Antigen-binding cells may be studied by various techniques. The best adapted to morphological studies is the rosette test in which the antigen considered is borne by red cells. One may thus study the rosette response to sheep red blood cells (SRBC), as has been done extensively in the mouse, at both immunological and morphological levels. It has been observed in the course of these studies that the unimmunized animal presents a small background (0.3-1%) of rosette-forming cells (RFC) in the various lymphoid organs. To avoid any possible confusion, one should note at this point that mouse spontaneous RFC are very different from human spontaneous rosettes, which represent a non-antigen-specific T-cell marker present on the majority of peripheral blood lymphocytes.

Mouse spontaneous RFC include macrophages, natural antibody-producing cells, and antigen-sensitive cells. The existence of the latter cells is demonstrated by the induction of specific unresponsiveness towards SRBC induced by removing SRBC rosettes. In addition, spontaneous RFC have been used extensively for the study of the...
circulating thymic hormone (1), whose target cell is found in high concentration in normal bone marrow.

Little is known in fact about mouse bone marrow lymphoid cells. It has been established that they include lymphoid stem cells, but the morphology of stem cells and other precursor cells is still largely unknown. Hence the idea was presented of examining bone marrow spontaneous RFC which include T-precursor cells (1). The results obtained will be compared with the description of the spontaneous rosettes found in normal spleen (4).

MATERIALS AND METHODS

Bone marrow from femurs and tibias of C57BL/6 mice was suspended in Hanks' solution; 1.5 \times 10^6 cells were washed twice at 200 g for 5 min and mixed with 6 \times 10^6 SRBC (in a total volume of 0.5 ml of Hanks' solution). The tubes were then centrifuged for 5 min at 200 g and then resuspended slowly for 7 min in a roller rotating vertically at 10 rpm.

Two series were prepared: the first, with cell preparations incubated for 1 h at 37°C before being mixed with SRBC, and the other, without incubation. The cell suspension containing rosettes was fixed in 1% glutaraldehyde in phosphate buffer for 30 min and then washed in the same buffer. The weak proportion of rosettes justified their previous isolation by micromanipulation before their inclusion, as described by Reyes and Bach (4). The serial sections were collected on wide-mesh grids covered with Formvar and were then impregnated with uranyl acetate and lead citrate for examination with a Philips EM 200 electron microscope.

Rosette preparations for scanning electron microscopy were fixed in buffered isotonic glutaraldehyde and isolated as previously described. They were then placed on a glass plate and dehydrated before being passed in the critical-point drying apparatus, gold-metallized by cathodic pulverization, and observed in the scanning electron microscope (Cameca Instruments, Inc.).

70 rosettes obtained from several mice were studied in serial sections to determine the largest number of contact points between RBC and lymphocytes.

RESULTS

When cells were examined in ultrathin sections after previous incubation at 37°C, all RFC proved to be lymphocytes. However, when the suspension was done without previous cell incubation, a proportion of macrophages was observed, similar to that found in normal spleen (4). Evaluations were made on 35 RFC: the percentage of bone marrow rosette-forming macrophages was 25% vs. 75% lymphocytes.

Lymphocytes

Lymphocyte ultrastructural characteristics were well defined. The cells were small and round with a high nucleo-cytoplasmic ratio in most sections. The nucleus was large and usually deeply indented. It showed a coarse chromatin pattern, which is characteristic of interphase cells. Electron-dense heterochromatin formed heavy clumps throughout the nucleoplasm and along the nuclear membrane. The lighter area was composed of euchromatin. The nucleolus was small and could not be seen in all section planes. The cytoplasm showed few organelles, one or two mitochondria, and sometimes one centriole in the Golgi area. Ribosomes were not clustered and the Golgi apparatus was relatively small. Only a few short lamellae of the endoplasmic reticulum were seen. Ribosome density, number, and size of mitochondria and the Golgi apparatus area varied from cell to cell, even in the same section plane. These aspects are characteristic of the so-called "inactive" small lymphocytes (7). They were found in all lymphocytes present in the center of the rosettes. Some sections allowed precise determination of the contact established between lymphocytes and erythrocytes. Microspikes and/or short and fine extensions of the red cell body were attached in a small area to, in some cases, short lymphocyte cytoplasmic processes (Figs. 1, 3).

Macrophages

Macrophages were easily recognized by their extended cytoplasm containing numerous organelles. Macrophage cytoplasmic processes were found inserted between erythrocytes (Fig. 2). In several cases, lysosomes, myelin figures, phagocytized erythrocytes, and crystal-like inclusions were seen inside the cytoplasm.

In rosettes seen in scanning microscopy, the lymphocyte surface is irregular with slightly shallow undulations and rare and short microvilli. These microvilli provide contact points between lymphocytes and SRBC, with multiple contacts for each lymphocyte. Lymphocytes maintain their spherical shape, and SRBC are rarely distorted but some of them show fine filaments at the site of contact with the lymphocyte. Other aspects are also observed: lymphocytes completely covered with erythrocytes, or "caps" formed by erythrocytes collected at a pole of the lymphocytes.

