Artifacts from agar-protein interaction simulating a bacterial growth in the haemocultures

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Summary. Some morphological, histochemical and biochemical properties of the particles taking origin from the polysaccharide-proteins interaction in the presence of platelets and lymphocytes are described.

Particles which increase in number and size in the haemocultures have frequently been wrongly interpreted as microbial forms (for review see Clasener[1] and Mattman[2]). On phase contrast examination in the course of the incubation of whole blood or of suspensions of erythrocytes or of products of haemolysis, the presence of Heinz bodies and/or particles taking origin from haemoglobin-proteins interaction may give rise to misinterpretation.

Even in the absence of erythrocytes and leucocytes, the platelets remaining in suspension in the plasma can produce, in culture media containing agar, an increasing turbidity. This turbidity is due to the formation of particles which, not only on phase contrast examination but also after the usual staining methods or electron microscopy on shadow-cast preparations or on sections, may, in the absence of a sufficiently critical evaluation, cause erroneous interpretations. We have proved that this situation can occur even with the simple incubation of platelets or lymphocytes and plasma in saline with added agar, and indicate here some data relating to the properties of these particles.

Methods. The media for the incubation carried out at 37 °C consisted of 0.9% NaCl solutions containing in various concentrations either Bacto agar Difco, or 1 of the following media: starch, agarose, jaluronic acid, chondroitin sulphate, gelatin.

To these media, various quantities of platelet-rich plasma supernatant (centrifuged at 250 × g for 10 min) of human, dog, rabbit or rat blood mixed with ACD (1:10) were added. In other tests, the following additions were used: the supernatant of the centrifugation at 4000 × g for 20 min of plasma or of non-inactivated or inactivated serum; serum albumin or serum globulin or egg albumin solutions, with or without the addition of platelets collected by means of centrifugation and washed with saline, whole or lysed with Triton × 100, or of human lymphocytes prepared by stratification on Ficoll[3]. Plasma supernatant 4000 × g was also added to the platelet suspensions incubated in agar-saline for various lengths of time at 37 °C and then heated for 20 min at 80 °C.

When, after the incubation, an uniform opacity or a stratification of the particles was seen, serial subcultures were attempted in or on various media: Difco nutrient broth and agar, Difco PPLO broth and agar, Difco brain-heart infusion, Difco trypticase soy broth and agar, Brewers thioglycollate medium (oxoid Brewers' thioglycollate broth + 0.05% oxoid agar No. 1). The solubility of the particles was checked in HCl and NaOH solutions at various concentrations, and, with the suspensions as such, preparations were made for phase contrast microscopy and for examination following staining with Giemsa, basic fuchsin, eosin, Dienes' stain, acridine orange for the evaluation of the fluorescence before and after DNase and RNase incubation. For electron microscopy, the sediments of the centrifugation at 4000 × g 20 min were either suspended in distilled water and chrome shadowed on the grid, or prefixed in glutaraldehyde, fixed in 1% OsO_4 in 0.1 M phosphate buffer pH 7.4 and embedded in araldite: ultrathin sections were stained with uranil acetate and lead acetate. The preparations were examined with a Philips EM 201 electron microscope. The electrophoretic examination in Tris-Veronal buffer pH 8.8, ionic strength 0.06, on cellulose acetate was carried out before incubation on plasma alone and on the mixture of saline, plasma, agar and platelets; and following incubation, on the supernatant after the elimination of the particles.

Results. The formation of masses of particles which remain suspended in the medium or are stratified at the bottom, or at a certain depth in the test-tube, was observed only following the incubation of preparations containing agar, plasma or serum or protein solutions, and platelets, either as such or lyzed, or lymphocytes (tests on other cells were not carried out). In the presence of plasma or serum or globulin solution, the quantity of particles seemed sensibly greater than in the presence of solutions of albumin alone. Agar with proteins in the absence of platelets or lymphocytes, agar with platelets or lymphocytes, agar with platelets or lymphocytes in the absence of proteins, and the addition of plasma to the platelet suspension in agar preincubated at 37 °C and successively heated 20 min at 80 °C did not give rise to the appearance of particles. Tests with other polysaccharides or gelatin gave negative results. The formation of the particles needed different lengths of time: from 12 to 24 h to several days. The speed of the formation of the particles was in descending order following the incubation of: plasma supernatant 4000 × g with the addition of lyzed platelets, plasma with the addition of washed platelets, platelets in suspension in their own plasma. The number and size of the particles was related to the number of platelets and to the quantity of plasma. A relevant formation of particle aggregates was obtained by incubating in the ratio 9:1:1 (v:v:v) agar 0.15% in NaCl 0.9%, plasma

