However, the most evident morphological differences between the 2 types of rosettes were seen in the interaction between lymphocytes and erythrocytes. In E rosettes, this consisted only of limited areas of membrane attachment between lymphocytes and sheep red cells (Figure 1). In EA rosettes, on the contrary, sensitized ox red cells appeared gathered around one pole of the lymphocyte, and numerous cytoplasmic pseudopods protruded from the red cells and came into contact with that part of the lymphocyte (Figure 2). Ox red cells which were not part of a rosette did not show pseudopods and non-rosetting lymphocytes had not the shape of uropods.

Discussion. Our observations indicate that relevant morphological differences exist between rosetting lymphocytes and lymphocytes-erythrocytes interaction in E and EA rosettes. In E rosettes, lymphocytes are grossly round in shape and the interaction between them and sheep erythrocytes consists only of limited areas of membrane attachment, as already described by others[7]. In EA rosettes, lymphocytes are mostly in the shape of uropods[6] and an evident interaction occurs between one of their poles and sensitized ox red cells. We do not yet know the significance of these morphological differences between E and EA rosettes. The morphological phenomenon of lymphocyte-erythrocyte interaction observed in EA rosettes is very similar to that described in the course of antibody-mediated cytotoxicity[2]. It is tempting to speculate that the same population of lymphocytes is responsible for the same phenomenon in the two experimental models. This population could be composed of lymphocytes with receptors for the Fc fragment of IgG. The different manifestations of the interaction between lymphocytes and erythrocytes, i.e. EA rosettes formation and antibody-mediated cytotoxicity, would depend only on the experimental conditions in which the interaction occurs. Our results confirm, on morphological grounds, that the formation of different types of rosettes is not due in all cases to the same type of lymphocyte-erythrocyte interaction. In fact, it seems likely that different morphological types of interaction are indicative of different types of links between the two types of cells.

Summary. The ultrastructural comparison between E and EA rosettes showed that, in the former, the rosetting lymphocytes are mostly round in shape and their interaction with sheep erythrocytes only consists of limited areas of membrane contact, in the latter, rosetting lymphocytes are mostly in the shape of uropods and surrounding ox red cells show pseudopods protruding towards the lymphocyte and coming into contact with it.

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The Electrophoretic Mobility of Serum Lysozyme

Since the publication of the original studies of Osserman and Lawlor[1], the unique cathodal electrophoretic mobility of human lysozyme has been considered as one of its most important characteristics. Distinct lysozyme fractions have been observed in conventional electrophoretic separations of the urinary proteins from patients with monocytic leukemia[1], but have never been found in separations of serum proteins from the same patients, even in the presence of considerably increased levels of the enzyme. The present report demonstrates that serum lysozyme has a different mobility from urinary lysozyme, perhaps as a consequence of complex formation with serum glycoproteins.

Material and methods. Serum and urine were obtained from patients with monomyelocytic leukemia, monocytosis, and hypogammaglobulinemia[2]. Lysozyme levels were determined with the lysoplate method[1] using egg white lysozyme[3] as standard.

Human lysozyme was isolated from the urine of a patient with monomyelocytic leukemia by ion-exchange chromatography in DE-52 (Whatmann) using sodium phosphate buffer, pH 6.5, 0.01 *M* for the elution of the enzyme. Lysozyme obtained by this procedure appeared to be pure by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and to have a molecular weight of 15,000 daltons[4,5].

The electrophoretic mobility of serum lysozyme was studied by obtaining imprints of the proteins separated on cellulose acetate membranes in agar plates to which a suspension of killed *Micrococcus lysodeikticus*[3] had been incorporated. For this purpose, unfixed cellulose acetate membranes were laid over the surface of the agar plates, and diffusion of proteins was allowed to proceed overnight. At the end of the incubation period, the membranes were removed and the agar plates inspected for lytic areas. All electrophoretic studies were performed with Sartorius cellulose acetate strips, using the barbital-boric acid described in a previous publication[6] and Ponceau S[3] for total protein staining, according to Neremberg[7].

