IN VITRO BIOSYNTHESIS OF COLD INSOLUBLE GLOBULIN (FIBRONECTIN) BY MOUSE PERITONEAL MACROPHAGES

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1. Introduction

Previous studies [1,2] have implicated that cold insoluble globulin (CIG) [3,4] plays an important role in the phagocytic function of the reticuloendothelial system (RES). It has been reported that the phagocytic dysfunction of the RES often seen in patients after trauma, surgery or in sepsis, is concomitant with a decreased plasma concentration of CIG [5,6] and can be reversed by the intravenous infusion of plasma cryoprecipitate [7,8] which contains CIG as a major component.

CIG is the plasma form of fibronectin, a glycoprotein found in connective tissue and reported to be produced by fibroblasts, endothelial and glial cells grown in vitro (reviewed [9]). Most of the fibronectin produced by fibroblasts in vitro is deposited as a matrix adherent to the tissue culture dish [10]. Previous studies [11] have demonstrated that peritoneal macrophages do not synthesize a fibronectin-containing adhesive matrix. However, here we present evidence that CIG is produced by macrophages, and is secreted to the culture medium.

2. Materials and methods

CIG was isolated from bovine plasma by affinity chromatography on gelatin-substituted Sepharose [12] followed by chromatography on DEAE-cellulose. Immunization of rabbits with CIG and isolation of rabbit IgG were performed as in [13].

CIG and anti-CIG antibodies were coupled to CNBr-activated Sepharose (Pharmacia, Uppsala) following the recommendations of the manufacturer.

CIG-depleted foetal calf serum was prepared by subsequent passage through columns of Sepharose substituted with gelatin and anti-CIG antibodies, respectively. The CIG-depleted serum was used in growth media at concentrations corresponding to the protein content of native foetal calf serum.

2.2. *Isolation and cultivation of macrophages*

Cells were obtained by peritoneal lavage of outbred NMRI mice by the method in [14], and were seeded in Linbro tissue culture plates (Linbro Sci., Hamden, CT) with glass cover slips at 0.5×10^6 cells/dish or in 35 mm Falcon plastic petri dishes (Gateway Int., Los Angeles, CA) at 3×10^6 cells/dish. After 1 h incubation at 37°C in humidified air supplemented with 5% CO_2 the cell layer was washed twice with 0.5 and 1 ml, respectively, of incubation medium to remove cells not attached to the culture dish. The incubation was continued in 0.5 and 2 ml, respectively, of F-10 medium supplemented with 10% foetal calf serum depleted of CIG. Macrophages were identified by morphology as well as by their ability to ingest IgG-opsonized red cells (fig.1). More than 98% of the cells were identified as macrophages by these criteria.

2.3. *Immunological quantification of CIG*

The concentration of CIG in the growth media was quantified by use of a radioimmunoassay. The assay will be detailed elsewhere. The immunoadsorbent contained monospecific rabbit antibodies directed against bovine CIG purified by affinity chromatog-

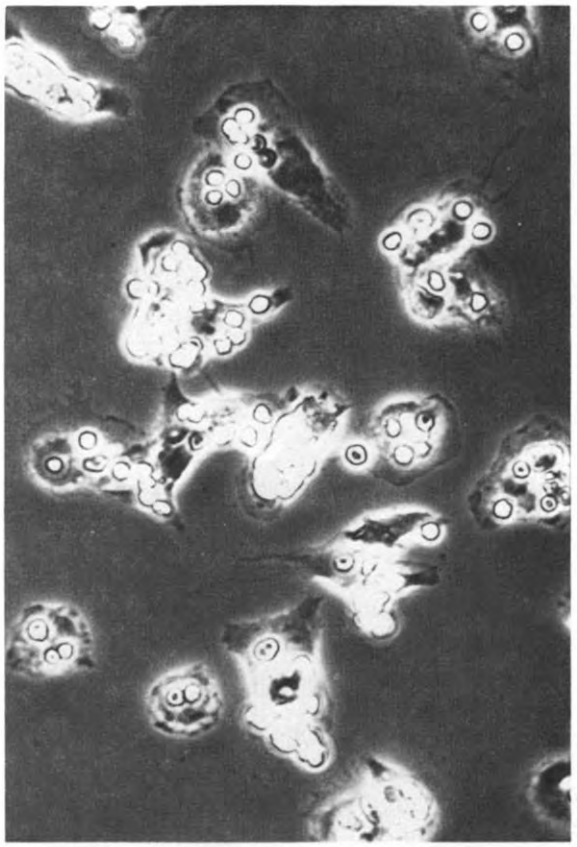

Fig.1. Phagocytes of IgG-coated erythrocytes by mouse peritoneal cells. Sheep erythrocytes were treated with rabbit anti sheep red cell IgG (Cordis Lab., Miami, FL) at 10 μg/ml in F-10 with Hepes buffer at 37°C for 30 min. The erythrocytes were washed 3 times in the buffer and added to the macrophages at a final concentration of 5×10^7 erythrocytes/10^6 macrophages. After 30 min incubation, non-attached erythrocytes were rinsed off and the cells fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. The cover slips were mounted and examined in phase contrast × 1200. Examination showed that erythrocytes were associated with > 98% of the cultured cells.

raphy on protein A–Sepharose and CIG–Sepharose and coupled to CNBr-activated Sephadex G-25, kindly provided by Pharmacia Fine Chem., Uppsala. The amount of mouse CIG was determined relative to CIG purified from human plasma purified.

2.4. *Gel electrophoresis*

Gel electrophoresis of proteins was carried out on 5% polyacrylamide slab gels using the buffer system in [15]. Before analysis the samples were boiled with 5% SDS and 10% β-mercaptoethanol for 3 min. The gels were stained with Coomassie brilliant blue and subjected to autoradiography as in [16]. Reference proteins (mol. wt): bovine CIG (220 000) I; transferrin (80 000) II; human IgG (52 000, heavy chains) III; (22 000, light chains) IV; lysosyme (14 300) V.

To indicate the positions of unlabeled reference proteins in the autoradiogram the stained bands were spotted with radioactive ink prior to autoradiography.

3. Results

By growing the macrophages in a medium containing serum depleted of CIG it was possible to detect the secretion of CIG by immunological techniques, i.e., a radioimmunoassay. During cultivation an increasing amount of CIG could be demonstrated in the culture media (fig.2) and after growing the cells for 24 h the CIG concentration in the media corresponded to 0.3 μg human CIG/ml.

To exclude that the CIG detected by the radioimmunoassay originated from other cells in the animal and was merely stored by the macrophages and released to the medium on in vitro culture, experi-

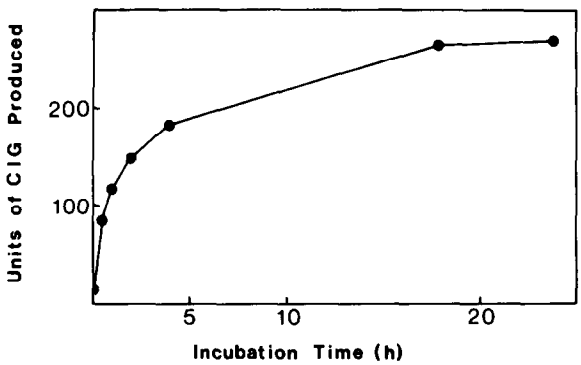

Fig.2. Secretion of CIG by mouse peritoneal macrophages in vitro. Mouse peritoneal macrophages were grown in F-10 medium supplemented with 10% calf serum depleted of CIG. The media of individual cultures were removed at the indicated points and the concentration of CIG in the media quantified by use of a radioimmunoassay. Mouse CIG was determined relative to CIG purified from human plasma; one unit of mouse CIG corresponds to 1 ng human CIG/ml medium.

