Immune Lysis of Normal and AET-treated Lymphocytes: A Study by Transmission and Scanning Electron Microscopy

By GIORGI LAMBERTENGHI-DELILIERS, SOLDANO FERRONE, TULLIO RANZI, AND GIROLAMO SIRCHIA

A morphologic study of normal and AET-treated lymphocytes exposed in vitro to cytotoxic antibody and complement (C) has been carried out using both transmission and scanning electron microscopy. The abnormalities observed were of the same kind for the two types of cell, but they were more marked in AET-treated lymphocytes than in their untreated counterparts. These results confirm the serologic finding that AET-treated lymphocytes possess a higher than normal susceptibility to the damaging action of antibody and C in the lymphocytotoxicity reaction.

THE LYMPHOCYTOTOXICITY TEST is an immune lysis reaction widely used for the purpose of tissue typing for organ transplantation. In this respect a major concern is the false negative results of the reaction that sometimes occur because of anticomplementarity of the antisera or other causes that make the antigen-antibody (Ag-Ab) reaction ineffective in determining cell lysis. This drawback could be overcome, at least in part, by increasing the sensitivity of the test system. For this purpose Mittal et al.1 used ficin-treated lymphocytes, and Yunis et al.2 used cells treated with neuraminidase. Recently in this laboratory3 it has been observed that in vitro treatment with the sulfhydryl compound AET (2-aminoethylisothiouronium bromide) also renders normal human lymphocytes more sensitive than untreated cells from the same subject to the lytic action of Ab and complement (C) in the dye-exclusion lymphocytotoxicity test, as indicated by an increment of both the percentage of stained cells and the antiserum titers. Moreover, some antisera giving false negative results with untreated lymphocytes react readily with the same cells treated with AET. These observations prompted an investigation into the appearance of normal and AET-treated human lymphocytes exposed to cytotoxic Ab and C by transmission and scanning electron microscopy to determine whether the above serologic observations could find a morphologic counterpart.

MATERIALS AND METHODS

Two normal subjects were employed as a source of lymphocytes and human C.

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Fig. 1.—Untreated lymphocytes. × 5,700.
Lymphocyte isolation and treatment of the cells with AET were performed as previously described. ABO compatible fresh serum containing leukocyte cytotoxic isoantibodies was obtained from a polytransfused patient. When tested in the dye-exclusion cytotoxicity test described by Engelfriet and Britten it gave a percentage of colored cells varying from 50% to 95% according to the lymphocytes used. The antiserum did not contain leukoagglutinins.

The suspensions of both the untreated and AET-treated lymphocytes were incubated for 1 hr at 37°C with the antibody-containing serum and, for control, with autologous normal serum.

For transmission electron microscopy (TEM), specimens were prepared as follows. At the end of the incubation period lymphocytes were washed three times with saline. After the last washing, the cell pellet less than 1 mm in depth was covered with a cold 2% solution of glutaraldehyde. Glutaraldehyde Biological Grade 50% obtained from Fisher Scientific, New York, N. Y.) in Millonig’s phosphate buffer pH 7.4. After a 20 min contact with the fixative, the pellet was gently detached from the bottom of the tube and cut into small pieces. These were kept in the cold glutaraldehyde solution for another 15 min, briefly rinsed with phosphate buffer, pH 7.4, supplemented with 0.54 g of sucrose/100 ml, then placed for 60 min in a cold 2% solution of osmium tetroxide in the supplemented phosphate buffer. The samples were rinsed with cold Ringer’s solution for 10 min and post-treated with a cold 0.5% aqueous solution of uranyl acetate adjusted to pH 5.0 with 1 N NaOH and supplemented with 4.5 g of sucrose/100 ml. After 60 min the samples were dehydrated first through a graded series of alcohols then with propylene oxide and finally embedded in Epon 812 according to Luft. Thin sections were cut with an L. K. B. Ultrotome equipped with a diamond knife, double-stained with uranyl acetate and lead citrate, stabilized by evaporation of a thin-carbon film, and examined with a Hitachi H-11A electron microscope at 75 kV.

For scanning electron microscopy (SEM), specimens were prepared as follows. At the end of the incubation period lymphocytes were washed three times with saline. To the cell pellet a cold 2% solution of glutaraldehyde in Millonig’s phosphate buffer was added, and the cells were immediately resuspended with a Pasteur pipette. Lymphocytes previously incubated with the antiserum did not resuspend uniformly in the fixative. In fact they formed clumps that were more evident for AET-treated than for untreated lymphocytes and could be only partially dissolved. After fixation for 30 min, distilled water was used to wash the cells three times and to resuspend them; one drop of this suspension was allowed to dry on a 10 mm diameter cover glass and was then coated with gold-platinum in a Hitachi vacuum chamber. The specimens were examined in a “Stereoscan” electron microscope (Cambridge Instruments, Model 2A) at an angle of 45° at 20 kV using Tri-X Kodak film.
Fig. 3.—Untreated lymphocytes incubated with cytotoxic antibody and complement. In addition to normal cells, some lymphocytes (A) show rounded nucleus in which heterochromatin predominates and is (Legend continued on facing page.)
RESULTS

Untreated Lymphocytes

TEM showed about 90% of the cell population to be made up of lymphocytes (Fig. 1). These exhibited an indented nucleus with heterochromatin mainly clumped along the nuclear membrane and some interchromatin granules scattered through the interchromatinic areas. The nucleolus was small and could not be seen in every section; the perinuclear space was narrow. In the cytoplasm the ribosomes were mainly free, the Golgi apparatus was small, the mitochondria were generally grouped at one pole of the cell, and rough endoplasmic reticulum profiles were rarely seen. The cytoplasmic membrane was always intact and showed projections.

