Characterization by Immunoperoxidase of the Lymphocytes With Bundle-Shaped Tubular (BST) Inclusions in Human Normal Peripheral Blood

By Marie-Dominique Appay, Jean Bariety, and Rosine Bretton

In six normal subjects, no surface immunoglobulins were detected on blood lymphocytes containing bundle-shaped tubular (BST) inclusions, after incubation with anti-human IgM peroxidase-labeled Fab\textsubscript{2} fragments. These Fab\textsubscript{2} fragments from pepsin-digested sheep anti-human immunoglobulins revealed human \(\mu\), \(\kappa\), and \(\lambda\) chains. These results suggest that the BST inclusions do not belong to B lymphocytes but belong to T cells and/or a third lymphocyte population, including K cells and precursors of T and B cells.

**Materials and Methods**

The lymphocytes and mononuclear cells of six healthy persons were investigated. In order to test the specificity on cells of the used conjugate, the blood of one patient with Sezary's syndrome and the blood of one patient with chronic B-lymphoid leukemia were investigated. The circulating lymphocytes of Sezary's syndrome had the distinctive morphology and the membrane features of thymus-derived T lymphocytes. In fact, the blood of the patient with Sezary's syndrome contained 90% T cells identified by the sheep erythrocyte rosette method using optical microscopy.

**Anti-IgM Antibodies**

The sheep anti-human IgM antiserum was provided by Dr. Druet. Human IgM used for immunization was isolated from a pool of human sera rich in polyclonal IgM. Pure sheep anti-human IgM antibodies were isolated as previously described.

The Fab\textsubscript{2} fragments of sheep anti-human IgM immunoglobulins were prepared using pepsin digestion. After precipitation by a 40% ammonium sulfate solution, Fab\textsubscript{2} fragments were separated from nondigested immunoglobulins by gel filtration through a Sephadex G-100 column. The fraction containing Fab\textsubscript{2} fragments (second peak) was concentrated and coupled with activated peroxidase. Fab\textsubscript{2} fragments were chosen to avoid antibody fixation on the Fc receptor of some T lymphocytes and also because, in preliminary experiments, the cell labeling with Fab\textsubscript{2} fragments was found to be better than with Fab fragments.

After peroxidase coupling, it was ascertained by Ouchterlony's method that the conjugate revealed human \(\mu\), \(\kappa\), and \(\lambda\) chains.

**Normal Sheep Fab\textsubscript{2} Fragments**

Thirty-two milliliters of pooled normal sheep sera were precipitated by a 40% ammonium sulfate solution, digested by pepsin, separated from nondigested immunoglobulins by gel filtration through a Sephadex G-100 column, and coupled to activated peroxidase. The pepsic fragments were tested against an anti-IgG sheep rabbit antiserum provided by Dr. Druet.

**Blood Cells**

Sixty milliliters of venous peripheral human blood were collected onto 12 IU/ml of lyophilized calcium heparinate (Choay, Paris). The blood was diluted with 60 ml of freshly prepared Hanks' balanced salt solution (HBSS) (Institut Pasteur, Paris) and treated as previously described by Büyum. The pellet of mononuclear cells was washed 3 times with 35 ml of HBSS and fixed in 1.25% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for 0.5 hr at 4°C. The cells were separated into 3 samples. The first sample was postfixed in a 2% OsO\textsubscript{4} phosphate buffer 0.1 M, pH 7.4, solution dehydrated in graded ethanol solutions infiltrated with an Epon-propylene oxide mixture at room temperature and embedded in Epon. Ultrathin sections of the cells were stained with uranyl acetate and lead citrate and examined with an EM 10 Zeiss electron microscope to determine the percentage of cells containing the BST inclusions. The second sample was incubated with Fab\textsubscript{2} fragments of normal sheep immunoglobulins and used as control. The third sample was incubated with the Fab\textsubscript{2} fragments of sheep anti-human immunoglobulins.

**Detection of Surface Immunoglobulins**

Ten million fixed cells were incubated in a 37°C water-bath in 0.5 ml of labeled anti-IgM Fab\textsubscript{2} for 1 hr. The cells were resuspended every 5 min during the incubation. They were washed 3 times with HBSS, fixed again in 1.25% glutaraldehyde in phosphate buffer 0.1 M, pH 7.4, washed 3 times, and put overnight in the same buffer. The next day, the cells were incubated in a mixture of 3,3'-

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diaminobenzidine tetrahydrochloride (Sigma, Grade II) 2 mg/ml in Tris HCl buffer 0.2 M, pH 7.6, and hydrogen peroxide (Merck) at a 1% final concentration and washed 3 times in the phosphate buffer. The cells were postfixed in a 2% OsO_4-phosphate buffer 0.1 M, pH 7.4, solution, dehydrated in graded ethanol solution, and embedded in Epon. Ultrathin sections were counterstained with uranyl acetate and lead citrate, or not counterstained at all. At least 200 cells were examined in each case.

RESULTS

In each of the six normal studied subjects, BST inclusions were observed in at least 20 lymphocytes. The BST inclusions were not detected in the lymphocytes with continuously labeled plasma membrane (Fig. 1). They were always detected in lymphocytes with a completely negative plasma membrane (Fig. 2).

The BST inclusions were not observed in monocytes. In the patient with B-lymphoid leukemia, all the lymphocytes were labeled. In the patient with Sezary’s syndrome, all the cells with ultrastructural pattern of Sezary’s cells were completely negative. In these two patients, no BST inclusions were observed.

DISCUSSION

The cell specificity for the surface immunoglobulins of the labeled anti-human IgM Fab_5 fragments was ascertained by the labeling of all the lymphocytes in the patient with chronic B-lymphoid leukemia and by the no-labeling of the Sezary’s cells in the patient with Sezary’s syndrome.

Our results show that the BST inclusions do not belong to cells covered with immunoglobulins (presumably B cells) but that they belong to nonimmunoglobulin-bearing cells, i.e., T cells and/or K cells and/or precursors of T and B cells. Payne et al. have observed BST inclusions both in E-rosette-forming cells, i.e., T cells, and in EAC-rosette-forming cells, but they could not conclude if the BST inclusions belong to B or K cells, since EAC-rosette-forming cells can be either B or K cells.

On the other hand, our results are consistent with those of Brunning et al., who demonstrated that BST inclusions are present in over 90% of the lymphocytes of a patient with chronic T-lymphoid leukemia (94% T lymphocytes). In another patient with chronic B-lymphoid leukemia, no B lymphocyte was found to contain BST inclusions.

Our data suggest that the BST inclusion is a natural marker of lymphocytes without surface immunoglobulins. Additional studies are necessary to further type the nature of BST inclusions containing lymphocytes.
Fig. 2. Normal human lymphocyte without labeling (incubation in the same way as in Fig. 1). Note the bundle-shaped tubular inclusions (arrows) (uranyl acetate, lead citrate, × 21,000). Inset: A higher power view of Fig. 2 BST inclusions, (1) in longitudinal section and (2) in cross-section (uranyl acetate, lead citrate, × 50,000).

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