Before incubation	Following incubation
Albumin 48.0;	Albumin 57.9;
Globulins: α_1 2.9; α_2 10.8;	Globulins: α_1 3.8; α_2 7.8;
β 12.3; γ 26.0;	β 8.1; γ 22.0;
Globulins/albumin 1.08;	Globulins/albumin 0.73

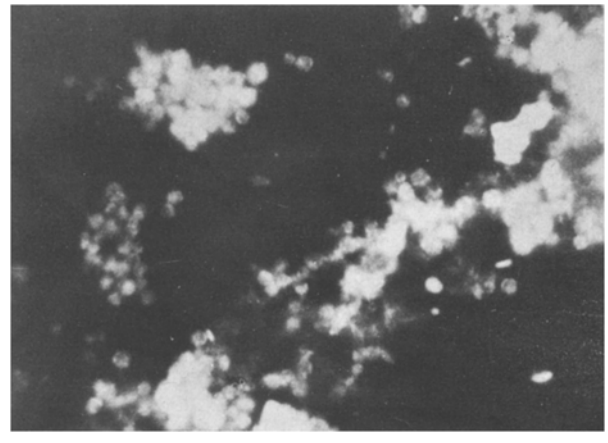
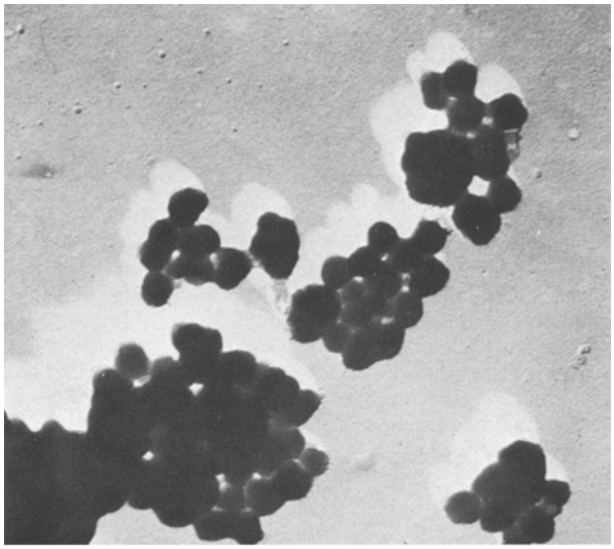
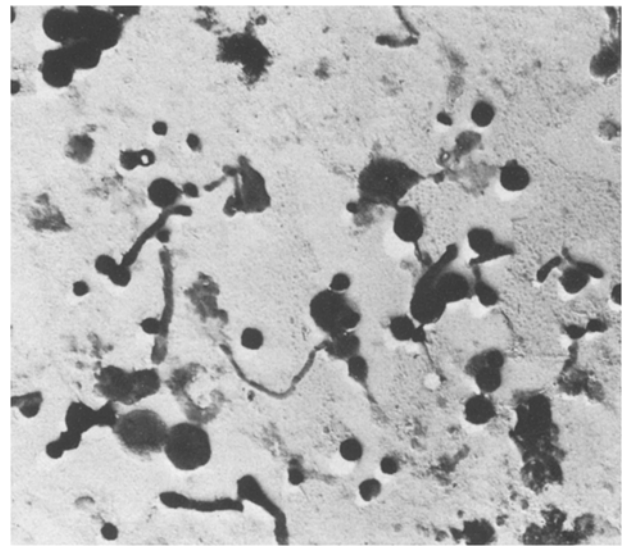
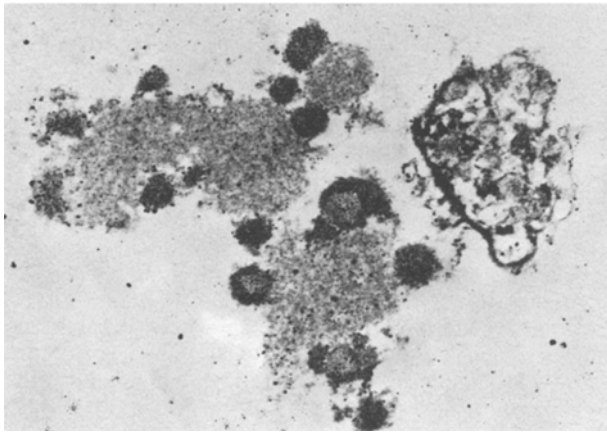