Results and discussion. Routine cellulose acetate electrophoresis of concentrated urine from a patient with suspected mono-myelocytic leukemia (Z.V.) showed a distinct post-gamma fraction that raised the suspicion of lysozymuria. Lysozyme assays were consistent with this interpretation: 19 mg/ml in 65× concentrated urine (corresponding to a lysozymuria of 318 mg/24 h) and 360 µg/ml in serum. Trying to be completely certain about the identity of the urinary post-gamma fraction, we attempted to obtain an imprint of the proteins separated from serum and urine samples of this patient on a *M.*

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lysodeikticus-containing agar plate. As shown by Figure 1, a lytic area spreading from the position of the post-gamma fraction to the application point could be observed in the zone corresponding to urinary proteins. In contrast, only discrete lysis was evident in the area corresponding to serum proteins, showing a localization and shape similar to those of the γ-globulin fraction. At the time of this observation, it was thought to correspond to a non-specific lytic effect of serum γ-globulin.

Later, similar investigations were conducted in a patient with monocytosis and non-confirmed diagnosis of myelomonocytic leukemia (J.M.). No post-gamma fraction was evident in the urine, and lysozyme assays were of 12.5 µg/ml in 100 × concentrated urine (corresponding to a lysozymuria of 156 µg/24 h) and of 150 µg/ml in serum. These values were contradictory – the serum level compatible with the diagnosis of myelomonocytic leukemia, but the urinary level well within normal limits[1,8,9]. This led to the investigation of the existence of free lysozyme in serum, as a way to confirm the results of quantitative determinations. The imprint studies reproduced in Figure 2 evidenced, once again, the coincidence of lytic activity with the γ-globulin fraction.

This second observation suggested the possibility that serum lysozyme could circulate in the form of enzyme-γ globulin complexes. To explore this possibility, incubation studies were undertaken, using egg white or human lysozyme. The enzymes were incubated with normal human serum for a 3 h period at 37 °C, followed by electrophoretic separation of the mixture and study of lysozyme by the imprint technique. When high lysozyme concentrations were used (around 1,000 µg/ml) widespread lytic areas, similar to those observed with the urine of patient Z.V. (Figure 1), were obtained. This led to the lowering of enzyme concentrations to 250 µg/ml – a value within the upper range of the levels assayed in monocytic and monomyelocytic leukemias. It was then found that increasing dilution of lysozyme resulted in progressive reduction of the enzyme's electrophoretic mobility. At concentrations below 500 µg/ml free egg white lysozyme had a mobility similar to that of serum γ-globulins. The decrease in mobility was still more evident with human lysozyme, hardly moving from the application at a 250 µg/ml concentration (Figure 3). It must be noted that human lysozyme concentrations are underestimated when egg white lysozyme is used as standard, as in the present study.

Incubation of human lysozyme at an apparent concentration of 250 µg/ml with normal human serum resulted in an increase of the electrophoretic mobility of the enzyme, that became close to that of serum γ-globulin. However, as shown in Figure 3, the lytic area obtained with the mixture of normal serum and human lysozyme spread further towards the cathode than the γ-globulin fraction, stained in the reference strip. Several hypotheses

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[9] D. CATOVSKY, D. A. G. GALTON and C. GRIFFIN, Br. J. Haematol. *21*, 565 (1971).

