L- and Conventional Forms of Micrococci in the Circulating Blood of Thrombocytopenic Patients

An incorporation of ^{14}C-thymidine not related to the synthesis of mitochondrial DNA, and strongly inhibited by oxytetracycline, has been observed in suspensions of platelets from normal human subjects following incubation in various culture media in the presence of plasma or serum: such incorporation has been attributed to the metabolic activity of bacterial L-forms[1]. The growth and multiplication of L- and conventional bacterial forms (provisionally considered as micrococci) originating at the level of the platelets, has also been obserevd: the microorganisms could be subcultured in many but not in all cases[2].

Here some observations are described which concern the presence of bacterial forms in the circulating blood of patients affected by thrombocytopenia from autoimmune disease, and the bacterial growth in cultures from blood of the same patients.

Materials and methods. The assays have been repeated many times using the blood from 4 patients whose idiopathic thrombocytopenic purpura has been attributed, on the basis of the positivity of indirect antiglobulin consumption tests, to autoimmune condition. Two of them had been treated at intervals with immunodepressant drugs before and during the period where they were kept under examination, and one had been splenectomized without improvement of the thrombocytopenic situation. The platelets count in the circulating blood varied between 3000 and 30,000/µl. The megakariocytes were recognizable in the bone marrow in abnormally large number and showed evidence of hindered maturation and impaired platelet production.

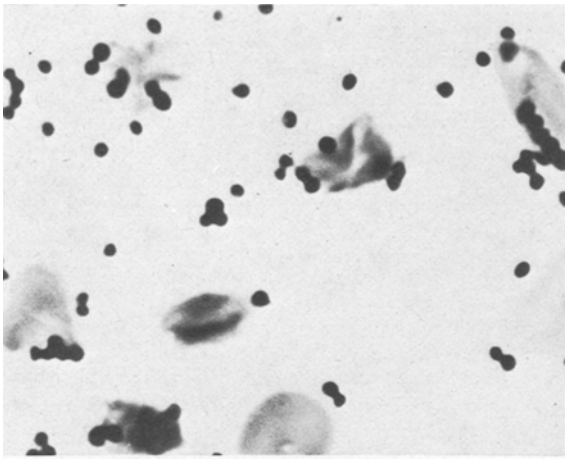


Fig. 1. Conventional bacterial forms in the fluffy layer from blood of thrombocytopenic patients. Giemsa, ×2200.

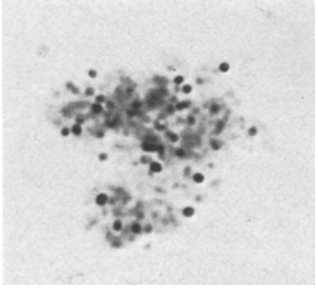


Fig. 2. Circulating platelets undergoing lysis carry bacterial forms of various size and staining affinity. Giemsa, ×2200.

After preparation of blood smears, blood specimens were collected under strictly sterile conditions, mixed with ACD (1:10) and centrifuged 20 min at 225 *g*. Following the centrifugation, samples for optical and electron microscopy were prepared from the supernatant and from the fluffy layer. For the incubation assay, carried out at 37 °C, 2 ml of the supernatant were added to 8 ml of Difco PPLO broth in Erlenmeyer flasks of 100 ml capacity; 0.2 ml of the supernatant were also scattered on Difco PPLO agar in Petri dishes 10 cm ⌀.

When bacterial growth had been verified in broth suspensions on the basis of increasing turbidity, subcultures were attempted on Difco nutrient agar. The primary cultures on PPLO agar plates were examined after 7 days incubation.

For optical microscopy, conventional methods were utilized. For electron microscopy, the material was prefixed in glutaraldehyde, fixed in 1% OsO_4 in 0.1 *M* phosphate buffer pH 7.4 and embedded in araldite; the specimens were stained with uranyl acetate and lead acetate.

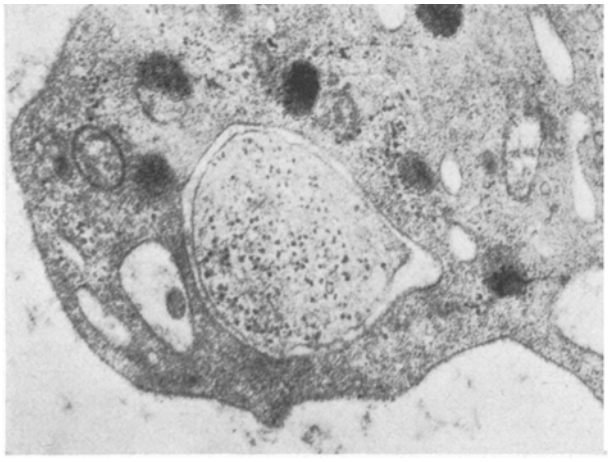
Results and discussion. The blood specimens obtained at frequent intervals through prolonged periods (1 to 3 years) from the 4 patients under study, gave constantly comparable results.