Fig. 1. Platelet rich human plasma incubated with 0.15% agar in NaCl 0.9%. UV-examination following acridine orange staining. Groups of particles showing reddish-greenish fluorescences. × 1160.







Figures 2, 3 and 4. Product of incubation as in figure 1. Particles looking like coccoidal and cell wall deficient bacterial forms in shadow-cast preparations may be recognized in ultrathin sections as aggregates of amorphous material. *2*× 22,200; *3*× 8590; *4*× 12,100.

supernatant 4000× g and NaCl 0.9% containing 2–5× 10^7 platelets collected and washed by centrifugation. The increase beyond such a limit of the quantity of plasma did not give rise to a greater production of particles.

The particles collected and washed were insoluble in acid or in 0.001 N NaOH solutions, but soluble in 0.01 N NaOH. Bringing back the solution to neutrality or to pH 3 with HCl, the material precipitated only partly and it had a gel consistency in which the particles were no longer clearly defined.

Attemps at serial subcultures of the particles in or on various culture media gave negative results. Following phase contrast examination, the particles of various sizes isolated or grouped together could be confused with evoluting bacterial forms; following fixation and staining a strong affinity for eosin was evident; with acridine orange, reddish and greenish fluorescences were detected which without the check with RNase and DNase could be attributed to RNA and DNA (figure 1).

The electron microscope examination of the shadow-cast preparations, or of not sufficiently thin sections, showed the presence of isolated or grouped, rounded or elongated, particles apparently interpretable as conventional or evoluting cell wall deficient (CWD) bacterial forms. The ultrathin sections showed without doubt that in the suspensions there were present, together with a small number of platelets undergoing lysis and setting free their own more or less deteriorated granules, various aggregates of amorphous material of different opacity under the electronic beam (figures 2–4).

Before incubation, the electrophoretic behaviour of the plasma with the addition of agar and platelets was the same as that of plasma alone. After incubation and elimination by centrifugation of the particles, the ratio total globulins/albumin decreased. The percentage data of the protein fractions relative to a typical experiment are given in the following table.

In the particles the protein content was very scarce with the prevalence of globulin fraction. This confirmed the results obtained following the incubations carried out with different protein fractions.

The results of the experiments indicate that the origin of the particles which increase in number imitating bacterial multiplication, must be recognized as the product of an interaction between the proteins and the agar dependent on an activity, probably enzymatic, related to the presence of the platelets or of cellular elements (lymphocytes). Analogously with these observations, in previous studies concerning the mesenchymal reactivity[4], it has been observed that the agar provokes a characteristic granulomatous reaction and assumes an intense affinity for eosin in the zones of confluence with the cytoplasm of syncytial cells deriving from the evolution of the histiocytes.

1 Clasener, A. Rev. Microbiol. *26*, 55 (1972).
2 L.H. Mattman, in: Cell Wall Deficient Forms, p.367. CRC Press, Inc., Cleveland, Ohio 1974.
3 A. Boyum, Scand. J. clin. Lab. Invest., Suppl. *21*, 9 (1968).
4 S. Armenio and G. Tedeschi, Tumori *39*, 147 (1953).