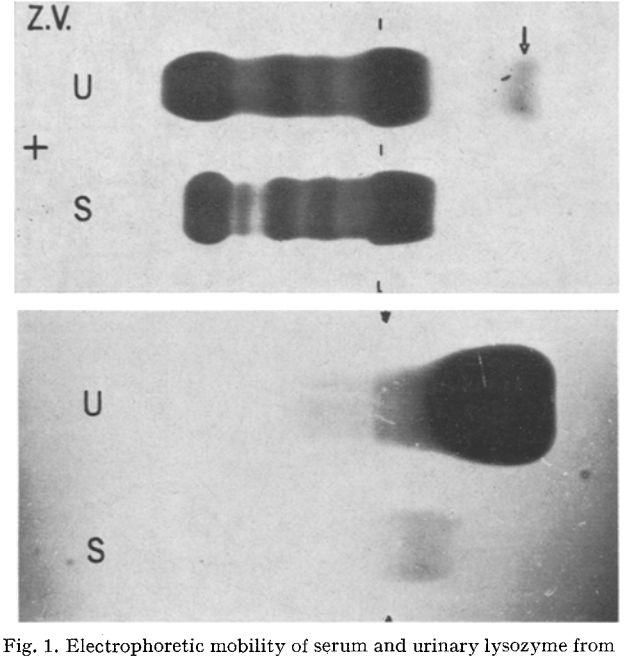

Fig. 1. Electrophoretic mobility of serum and urinary lysozyme from patient Z.V. The upper part reproduces the electrophoretic separation of serum and urine samples, stained for total protein. Application points are indicated by the short vertical lines and the arrow points to the post-gamma fraction detected in the urine. The lower part reproduces the imprint obtained from an unfixed cellulose acetate strip, where the same samples had been simultaneously separated, by laying it over the surface of an agar-*M. lysodeikticus* plate. The agar plate was photographed with indirect light over a dark background so that lytic areas appear as dark spots. Triangular notches indicate the position of the application points on the unfixed cellulose acetate membrane after it was laid over the agar plate.

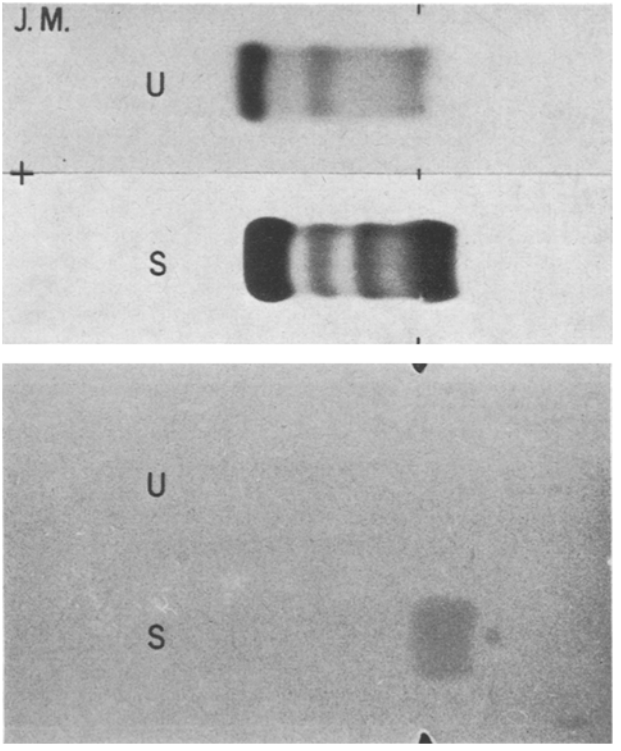

Fig. 2. Electrophoretic mobility of serum lysozyme in patient J.M. The upper part reproduces the reference cellulose acetate strips where the proteins separated from serum and urine samples were stained with Ponceau S. The lower part reproduces the imprint of a simultaneously electrophoresed strip, to which the same samples had been applied, over an agar-*M. lysodeikticus* plate. Dark spots correspond to lytic areas. Position of the application points is indicated by short vertical lines in the stained cellulose acetate strip and by triangular notches in the agar plate.

could account for this discrepancy, but the possibility that we were dealing only with a change in the electrophoretic mobility of free lysozyme resulting from dilution in a protein-rich medium could not be ruled out.

As a way to determine whether γ-globulins were or were not involved in complex formation with lysozyme, incubation studies were conducted using human and egg white lysozyme and the serum from an hypogammaglobulinemic patient (A.S., total γ-globulin level of 36 mg/ml). It was unexpectedly found that lytic activity became localized in 2 different areas (Figure 4): a major fraction of lysozyme showed α_2-mobility, while a minor part of the enzyme stayed in the γ-globulin region. Non-incubated samples of this hypogammaglobulinemic serum showed very slight lytic activity in the α_2 region. These findings suggest that lysozyme formed complexes with residual γ-globulins, but due to the low concentration of these globulins most of the added enzyme was not bound and eventually formed complexes with α_2-globulins.

