (area 25 cm^2) coated with polylysine. After 60 min flasks were fixed with alcohol and stained by Giemma's method. The bone marrow cells were cultured on HAM medium with 20% embryonic calf serum. To determine the efficiency of stromal colony formation (ECF-f) [4, 7] and to obtain colonies for passage, between 10^4 and $3 \cdot 10^4$ bone marrow cells were explanted into flasks with an area of 20-40 cm^2.

For passage of single colonies primary cultures were treated for 1-3 min with a 0.25% solution of trypsin, and during the next 10-15 min the wet flasks were kept at 37°C, after which the single colonies were removed with gelatin sponges. The cells were washed out of the sponges by pipeting and subjected to further culture. Repeated passages were carried out when the cultures reached confluence. X and Y chromosomes were identified [9] in dividing cells of the colonies in mixed cultures of male and female bone marrow and in cells of strains obtained by passage of colonies derived from mixed cultures.

Cells of the 2nd or 3rd passages, taken from the cultures, were transplanted allogeneically into two types of diffusion chambers: type A (volume of chamber 0.015 mm^3, height 0.1 mm [4], and type O (volume of chamber 0.15 cm^3, height 2 mm) [8].

The diffusion chambers were fixed on the 30th-90th day with alcohol-formol, calcium-formol, or 96° alcohol, and subjected to histologic treatment, including preliminary decalcification in 5% HNO_3. Series of paraffin sections were stained with hematoxylin and eosin, by Cossa' method, and by Gomori's method for alkaline phosphatase.

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