SEM showed the lymphocytes to be evenly distributed. They had a roughly spherical shape and a coarse surface with many blebs (Fig. 2), as previously reported by other authors.14-16

AET-Treated Lymphocytes

The morphology of these cells did not differ in any respect from that of untreated lymphocytes, both under TEM and SEM.

Untreated Lymphocytes Incubated With Antiserum

Under TEM a proportion of lymphocytes revealed degenerative changes (Fig. 3). In some of these cells the nucleus had a round shape with a predominance of heterochromatin substance condensed into compact masses. In the interchromatinic areas numerous interchromatin granules and, in a few cells, bundles of fibers were seen. The perinuclear space was dilatated and occasionally expanded into large sacs. The cytoplasm was electron transparent because

![Fig. 4.—Untreated lymphocyte injured by cytotoxic antibody and complement. Cell surface is smoother than normal and shows irregular craters. × 10,000.](image)

more compact than normal, dilatation of perinuclear space (S), degeneration of cytoplasm, gaps in cytoplasmic membrane. In other lymphocytes (B) alteration of nucleus and cytoplasm seem more marked; note condensation of nucleolus (arrows). × 5,700.
Fig 5.—AET-treated lymphocytes incubated with cytotoxic antibody and complement. Degenerative changes of nucleus and cytoplasm are visible in every cell. Much cellular debris is evident in extracellular space (E). × 5,700.
of the almost complete absence of the ribosomes. The mitochondria were swollen and their cristae fragmented. Rough endoplasmic reticulum profiles were not observed. In the majority of these degenerated lymphocytes small electron-opaque bodies were seen, usually in close contact with the membranous systems. The cytoplasmic membrane had no projections and presented some gaps. Other lymphocytes showed even more marked abnormalities, with more evident alteration of nuclear structure, nucleolar condensation, and extensive fragmentation of the cytoplasmic membrane.

Using SEM, small cell clumps were observed in which most of the lymphocytes were joined by a threadlike material. A proportion of both clumped and isolated lymphocytes (Fig. 4) showed an abnormal shape, irregular craters, and cracking of the surface; in some cells the surface appeared smoother than normal. Collapsed cells and cellular debris were also seen, although rarely.

**AET-Treated Lymphocytes Incubated With Antiserum**

TEM showed marked degenerative changes in almost every cell (Fig. 5). The chromatin condensation, the intranuclear fibers, the dilatation of the perinuclear space, the absence of the ribosomes, the swelling of the mitochondria, the presence of small electron-opaque bodies close to the membranes, and the fragmentation of the cytoplasmic membrane were of the same kind as those previously described for untreated lymphocytes incubated with the antiserum. However, the degenerative changes were more pronounced and present in a far higher proportion of cells. Cellular debris, mitochondria, vacuoles, and "naked" nuclei were present in the extracellular space.

SEM showed cell clumping to be extensive; inside the clumps lymphocytes were embedded in amorphous material so that a large part of their surface was hidden, and evaluation of their abnormalities was difficult. Nonclumped
cells showed alterations (Figs. 6, 7) ranging from craters and cracking of the surface to gross deformation and collapse of the cell. Much cellular debris was visible (Fig. 6).

**DISCUSSION**

The lymphocytotoxicity test is an immune cytolysis reaction causing lymphocytes to undergo morphologic alterations made visible in detail in the present investigation. Under SEM, lymphocytes damaged by Ab and C show definite abnormalities of cell surface, shape, and size. Although probably an artifact induced by glutaraldehyde, their clumping could be an expression of membrane alteration caused by Ab and C. In fact this phenomenon was not shown by lymphocytes, both normal and treated with AET, not exposed to the antiserum. Under TEM, the damaging action of Ab and C on normal lymphocytes is more evident; moreover, it does not appear to be limited to the membrane, since alterations of nucleus and cytoplasm were also seen. These may be considered as a pyknotic type of cell necrosis. Similar changes have previously been observed by Walford et al.\(^{17}\) in similar experimental conditions, and by Click et al.\(^{18}\) in human granulocytes incubated with rabbit antihuman granulocyte agglutinins. However they have also been observed in nonimmunologic conditions,\(^{19,20}\) so that they may represent a general consequence of cytoplasmic membrane injury rather than a specific effect of antisera.\(^{18}\)

In addition to the above results on the morphology of normal lymphocytes undergoing immune lysis, the present investigation has made visible in detail AET-treated lymphocytes acted upon by cytotoxic Ab and C. These cells exhibit striking abnormalities. Under SEM, they affect, in various degrees and combinations, the surface, shape, and size of almost every cell; cell clumping is
also more marked. Under TEM, not even a single AET-treated lymphocyte escaped immunologic injury, and the alterations are extreme, making the cells notably different from their immunologically injured untreated counterparts on a quantitative ground.

It is difficult to say to what extent the morphologic abnormalities displayed under TEM and SEM by untreated and AET-treated lymphocytes exposed to cytotoxic Ab and C are due to a technical artifact occurring during specimen preparation. However, the conclusion seems to us inescapable that AET-treated lymphocytes possess a higher than normal susceptibility to the damaging action of Ab and C in the lymphocytotoxicity reaction. This confirms what has been previously observed at a serologic level.

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