In Vitro, Cell Protective Effects by Certain Antiproteases of Human Serum

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Although a cell growth-promoting activity is usually attributed to the serum globulin fraction [1] added as a supplement of animal tissue culture media, little information is given as to the specific role when purified protein components are used instead. The present study was undertaken to correlate the growth of an established insect cell line [2] in a serum-free medium with the antiprotease activity of certain purified human plasma proteins.

Periplaneta americana L. fibroblastic cells (EPa strain) were grown at 30 °C in 50 ml tightly closed glass flasks containing 4 ml of a medium reproducing the features of insect hemolymph [2] and including 2 – 5% of fetal calf serum. This medium assures a satisfying cell multiplication rate, but the cells have to be transferred at weekly intervals. With increasing cell density their attachment to glass is diminished; transferring is best realized when the cells are detached in the metabolized medium and then resuspended in an equal volume of fresh medium. The cells adhere again to the glass surface and the debris can be eliminated with the supernatant. An alternative is the replacement of the culture medium immediately before transferring the cells.

When our basal medium is used without any fetal calf serum, the EPa cells do not survive for long, and they cannot be transferred from one culture vessel to the other. When the medium is only supplemented by human serum albumin (0.4%), a good cell proliferation rate is obtained. However, difficulties are arising during transfer operations. The medium present during cell detachment has to be carefully eliminated together with cell debris to allow normal cell growth of the newly explanted material (washing of the intact cells may be indicated). With increasing cell density, lysis areas can be observed spreading over the whole culture. No such inconvenience is observed when 2 – 5% of fetal calf serum are added to our medium.

As part of the cells are already destroyed during transfer, it seems obvious that the cell-damaging material is liberated on that occasion. It has been shown that lysosomal enzymes play an important part in the regression of many tissues, especially with respect to their catheptic activity [3]. In insects, a chymotrypsin-like protease has already been shown in hemolymph by LAUFER [4], while LECADET and DEDONDER [5] identified a trypsin-like enzyme in *Pieris brassicae*. If proteolytic enzymes are at the origin of the observed cell culture damage, the protective effect of fetal calf serum may be attributed to the well known antiproteases of mammalian sera.

The cell protective effect of fetal calf serum can be replaced by a commercial α-globulin fraction (Mann, Calbiochem) of bovine, equine or human origin. This partly denatured preparation is not completly soluble and 0.1% of the soluble material is necessary to obtain a sufficient cell protection.

The known antiproteases of serum like α_1-antitrypsin, α_2-macroglobulin, protein π [6] (or inter-α-trypsin inhibitor [7]) as well as the inhibitor of C_1'-esterase are all α-globulins. Serum heated to 56° for decomplementation still possesses its protective capacity, whereas α_1-antitrypsin is known to be a very heat-labile protein [8] and the heat-lability of the inhibitor of C_1'-esterase is nearly as important [9]. For this reason, we limited or studies to α_2-macroglobulin (α_2-M) and protein π isolated from human plasma by techniques already published [10, 6a, 6c].

Both these proteins proved to be highly active to assure the normal maintenance of our EPa cell strain. The optimal concentration of protein π is less than 10 mg% and an even smaller amount of 3 mg% of α_2-M is sufficient to promote the expected effect. The cell protection is well correlated with the antiprotease activity of the proteins under consideration, because a similar effect can be obtained with commercial soybean trypsin inhibitor (Mann) and with a pancreatic inhibitor (Iniprol-Choay) used in equal amounts.

Furthermore a definite proteolytic activity is found in the medium present during cell detachment 1. with CBZ-α-glutamyl-L-tyrosine [11] and 2. with hide-powder labelled by Remazolbrilliant Blue (RBB-Hide) [12]. The

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medium is concentrated by ultrafiltration then dialysed prior to testing. A 16 h. incubation time is used with RBB-Hide, a higher proteolytic activity being observed at pH 5 (acetate buffer) than at pH 7.5 (phosphate buffer). α_2-M like with other proteases and unlike protein π did not inhibit the esterase activity detected by the synthetic dipeptide substrate; whereas both serum antiproteases completely inhibit the enzymatic digestion of RBB-Hide.

