ANG and KOS without saline treatment of the rear footpads were as follows: 14 of 27 (52%) mice died at a dose of 10^7 PFU; 11 of 12 viruses isolated from different brains of dead mice were recombinants; and 2 of 12 recombinant viruses were virulent when reapplied to the footpads of mice.
17. L. J. Reed and H. Muench, *Am. J. Hyg.* **27**, 493 (1938).
18. J. M. M. Walboomers and J. Ter Schegget, *Virology* **74**, 256 (1976).
19. Rabbit skin cells were infected (multiplicity of infection, 0.1 to 0.01), and when a 100% cytopathic effect was observed, cells were harvested and lysed in 10 m*M* tris (*p*H 7.4) containing 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 0.5% NP40. The nuclei were removed by centrifugation at 1500*g* for 5 minutes, and the cytoplasmic fraction was incubated in the presence of 1 mg/ml of Pronase and 1% SDS at 56°C for 2 hours. The DNA was then purified in sodium iodide at 44,000 rpm in a Ti-50 rotor for 44 hours. The DNA band was collected by side puncture, extracted with butanol to reduce the volume and remove ethidium bromide, and dialyzed against TE buffer (10 m*M* tris, *p*H 8.0, 1.0 m*M* EDTA).
20. T. Maniatis, W. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
21. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
22. G. M. Wahl, M. Stern, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3683 (1979).
23. P. W. J. Rigby, M. Dieckmann, C. Rhoder, P. Berg, *J. Mol. Biol.* **113**, 237 (1977).
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Intracellular Accumulation of T-Cell Receptor Complex Molecules in a Human T-Cell Line

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This work was aimed at understanding the mechanisms of T-lymphocyte function by studying the cellular distribution and traffic of molecules of the T-cell receptor complex. The accumulation of specific molecules in intracytoplasmic vesicles is related to the activation of T lymphocytes. Some of these molecules include acid hydrolases, the transferrin receptor, and class I antigens of the major histocompatibility complex. Molecules of the T-cell receptor complex have now also been found in intracytoplasmic vesicles in a human T-cell line derived from a lymphoblastic leukemia. Such vesicles were tightly associated with the cytoplasmic microtubule network. One functional aspect of this association is a cellular pathway by which vesicles traveling to and from the cell surface converge in an area of the cells that is rich in processing enzymes.

THE SPECIFIC ACTIVATION AND function of regulatory and effector T cells is mediated through a receptor that recognizes foreign moieties in the context of self antigens of the major histocompatibility complex (MHC) (*1, 2*). This receptor is a multimolecular complex on the surface of human and murine lymphocytes (*3, 4*); it consists of a clonotypic molecule (Ti) that is a glycosylated heterodimer tightly associated with the oligomeric T cell–specific T3 antigens.

Two clonotypic monoclonal antibodies have been prepared by independent laboratories against HPB-ALL, a human T-leukemic cell line (*5, 6*). The use of these antibodies with indirect immunofluorescence microscopy revealed, in addition to the anticipated Ti reaction on the cell surface, the presence of Ti molecules within intracytoplasmic vesicles in HPB-ALL cells (Fig. 1A). Other membrane glycoproteins that were also located intracellularly in the HPB-ALL cells included T9 and class I MHC antigens as well as T3. In contrast, T4, T8, and T11 antigens were all expressed on the cell surface as determined by fluorescence-activated cell sorting (FACS) analysis and visualized by fluorescence microscopy (Fig. 1A); but T4 and T11 were present only at low levels in intracytoplasmic vesicles, and T8 was undetectable.