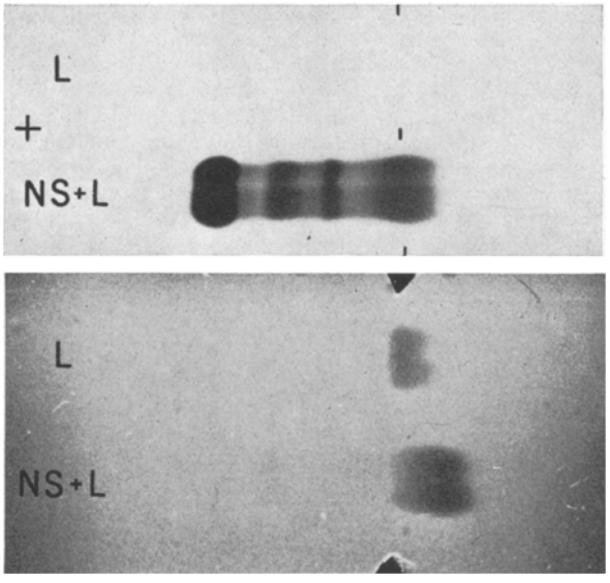

Fig. 3. Comparison of the electrophoretic mobility of free human lysozyme (L) and of the same enzyme after incubation with normal serum (NS+L). The reference cellulose acetate strip, stained for total protein, is shown above. Lysozyme is not detectable by protein stains at the concentrations employed. The imprint of an identical strip over an agar-*M. lysodeikticus* plate is shown below. Dark spots correspond to lytic areas. Position of application points is indicated by short vertical lines in the stained cellulose acetate strip and by triangular notches in the agar plate.

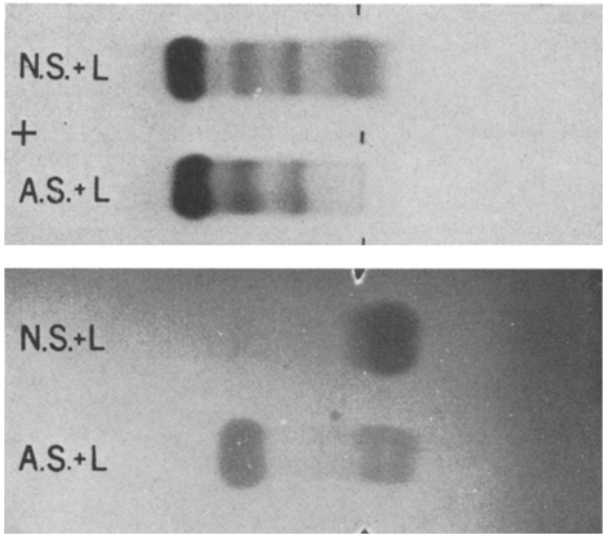

Fig. 4. Comparison of the electrophoretic mobility of human lysozyme incubated with normal serum (NS+L) or with an hypogammaglobulinemic serum (AS+L). The reference cellulose acetate strip is shown above. The imprint of an identical strip over an agar-*M. lysodeikticus* plate is shown below. Position of application points is indicated by short vertical lines in the reference strip and by triangular notches in the agar plate. Dark spots represent lytic areas.

Since one of the common features of the γ- and α_2-globulin fractions is their richness in glycoproteins, such as immunoglobulins, and considering the strong affinity of lysozyme for the N-acetyl-d-glucosamine residues[10], present in the carbohydrate side-chain of immunoglobulins[11] and in most of the characterized α_2-glycoproteins[12], the formation of complexes between lysozyme and these proteins appears as the most likely explanation for our findings. At the same time, our results suggest that complexes formed between monoclonal IgG proteins and lysozyme, as reported by FINKLE et al.[13], may result from this type of interaction rather than from an antigen-antibody reaction.

Summary. The electrophoretic mobility of serum lysozyme in 2 patients with raised enzyme levels was identical to that of γ-globulins. Similar mobility was observed after incubation of lysozyme and normal serum. Incubation with one hypogammaglobulinemic serum showed that lysozyme could also acquire α_2 mobility.

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[14] Acknowledgments. I wish to acknowledge fruitful discussions held with Dr. M. CELESTE LECHNER and the help of Dr. R. BOACKLE in the preparation of the manuscript. Ms. MARIA JULIA GONÇALVES DA SILVA and Mr. A. COSTA provided excellent technical assistance.
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[16] This work was partially supported by NIH General Research Support Grant RR054020 and by NIH Grant HD-09938. This is publication no. 15 from the Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina.