RAPID COMMUNICATION

Cytoplasmic Transfer Between Endothelium and Lymphocytes:

Quantitation by Flow Cytometry

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Interactions between human lymphocytes and vascular endothelial cells in coculture have been examined using a newly developed fluorescence flow cytometric technique. Endothelium and blood lymphocytes are capable of bidirectional transfer of a cytoplasmic fluorescent dye, suggesting a novel path of communication between circulating blood cells and the vessel wall. This method also may be applicable to the analysis of other heterocellular interactions. (Am J Pathol 1988; 132:406-409)

CELL-MEDIATED IMMUNE inflammation depends on the recruitment and local activation of T lymphocytes. T lymphocyte activation is, in turn, dependent on signals delivered by nonlymphocyte cell types, collectively called immune accessory cells. Accessory cells perform a number of critical functions, including processing and presenting antigens in a form recognizable by the T cell antigen receptor and generation of various T cell activating factors (eg, interleukins 1 and 6).1

Although accessory cells have traditionally been considered to be of bone marrow origin (eg, macrophages, dendritic cells, B lymphocytes), fixed tissue cells can also influence the T cell activation process.2 In particular, vascular endothelial cells (EC) are uniquely positioned to both activate circulating T cells and to recruit such T cells into a developing immune response. Furthermore, EC, at sites of immune inflammation, also appear to be activated by the infiltrating cells.3,4

It has long been appreciated that EC communicate among themselves and with other cell types (eg, vascular smooth muscle) by formation of intercellular gap junctions5-7 that permit transfer of small cytoplasmic molecules and membrane potential. The present study examines the possibility that EC could also communicate with lymphocytes in a similar fashion. It was found that human peripheral blood lymphocytes (PBL), while bound to EC, participate in bidirectional exchange of low molecular weight cytoplasmic molecules with these cells, but not with the canine epithelial cell line MDCK. Furthermore, T cell populations are enriched for cells able to participate in such transfer.

For these experiments, a new technique was developed that allows for the evaluation of bidirectional fluorescent dye transfer between two cell populations. Briefly, one population of cells is labeled with the membrane permeable fluorescent dye 2',7'-bis(car-

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boxyethyl)-5 (and -6) carboxyfluorescein acetoxymethyl ester (BCECF-AM, Molecular Probes, Eugene, OR). Intracellular esterases rapidly cleave the molecule to the membrane impermeant acid (BCECF, molecular weight 520), which is trapped in the cytoplasm and can be measured fluorimetrically. To detect transfer, labeled cells are cultured with unlabeled cells and, at predetermined intervals, suspended and examined on a fluorescence flow cytometer (FACS Analyzer, Becton Dickinson, Mountain View, CA) for fluorescence intensity and for other parameters permitting distinction between the cell populations. This method is quantitative in that the percentage of the unlabeled population that acquires dye may be determined; however, this measurement will be affected if cells with acquired dye can redistribute it through homotypic interactions.

Materials and Methods

Cell Culture

EC were isolated from human umbilical vein by collagenase treatment and serially subcultured. EC from subcultures 3 through 9 were used in these experiments. Saphenous vein EC were provided by Dr. Peter Libby (Department of Medicine, Tufts University School of Medicine) and were cultured in the same way as umbilical vein EC. MDCK cells (American Type Culture Collection, Rockville, MD) were cultured according to instructions of the distributor.

Transfer from PBL to Monolayers

Peripheral blood mononuclear cells, isolated from heparinized venous blood obtained from donors after informed consent, were separated on Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) and labeled in RPMI 1640 (Gibco, Grand Island, NY) containing 1.5 μM BCECF-AM for 30 minutes at 37°C in 5% CO₂-air. Control cells were mock labeled. Cells were washed twice and then adherence-depleted of monocytes for 60 minutes. Adequacy of monocyte depletion was determined by quantitating the number of residual PBL expressing Leu M3, a monocyte/macrophage differentiation antigen (Becton Dickinson) by FACS analysis. Typically, less than 2% Leu M3 positive cells remained. Monocyte-depleted PBL were collected, washed, and added to confluent EC or MDCK monolayers (in Corning 2.0 sq cm wells, Corning, NY) in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Gibco). Serially-passaged human umbilical vein EC cultures received 16 × 10⁵ PBL/well whereas MDCK received 56 × 10⁵ PBL/well to maintain a constant PBL:EC or MDCK ratio of about 15:1. In some wells, BCECF 0.09 μM was added to unlabeled EC and PBL; at this concentration of BCECF-AM, EC become brightly labeled. Cells were then cocultured for 4 hours at 37°C in 5% CO₂-air, after which nonadherent PBL were removed by washing twice with CA²⁺/Mg²⁺-free Hank’s Balanced Salt Solution (HBSS, Gibco). Residual cells were made into unicellular suspensions with trypsin-versene (Gibco), washed twice in RPMI 1640, and examined on the FACS Analyzer. Electronic volume gates were set (using unlabeled PBL, EC, and MDCK as standards) to completely exclude PBL are much smaller than EC or MDCK.

Transfer from Monolayers to PBL

Confluent EC and MDCK monolayers in 2.0 sq cm wells (Corning) were washed three times with RPMI 1640 and then labeled with 1.5 μM BCECF-AM for 30 minutes at 37°C in 5% CO₂-air. Control monolayers were mock labeled. Monolayers were then washed 3 times, incubated 1 hour in RPMI 1640 containing heat-inactivated 10% fetal bovine serum to permit leakage of dye not de-esterified, then washed once again before use. PBL (prepared as above) were added to the washed EC monolayers at 2 × 10⁵/well and to MDCK monolayers at 8 × 10⁵/well to maintain a constant PBL:EC or MDCK ratio of about 1:5. BCECF was added to some wells at 0.09 μM; at this concentration of BCECF-AM, PBL become brightly labeled. After coculture for 3 hours at 37°C in 5% CO₂-air, unattached PBL were removed, and the monolayers and residual bound PBL were washed twice with HBSS and made into unicellular suspensions with trypsin-versene. All samples were then washed twice in RPMI 1640 and analyzed using the FACS. Electronic volume gates were set on the PBL population by analyzing control PBL, EC, and MDCK to exclude completely the much larger EC or MDCK. The PBL were analyzed for acquisition of fluorescence.

Results

Figure 1 shows that PBL were able to transfer BCECF to EC monolayers. The following criteria support this conclusion: 1) acquisition of dye by EC was not due to EC labeling by BCECF that had leaked from PBL because adding to PBL-EC cocultures of a BCECF concentration equimolar to a BCECF-AM concentration sufficient to label PBL 10-fold more intensely than recipient EC failed to result in any increased EC fluorescence; 2) EC did not artifactually appear fluorescent by adsorbing fluorescent PBL because the net fluorescence intensity of individual labeled PBL was 5-10-fold greater than that of the EC.
to which dye had transferred (whereas EC coupled to PBL would of necessity be at least as fluorescent as an unbound labeled PBL); and 3) PBL transfer of dye to EC appears to have some specificity as no dye was transferred to MDCK cells. These observations confirmed the results of pilot experiments by a less quantitative technique, using autoradiography to follow transfer of \(^{3}H\)-uridine. In addition, the data in Figure 1 indicate that EC acquired dye uniformly, suggesting either that transfer occurred from labeled PBL to each EC or that labeled EC transferred dye to their neighbors subsequently.

Cytoplasmic dye transfer between PBL and EC was bidirectional (Figure 2). When unlabeled PBLs were added to labeled EC monolayers, a generalized low level increase in fluorescence in all PBL was seen within 15 minutes; this low level increase remained unchanged during the remainder of the 3-hour coculture and may represent membrane transfer of residual BCECF-AM. After 2–4 hours of coculture, however, a discrete population of bright PBL could be identified. In different experiments, the number of bright cells ranged from 15–30% of the monolayer-bound PBL and from 5–15% of total PBL. Individual bright PBL could also be visualized by fluorescence microscopy of the same cell suspensions (not shown). No such population of PBL was detected by FACS or microscopy when PBL were cocultured with labeled MDCK monolayers. Such brightly fluorescent PBL could not be produced by incubating unlabeled PBL and EC in the presence of extracellular BCECF. The observation that some PBL remained unlabeled suggests both cell specificity (ie, only a subset of lymphocytes could be labeled by EC) and that PBL did not, under the conditions of the experiment, transfer dye to other PBL.

The ability of EC to exchange in bidirectional cytoplasmic transfer with PBL was not limited to neonatal (umbilical vein-derived) EC, as ECs derived from adult (saphenous vein) tissues were also able to interact with PBL in a similar fashion (data not shown). Several characteristics of the PBL populations participating in bidirectional cytoplasmic exchange with EC have been determined. First, the PBL that became brightly fluorescent were those bound to the monolayers. Such adherent PBL were operationally defined as those PBL still attached to the cell monolayer after vacuum aspiration and two serial washes of the well with HBSS. At 3 hours, nonadherent PBL were not brightly fluorescent (Figure 2, panel B). Further, MDCK do not bind PBL and do not transfer dye. Second, monocytes were not responsible for dye transfer. The percentage of monocytes in the fibronectin adherence-depleted PBL preparations was generally less than 2. Additionally, total peripheral blood mononuclear cells (ie, not fibronectin adherence-depleted of monocytes) did not differ from PBL in their quantita-
tive exchange of cytoplasmic dye. Finally, T lymphocytes, isolated from PBL by formation of E-rosettes, appeared enriched for the ability to accept transfer of dye from EC; eg, when Leu 4 (CD3) (Becton Dickinson) positive cells were increased by E-rosette selection from 80 to 96% of the PBL, the percentage of bright monolayer-bound PBL (after 4 hours of coculture with BCECF-labeled EC) increased from 20 to 30. These initial experiments do not exclude the possibility that non-T cells also can exchange dye.

**Discussion**

The studies reported here show that EC and lymphocytes in vitro are capable of exchanging cytoplasmic dyes. Possible mechanisms of cytoplasmic exchange include gap junction formation, cell-cell fusion, and formation and transfer of vesicles from one cell to another. Gap junction formation is the best documented of these potential pathways. The failure of MDCK cells to exchange cytoplasmic dye with lymphocytes may correlate with the inability of MDCK to form gap junctions under standard culture conditions; in fact, this property determined the selection of this cell for comparison. EC are known to form both homotypic and heterocellular junctions (with smooth muscle cells) in culture, and lymphoid cells have been reported to form homotypic junctions after activation. It will be of interest to learn what other cell types form such junctions with T lymphocytes and whether this ability correlates with immune accessory functions.

In summary, a new method of quantifying cytoplasmic dye exchange was developed to demonstrate that EC, but not MDCK cells, can participate in intercellular communication with PBL, including E-rosette selected T cells. It is speculated that this interaction could have immunologic significance, allowing endothelial cells to promote T cell activation by providing a second signal, such as membrane potential or cytoplasmic calcium, to augment a primary signal mediated through the T cell antigen receptor. These observations also raise the possibility that in other settings of lymphocyte interactions with tissue cells (eg, in granulomas or atheromatous plaques), cell contact-dependent pathways of communication involving cytoplasmic exchange may be important for the bidirectional delivery of a variety of signals, including signals for mitogenesis and differentiation. The quantitative flow cytometric method described in this report may be useful for such studies.

**References**


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