Antigen- or Mitogen-Provoked Spleen Cells Produce Factors that Stimulate the Secretion by Macrophages of a Neutral Proteinase Degrading Cartilage Proteoglycans

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Soluble products released by rabbit spleen cells on stimulation with either mitogen or antigen markedly stimulate the secretion of a proteoglycan-degrading neutral proteinase by rabbit bone-marrow macrophages.

The lysis of the proteoglycans and of the collagen of the cartilage matrix under the advancing edge of the inflammatory synovial pannus is a major cause of articular cartilage destruction in rheumatoid arthritis (Krane, 1974). Macrophages (Kobayashi & Ziff, 1975), as well as T- and B-lymphocytes (Ishikawa & Ziff, 1976) are abundant in the pannus, suggesting (Stastny *et al.*, 1975) that the lymphocytes control, through their secretory products, the activity of the macrophages.

In the present paper we demonstrate that the secretion of a proteoglycan-degrading neutral proteinase by rabbit macrophages (Hauser & Vaes, 1978a) is indeed considerably enhanced under the action of soluble factors produced by rabbit spleen cells on stimulation with antigen or mitogen. Moreover, these factors concomitantly stimulate the secretion of neutral collagenase, but not that of lysosomal enzymes nor of lysozyme.

Materials and Methods

Animals and materials

Concanavalin A (grade III), phytohaemagglutinin, ferritin (horse spleen) and bovine γ -globulins were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); complete Freund's adjuvant was from Difco (Detroit, MI, U.S.A.). The culture media, the other special chemicals and the animals (male rabbits of the Termonde white strain) were from the same suppliers as mentioned in our previous study (Hauser & Vaes, 1978a,b).

Preparation of spleen-cell factors

Sensitized spleen lymphoid cells were obtained from rabbits (2–3 kg) immunized by two subcutaneous injections (at 2-week intervals) of 5 mg of ferritin emulsified in 1 ml of complete Freund's adjuvant. The rabbits were killed 1 week later. The spleen of either normal or immunized rabbits was excised and teased with scissors in 10 ml of our (Hauser & Vaes,

1978a) 'basal' culture medium. Tissue clumps were allowed to sediment for 2–3 min. The supernatant was separated and the sediment treated again twice in the same way in 10 ml of fresh basal culture medium. The cells present in the pooled supernatants were sedimented by centrifugation (1300g-min at 4°C), resuspended in 10 ml of basal medium and counted in a haemocytometer. About 3×10^8 – 4×10^8 nucleated cells were usually recovered from one spleen. The suspension was adjusted to 6×10^6 nucleated cells/ml of serum-free basal medium and distributed in 10 cm-diameter polystyrene Petri dishes. Half of the preparation was cultured, usually for 3 days, with either mitogen (5 μ g/ml), usually concanavalin A, or antigen (100 μ g of ferritin/ml) to provide stimulated lymphocyte media. The other half was cultured for the same time, without additive to provide non-stimulated lymphocyte media to which the antigen or the mitogen was added at the end of the culture. The lymphocyte media were collected by centrifugation (2500g-min at 4°C), filtered on Millipore filters (0.22 μ m pore size) and immediately used.

Culture of macrophages with lymphocyte media

Rabbit bone-marrow macrophages were isolated and cultured as described elsewhere (Hauser & Vaes, 1978a,b). The nucleated non-adherent cells, collected after the first 6 days of culture of the marrow cells, were distributed into 6 cm-diameter Petri dishes (10^6 cells in 4 ml of medium/dish) and further cultured for 20 h in serum-free basal medium. About 75% of the cells became adherent under these conditions. The non-adherent cells were eliminated with the culture fluid and the adherent cells (macrophages) were further cultured, usually for 4 days, in 4 ml of basal medium containing 0–50% (v/v) of stimulated or non-stimulated lymphocyte media. The macrophage-conditioned media were then collected, supplemented with 25 μ l of 1 M-sodium cacodylate/HCl buffer (pH 7)/ml and assayed for enzymic activities.

Enzyme assays

Assays (and units of activity) of neutral proteinase (carried out with ^{35}S -labelled proteoglycans of cartilage discs or [^3H]acetylated casein as substrates), neutral collagenase [carried out after activation of latent collagenase by $4\mu\text{g}$ of trypsin/ml, by the method of Vaes (1972)], β -glucuronidase and lysozyme were as described previously (Hauser & Vaes, 1978a; Vaes *et al.*, 1978). *N*-Acetyl- β -D-glucosaminidase was assayed as described by Leaback & Walker (1961); 1 unit of its activity refers to the decomposition of 1 nmol of substrate/min.

Results and Discussion

As previously shown (Hauser & Vaes, 1978a), rabbit bone-marrow macrophages in culture secrete a metal-dependent neutral proteinase that degrades the protein core of the proteoglycan subunits present in cartilage; the enzyme is, however, not stored in the cells. Macrophage-conditioned culture media manifested simultaneously a proteolytic activity towards casein, and evidence was presented indicating that it was likely to be due to the same neutral proteinase. Such an enzyme could play an important role in chronic inflammatory processes where macrophages are abundant, particularly those where cartilage is being eroded, such as in experimental immune arthritis of the rabbit

(Dumonde & Glynn, 1962) or in human rheumatoid arthritis (Krane, 1974). The control of its secretion could possibly be exerted by factors produced by nearby lymphocytes under specific immune stimulation.

Testing this hypothesis, we first observed a considerable enhancement of the secretion of neutral proteinase by the macrophages on addition to their culture medium of supernatants (25%, v/v) of cultured spleen cells stimulated by concanavalin A; the macrophages then released 8.6 ± 1.0 units (mean $\pm$ s.d. for six cultures) of neutral proteinase (assayed on casein)/ 10^6 cells over 3 days of culture as opposed to 1.8 ± 0.7 units released in the presence of non-stimulated lymphocyte media ($P < 0.001$ with Student's *t*-test). The activation of the macrophages was maximal with media obtained after 2 or 3 days stimulation of the spleen cells by the mitogen, and it was best manifested after 3 or 4 days of culture of the macrophages with these media.

Increasing concentrations of concanavalin A-stimulated lymphocyte media caused a progressively increasing secretion of the neutral proteinase, assayed either on casein or on cartilage proteoglycans, by the macrophages (Fig. 1). Concomitantly, these media enhanced the secretion of neutral collagenase, as already observed by Wahl *et al.* (1975), but not of lysozyme nor of the lysosomal hydrolases, β -glucuronidase and *N*-acetyl- β -glucosaminidase. As

Fig. 1. Secretion of enzymes by macrophages as a function of the concentration of added supernatant obtained from cultures of mitogen-stimulated lymphocytes

Stimulated (●) or non-stimulated (○) lymphocyte media were obtained by culturing spleen cells from a normal rabbit respectively in the presence or in the absence of concanavalin A. Each value is the mean for two cultures. Abbreviations used: CAS, neutral proteinase assayed on casein; PRO, proteoglycan-degrading neutral proteinase; COL, collagenase; GLU, β -glucuronidase; NAG, *N*-acetyl- β -D-glucosaminidase; LYS, lysozyme.

Fig. 2. Secretion of enzymes by macrophages cultured with conditioned medium obtained from lymphocytes of a ferritin-immunized rabbit

Stimulated lymphocyte media (■) were obtained by culturing for 48 h the spleen cells with ferritin (F), heterologous antigen (100 µg of bovine γ -globulins/ml, G), concanavalin A (M) or phytohaemagglutinin (P). In the non-stimulated lymphocyte media (□), the antigen or the lectin was added at the end of the culture. Both media were used at a 25% (v/v) concentration. Other controls (▨, C) included macrophages cultured without lymphocyte medium. Each value is the mean $\pm$ S.D. for three cultures. The abbreviations identifying the enzymes assayed are as given in Fig. 1.

they have been reported to stimulate the secretion of lysosomal enzymes by macrophages under other experimental conditions (Pantalone & Page, 1975), it appears likely that different patterns of macrophage secretory responses may occur under the action of lymphocyte products. The lack of difference observed in the accumulation of lysozyme between the cultures containing either stimulated or non-stimulated lymphocyte media, strongly suggests that the former did not stimulate the proliferation of the macrophages under our culture conditions (Gordon *et al.*, 1974). This indication was supported by the lack of difference observed between these two types of cultures, either in the number of adherent cells counted under an inversion microscope after 3 days culture, or in the amounts of [³H]thymidine incorporated in the adherent cells during the last 24 h of culture.

Obviously our spleen-cell population is heterogeneous, and various types of cells could be sensitive to the mitogen and be at the source of the factors active on the macrophages. However, we observed that spleen cells from ferritin-sensitized rabbits produced, on challenge with ferritin, but not with an heterologous antigen such as bovine γ -globulin, soluble factors whose effects on the secretion of enzymes by the macrophages were similar to those obtained on challenge with the mitogens (Fig. 2). Therefore a lymphocyte origin for these factors appears highly probable. It may thus be concluded that stimulated lymphocytes release soluble factors that activate the secretion by macrophages of neutral

proteinases capable of degrading the main structural macromolecules of cartilage and other connective tissues.

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