DISCUSSION

Discussion of these data may be approached in
two ways: morphological and immunological. Our data show that only two cell types are responsible for rosette formation in normal bone marrow: small lymphocytes and macrophages. Macrophages are seen only when there has been no previous incubation. It should be noted, however, that in all immunological systems macrophages had been eliminated by a similar 90-min incubation at 37°C. In particular, rosettes considered in the thymic hormone studies were all examined after such incubation. It is likely that monocytes and macrophages disappear during the incubation time by adherence to plastic.

As far as macrophages are concerned, their significance is open to discussion. They may form rosettes under the influence of natural antisheep RBC antibodies or may take up red cells without the intervention of any antibody. This latter mechanism is not proven, however.

Bone marrow RFC are mainly inactive lymphocytes according to classical criteria (7) (high nucleocytoplasmic ratio, few monoribosomes, and especially nuclear aspects) in contrast to active lymphoblasts or lymphoid cells issued from cell differentiation after contact with antigens. The appearance of quiescent rosette-forming lymphocytes found in the bone marrow was similar to that reported previously for spleen spontaneous RFC (4).

In the present system the circulating thymic hormone target cells, which represent more than 70% of bone marrow RFC (1), are thus probably small quiescent lymphocytes since the totality of rosette-forming lymphoid cells examined in this work are small lymphocytes. It may be, however, that the thymic hormone acts at different stages of T-cell differentiation and that bone marrow lymphoid cells in normal animals include both primitive precursors and cells already somewhat differentiated in the T-cell lineage, as suggested by several pieces of evidence (2, 5, 6). Indeed, it has been shown by Stutman that normal bone marrow contains post-thymic cells which are capable of differentiating into competent T-cells under the
Details of rosette with small lymphocyte. In the lymphocyte cytoplasm: Golgi area ($G$) and mitochondria ($M$). Facing the lymphocyte are fine erythrocyte pseudopodia that come into contact with the lymphocyte membrane. Some of the pseudopodia appear to be cut in transverse sections forming isolated small fragments (arrows). $\times$ 35,000.
mere influence of a thymus graft insert in a Millipore chamber (Millipore Corp., Bedford, Mass.). One should note in that context that Stutman's post-thymic cells are relatively large and rapidly dividing cells (6), and that other data to be published indicate that autologous RFC, which are probably markers of immature T-cells (3), do show some younger aspects than do the quiescent lymphocytes studied here (in preparation). One may hope that work in progress will help to determine more precisely the relationships between circulating thymic hormone target cells (bone marrow RFC) and other prethymic cells (4).

SUMMARY
Mouse bone marrow contains spontaneous rosette-forming cells (RFC) which include more than 70% T-cell precursors, as assessed by their transformation into theta-positive cells after incubation with thymic hormone. Such spontaneous RFC, examined in C57Bl/6 mouse bone marrow by electron and scanning electron microscopy, have consistently been shown to be small, inactive mouse lymphocytes when macrophages have been eliminated by cell preincubation. These data suggest that thymic hormone target cells include small quiescent lymphocytes.

The authors wish to thank Dr. F. Reyes and M. Prenant for their advice and assistance. They also thank E. Lallemand, I. Andrianarison, M. Lillie, and I. Muller for their technical assistance.

Received for publication 1 July 1976, and in revised form 4 November 1976.

REFERENCES
1. Bach, J. F., M. Dardenne, J. M. Pleau, and M. A. Bach. 1975. Isolation, biochemical characteristics and biological activity of a circulating thymic hormone in the mouse and in the human. Ann. N. Y. Acad. Sci. 249:186–210.
2. Bach, J. F., H. Cantor, G. Roelants, and O. Stutman. 1975. T-cell subsets: terminology problems. In Biological Activity of Thymic Hormones. D. W. Van Bekkum, editor. Kooyber Scientific Publications, Rotterdam. 159–169.
3. Charriere, J., and J. F. Bach. 1975. Binding of autologous erythrocytes to immature T-cells. Proc. Natl. Acad. Sci. U. S. A. 72:3201–3205.
4. Reyes, F., and J. F. Bach. 1971. Rosette forming cells in the unimmunized mouse: morphological study with phase and electron microscopy. Cell. Immunol. 2:182–198.
5. Roelants, G. 1974. Quantification of antigen-specific T and B lymphocytes in the mouse. Nature (Lond.). 236:252–254.
6. Stutman, O. 1975. Humoral thymic factors influencing post-thymic cells. Ann. N. Y. Acad. Sci. 249:89–115.
7. Zucker-Franklin, D. 1969. The ultrastructure of lymphocytes. Semin. Hematol. 6:4–10.