MARR et al. [13] described a *growth-promoting activity* of α_2-M on HeLa cell strain, HEALY and PARKER [1] observed alike a beneficial effect on mouse embryonic cells, when their culture medium was supplemented by horse α_2-M. They further noticed that α_2-M could be replaced by Dextran (M.W. 100.000 – 200.000) or Ficoll. A much higher amount of α_2-M (100 mg%) than that used in our study (3 mg%) was necessary to obtain this protective effect. This can be explained by the following reasons: the α_2-M was prepared by these authors from horse *serum* by precipitation with ammonium sulphate. Now α_2-M is not only an antitrypsin, an anti-plasmin, an anti-elastase [14] but also an antithrombin [15]. Furthermore, plasminogen is a tenacious contaminant of α_2-M which has to be carefully adsorbed during the purification process [10]. It is not surprising that our α_2-M prepared from human *plasma* by a specific technique including adsorption of plasminogen shows a higher inhibitory activity. We noticed that α_2-M shows a higher affinity for our insect cell proteolytic enzyme than did protein π, whereas this inhibitor has a very high affinity for mammalian trypsin [6b, c].

Thus, the cell-protective effect of serum added to cell cultures might tentatively be attributed to its anti-protease activity and actually our serum-free medium, which we call S 19 [16], contains human α_2-M as a normal constituent.

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Polyribosomes in Thin Sections of *Leishmania Donovani*

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Polyribosomes were first demonstrated in rabbit reticulocytes and are known to be involved in the synthesis of haemoglobin [1–3]. Electron microscopic visualisation of the polyribosomal structure in rabbit reticulocyte was made using the shadow casting technique [1] and also the negative as well as the positive staining techniques [4]. It was found that the great majority of reticulocyte polyribosomes contained five ribosomal units. The association of five ribosomal units in the form of polyribosomal complex with the synthesis of haemoglobin led to a great interest in the study of the configuration of ribosomes in other cells where many different types of protein are synthesised. In the acinar cells of pancreas where a variety of digestive enzymes are synthesised, the ribosomes were found distributed individually [5]. In liver cells, on the other hand, polyribosomal complexes containing up to twenty ribosomal units [6] and in *Escherichia coli* up to forty ribosomal units [3, 7, 8] were detected by physico-chemical techniques. Electron microscopic studies of these structures in different cells should thus be of interest.

In the course of our investigation on the ultrastructural organisation of the promastigote of *Leishmania donovani*, we noted the abundance of polyribosomal complexes in the cytoplasm of the parasite. The configuration of these complexes in thin sections of the parasite has been studied and the findings are presented in this communication.

Materials and Methods

The primary culture from a nodular lesion of a case of dermal leishmanoid (DL) was made in N.N.N. medium [9]. This was then adapted to and cultivated on the surface of RAY's medium [10]. The leptomonads were harvested, washed in physiological saline and then fixed in 4% formaldehyde in phosphate buffer (pH 7.2) at 0 – 4 °C for one hour. They were then washed

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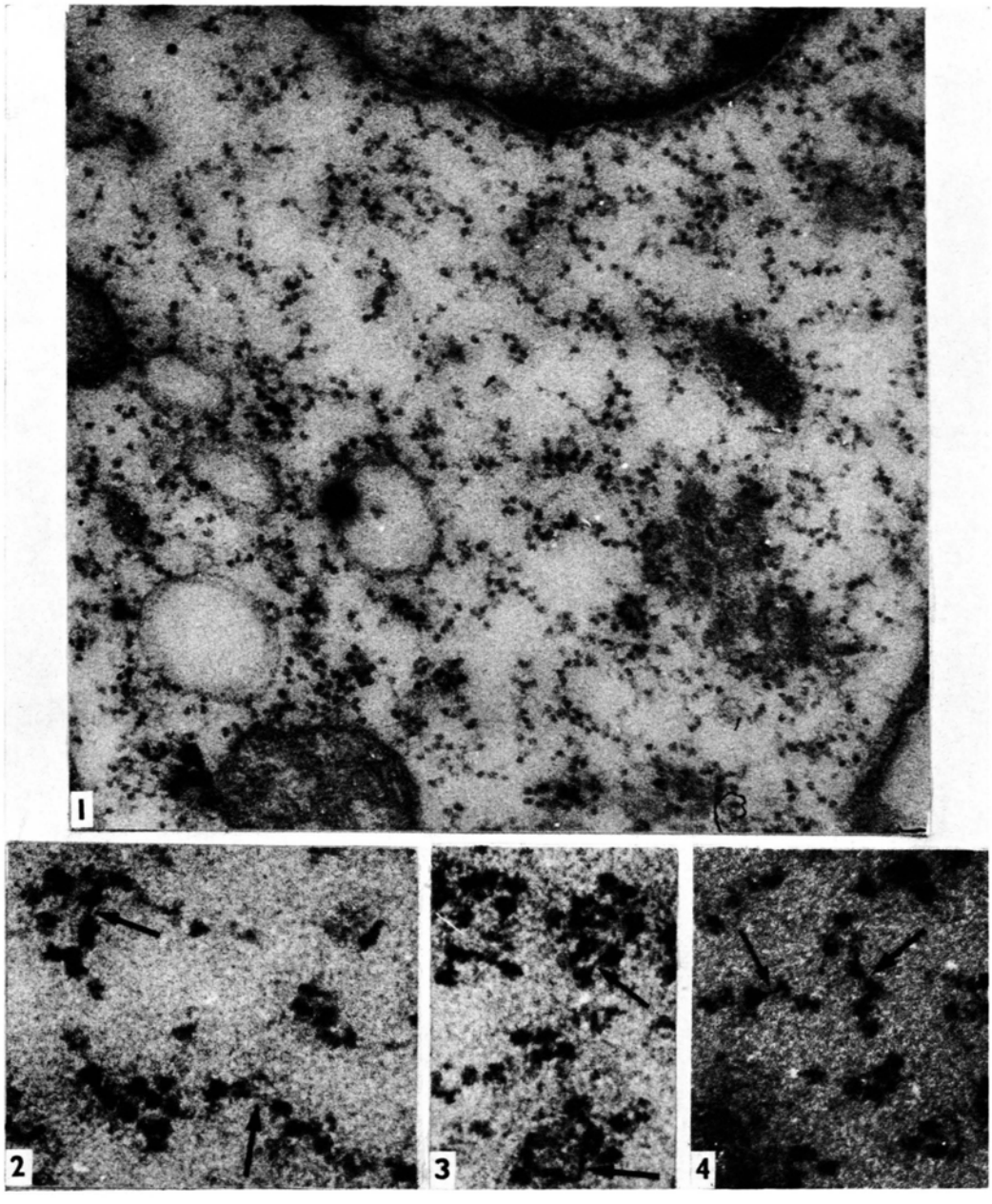

Fig. 1. Electron micrograph showing part of the nucleus and the cytoplasm of a promastigote of *L. donovani* in thin section. Abundance of polyribosomal complexes, mostly in the linear array configuration, can be seen. X 66,000.

Figs. 2, 3 and 4. Higher magnification reproductions of polyribosomal complexes in thin sections of the cell. Arrows indicate the thin thread like structures connecting the neighbouring ribosomes. X 120,000.

twice in 4.5% sucrose solution in cold and post-fixed in 1% osmium tetroxide in veronal acetate buffer for about an hour at 0–4 °C. The material was dehydrated in graded ethanol and embedded in a prepolymerised methacrylate mixture [11]. Ultrathin sections were cut with a Porter-Blum microtome, stained with 2% aqueous solution of uranyl acetate and subsequently overlaid by a thin film of carbon [12]. Electron micrographs were taken by a Hitachi HS-6 electron microscope at instrumental magnifications ranging between X10,000 and X15,000. Measurements were made from the photographically enlarged prints (X100,000 or more) by a micrometer eye piece (accuracy 0.1 mm.).

Results

In the promastigote of *L. donovani* ribosomes were found in plenty in the cytoplasm. The typical rough surfaced endoplasmic reticulum was not found in thin sections and the cytoplasmic ribosomes were seen as clusters or linear arrays which are conventionally termed as polyribosomes (Fig. 1 *). Ribosomes were also found in the nucleolus distributed individually and occasionally attached to the cytoplasmic surface of the nuclear membrane. The polyribosomes were found at no specific site in the cytoplasm. They were found immediately outside the nucleus and also beneath the pellicle of the parasite but never within or attached to the surface of any cytoplasmic organelle, e. g., mitochondria, kinetoplast etc.

The ribosomes forming the polyribosomal complexes of this parasite had an average diameter of 220 Å. Both types of configuration, clusters and linear arrays of ribosomes, were found even in the same section of the parasite. The frequencies of their occurence were comparable when a large number of polyribosomes counted from many different sections were considered. In the clusters, the number of ribosomes per polyribosomal complex was most frequently ten (Fig. 5) and the particles were often arranged as if to form a closed ring (Fig. 3). In the linear arrays, however, this number was found to be five (Fig. 6). The center to center distance of the ribosomes in the complexes was mostly found to range between 275 and 330 Å (Fig. 7). However, distances as large as 440 Å were also noted. Often a very thin thread like structure was found to connect the ribosomes in polyribosomal complexes (Figs. 2, 3 and 4).

Discussion

The presence of both types of polyribosomal configurations in *L. donovani* is of interest. SLAYTER et al. [10] also observed the presence of both types of configurations in the particulate preparations of the reticulocyte polyribosomes but suggested that the linear array might be the actual configuration *in vivo*.

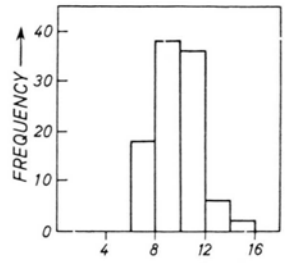

Fig. 5. Frequency distribution of the number of ribosomes per polyribosome in cluster configuration.

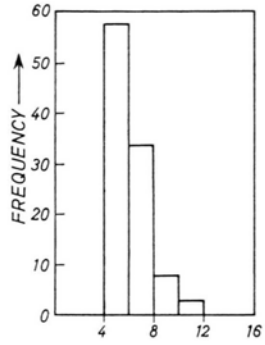

Fig. 6. Frequency distribution of the number of ribosomes per polyribosome in linear array configuration.

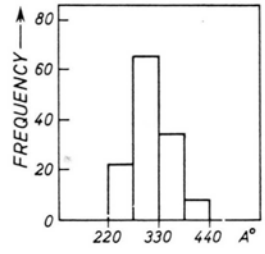

Fig. 7. Frequency distribution of the centre to centre distance of the ribosomes in polyribosomal complexes.

MATHIAS et al. [13] observed, on the other hand, that in ultrathin sections of the reticulocyte cells, the ribosomes were always in closed configurations. Whether the configuration of the complexes is specific for any functional aspect remains to be known.

The most frequent number of ribosomes found in association with each complex is ten in clusters and five in linear arrays. However, in case of ribosomes arranged in linear arrays, it is difficult to have an idea of their actual number per polyribosomal complex in thin sections because the chain may frequently go out of the plane of section. Up to twelve ribosomes have been detected in linear arrays in this investigation. It is thus

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unlikely that the most frequent number five represents the actual state of affairs in so far as the number of ribosomes present in each linear array is concerned. On the other hand it may not be unlikely that the most frequent number of ribosomes per polyribosomal complex is the same in both types of configuration in this parasite.

SLAYTER et al.[4] found that the inter-ribosomal separation in the polyribosomal complexes of rabbit reticulocytes depended to a great extent upon the conditions of specimen preparation. In the present case, the center to center distance of the ribosomes has been found to vary greatly although the most frequent value ranges between 275 and 330 Å. Apart from any other factor, the variation in the inter-ribosomal distance may occur simply because the ribosomes may easily lie in different planes in each section. It is therefore difficult to conclude whether the variation in the inter-ribosomal distance observed represents the actual state of affairs *in vivo*.

In the present investigation a very thin thread like structure has often been found to connect the ribosomes in the polyribosomal complexes. Although the chemical identity of the thread has not been established in this study, it is presumed that the thread represents portion of the messenger RNA. The thread has often been found to appear somewhat thicker than messenger RNA, but this might be due to the condensation of the uranyl stain.

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