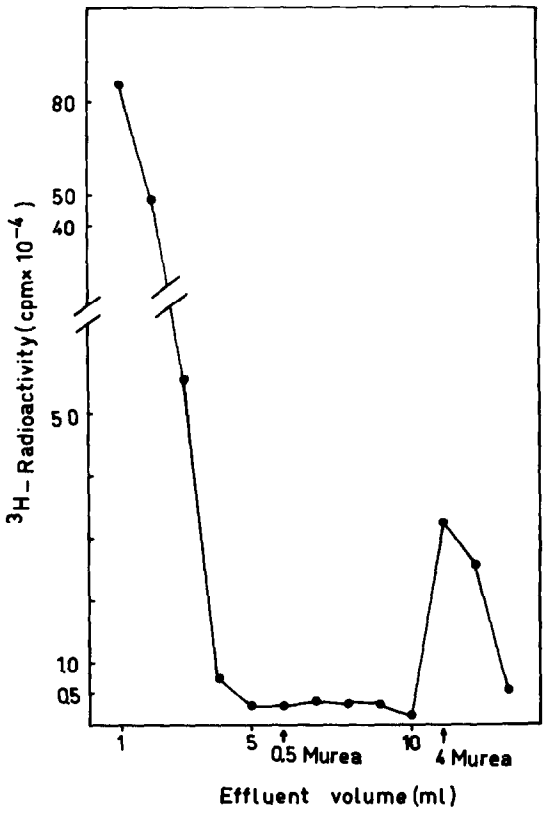

Fig.3. Chromatography on gelatin Sepharose of ^{3}H-labeled proteins produced by macrophages grown in vitro. Peritoneal macrophages (3 × 10^6 cells) from NMRI mice were seeded on 35 mm Falcon plastic petri dishes in 2 ml leucin-free F-10 medium supplemented with 10% foetal calf serum depleted of CIG. After 1 h incubation the cell layer was washed and the incubation was then continued in 2 ml fresh medium supplemented with 100 μCi [^{3}H]leucine (spec. act. 28 Ci/mmol). After 48 h the media was collected and particulate material removed by centrifugation. Labeled material of low molecular weight was removed by dialyses against phosphate buffered saline (PBS). Dialysed medium (1 ml) was supplemented with 50 μg CIG purified from bovine plasma as carrier and incubated with 0.5 ml gelatin Sepharose end-over-end for 30 min at 20°C. The mixture was transferred to a column and eluted with 5 ml each of PBS, 0.5 M urea in PBS and 4 M urea in PBS, respectively. Fractions (1 ml) were collected and analyzed for radioactivity.

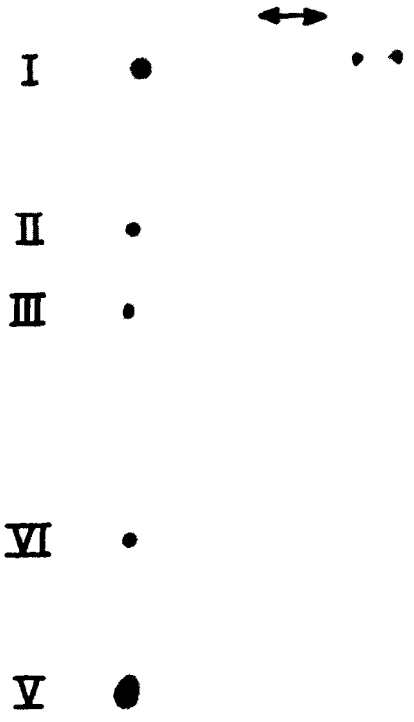

Fig.4. Gel electrophoresis of ^{3}H-labeled proteins binding to gelatin. Shown in the figure is an autoradiography of a slab gel electrophoresis of ^{3}H-labeled proteins produced by the macrophages and subsequently absorbed to Sepharose gels substituted with gelatin and anti-CIG antibodies (left track) and standard proteins (right track). The electrophoresis was carried out as in section 2. The arrow indicates the top of the gel.

ments were conducted to demonstrate the biosynthesis of CIG by macrophages grown in vitro. To this end cells were grown in a [^{3}H]leucine containing medium and after incubation of the macrophages for 48 h the medium was collected and analyzed by affinity chromatography on a Sepharose column substituted with gelatin. About 4% of the labeled macromolecules bound to the column and was eluted with 4 M urea (fig.3). Furthermore, 85% of this labelled material subsequently bound to a Sepharose substituted with anti-CIG antibodies and was shown to migrate in gel electrophoresis identically to CIG isolated from bovine plasma (fig.4).

4. Discussion

These results demonstrate that peritoneal macrophages in vitro synthesize and secrete a protein that

by immunological, biochemical and functional (binding to gelatin) criteria can be identified as CIG. CIG has been reported to affect the phagocytic function of RES by acting as an opsonin [4]. If this proves to be true one must assume that CIG is first released by the mononuclear phagocytes, then bound to appropriate particles, recognized by the cells again and ingested in complex with the particle. Such a mechanism has a parallel in the complement system where the component C_3 is reported to be produced by macrophages [17] and act as an opsonin after binding to particles and modification to C_3b [18].

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