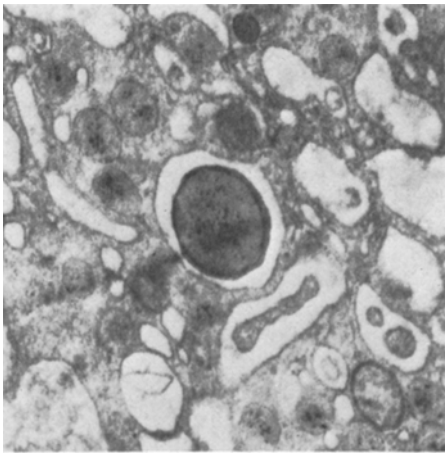
In the circulating blood, Gram-positive Cocci could easily be recognized in the smears and in relevant number in the preparations obtained from the fluffy layer; they were free (Figure 1) or phagocytized by leucocytes. Other microbial forms of various size and shape were detectable, showing variable staining affinities with fucsin and Giemsa; a yellow-green, orange or reddish fluorescence was observed following acridine orange staining (the conventional forms give a strong red fluorescence). The fact that immature microbial forms were carried by platelets often undergoing lysis was easily demonstrated by means of various staining methods (Giemsa, methyl green, acridine orange fluorescence, Figure 2). Electron microscopy confirmed the presence of L- (Figure 3) and reverting but still immature (Figure 4) bacterial forms inside some platelets, and also the presence of phagocytized forms inside the circulating leucocytes (Figure 5).

After 2 to 5 days incubation of the supernatant 225 g in PPLO broth, a bacterial growth was observed; the subcultures on nutrient agar gave constant and positive results; the variability in shape, size and staining affinity of the microorganisms disappeared in subcultures.

Primary cultures on PPLO agar plates did not give rise to colonies recognizable with the naked eye. In every case, microscopical examination demonstrated the presence of small groups of microorganisms in various stages of evolution from L- to conventional forms (Figure 6).

The presence of unstable L-forms inside the platelets cannot be related to the platelet-bacterial interaction which has been taken into consideration[3–5] as a possible mechanism of clearance of bacteria from the circulation. However, the possibility has been admitted[1,2] that after the conventional bacterial forms originating from L-forms carried by platelets are set free in the circulating blood, they may proceed in a sequence of events comparable to what some authors had interpreted as a clearing process. The large number of conventional bacterial forms free in





Figs. 3 and 4. Electron microscopy demonstrates the presence of L- (Figure 3) and reverting (Figure 4) bacterial forms inside platelets undergoing vacuolization. ×32,000.

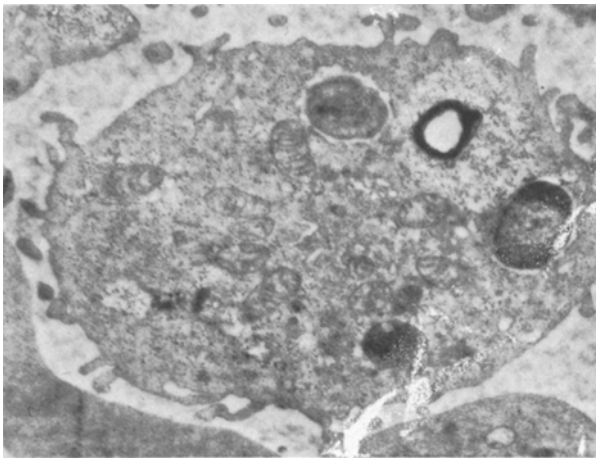


Fig. 5. Phagocytized bacterial forms are present inside the circulating leucocytes. ×18,000.

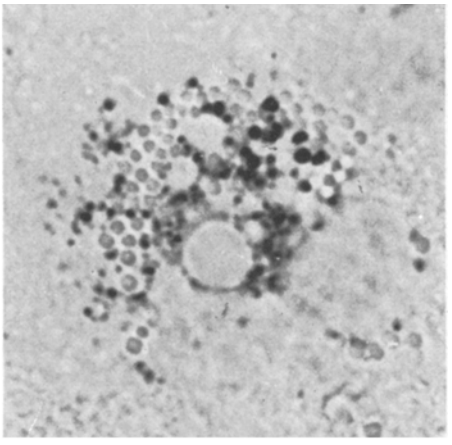


Fig. 6. Following incubation of plasma supernatant 225 *g* on PPLO agar, the evolution of microorganisms from L- to conventional forms may be recognized. Dienes staining method. ×2200.

the blood of the thrombocytopenic patients may in this way enhance the primary platelet damage deriving from the autoimmune condition.

Previous research (unpublished results) has demonstrated that the incorporation of ^{14}C-thymidine by platelets of normal and thrombocytopenic subjects is of the same order when evaluated in the presence of penicillin and referred to the number of platelets. The multiplication of a large number of conventional bacterial forms originating from the L-forms carried by a small number of platelets in the circulating blood of thrombocytopenic patients, may be interpreted as a consequence of the platelet lysis.

An observation which needs to be taken in account, and which gave rise to further research actually in progress, is the apparent lack of a specific immunological reactivity towards the conventional bacterial forms present in the circulation of the thrombocytopenic patients. No relationship has been established between splenectomy, immunodepressant therapy and the presence of bacteria in the blood.

Summary. The multiplication of Gram-positive Cocci originating from L-forms carried by platelets of autoimmune thrombocytopenic patients, may be attributed to the primary platelet damage enhanced following interaction with bacteria.

G. G. Tedeschi, D. Amici and I. Santarelli

Institute of General Physiology, University of Camerino, I-62032 Camerino (Italy), 18 April 1975.

[1] G. G. Tedeschi and D. Amici, Ital. J. Biochem. *24*, in press (1975).
[2] G. G. Tedeschi, D. Amici, I. Santarelli, M. Paparelli and C. Vitali, in *Microbial Ultrastructure*, Technical Series of the Society for Applied Bacteriology, No. 10, in press.
[3] C. C. Clawson and J. G. White, Am. J. Path. *65*, 367 (1971).
[4] C. C. Clawson and J. G. White, Am. J. Path. *65*, 381 (1971). R. A. Kahn and L. J. Flinton, Blood *44*, 715 (1974).