To confirm and quantitate intracellular Ti and other molecules, we treated viable HPB-ALL cells with protease until these membrane glycoproteins were no longer detectable on the cell surface by indirect immunofluorescence and FACS analysis. The cells were then lysed onto nitrocellulose, and the cell lysates were immunoblotted with the appropriate monoclonal antibodies and then with rabbit antiserum to mouse immunoglobulin (Ig) followed by radiolabeled protein A. Only those antigens that were shown by immunofluorescence microscopy to have an intracellular localization were protected from digestion by extracellular protease (Fig. 1B). Intracellular T3 and Ti were determined by densitometric scanning of the autoradiogram to be 60 to 70% of that expressed by the same cells mock-treated with bovine serum albumin (BSA). In contrast, only 10% of the T8 antigen expressed by these cells was insensitive to extracellular proteolysis.

The collection of Ti-containing vesicles in a central area, their juxtanuclear position, and the occasional appearance of cells in a log-phase culture with scattered vesicles suggested the association of these structures with the cytoskeleton (*7*). Costaining with monoclonal antibodies to tubulin by indirect immunofluorescence and isotype-specific antibodies to mouse IgG conjugated to different fluorochromes confirmed the association of Ti-containing vesicles with the cytoplasmic microtubule network (CMTN): vesicles containing Ti were clustered around the microtubule organizing center (MTOC) in interphase cells, but were scattered in cells undergoing mitosis (Fig. 2A). Treatment of HPB-ALL cells with colchicine led to dispersion of Ti-containing vesicles in the entire culture, further indicating their association with the CMTN.

The expression of acid hydrolases and lysosomelike organelles is a marker of T-cell activation and neoplasia (*8–10*). Cytochemical staining showed that HPB-ALL cells were positive for acid phosphatase, but negative for esterase and β-glucuronidase. Furthermore, acid phosphatase–positive vesicles collected in a central area of these cells, like Ti-containing vesicles, became dispersed upon treatment with colchicine (Fig. 2B), indicating their association with the CMTN. Several recent reports describe the association of Golgi elements with the CMTN in nonlymphoid cells (*11, 12*). Acid phosphatase is known to be localized not only in lysosomes and in the matrix of multivesicular bodies but also in *trans*-Golgi stacks and post-Golgi reticular elements (*11, 13, 14*). The post-Golgi region was found to be an acidic compartment (*15*) where uncoupling and sorting of recycling receptors may occur (*14, 16*), and there was evidence for the intracellular resialylation of transferrin receptors treated with extracellular neuraminidase (*17*).

When we costained for T9 [the transferrin receptor (*18*)] and Ti in HPB-ALL, these two molecules were found in the same vesicles, or at least in vesicles collected in the

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same area of the cells, that is, around the MTOC (Fig. 3A). We next investigated the functional significance of the association of vesicles containing a recycling cell-surface receptor with the CMTN. HPB-ALL cells were incubated with rhodamine-labeled human transferrin in the presence or absence of colchicine at 37°C for various lengths of time. The amount of transferrin on the cell surface was determined by restaining viable cells with antibodies to human transferrin labeled with fluorescein. Cell-bound rhodamine and fluorescein fluorescence were measured by FACS analysis. Colchicine had no statistically significant effect on the quantity, and a minimal effect on the rate, of rhodamine-transferrin uptake by HPB-ALL (Fig. 3B). In the presence or absence of colchicine, the amount of transferrin localized on the surface of these cells remained constant with time at 37°C and was not significantly different from that of cells incubated at 0°C (at which temperature endocytosis cannot take place). We conclude, therefore, that transferrin internalization was not affected by this microtubule-disrupting drug. However, in cells treated with colchicine, vesicles containing internalized transferrin appeared scattered in the cytoplasm, whereas in control cells they were centrally located (Fig. 3C).

We have demonstrated that the expression of intracytoplasmic vesicles containing class I MHC antigens is a tissue-specific, lineage-specific, and activation-specific phenomenon in T lymphocytes (*19, 20*). Furthermore, in ways that resemble the cellular dynamics of the T9 antigen (*18, 21*), class I MHC antigens internalize and recycle in T lymphoblasts (*22*). We have shown here that in a human T-cell line derived from an acute lymphoblastic leukemia, intracytoplasmic vesicles contain these two membrane glycoproteins as well as molecules of the T-cell receptor complex. The intracellular accumu-

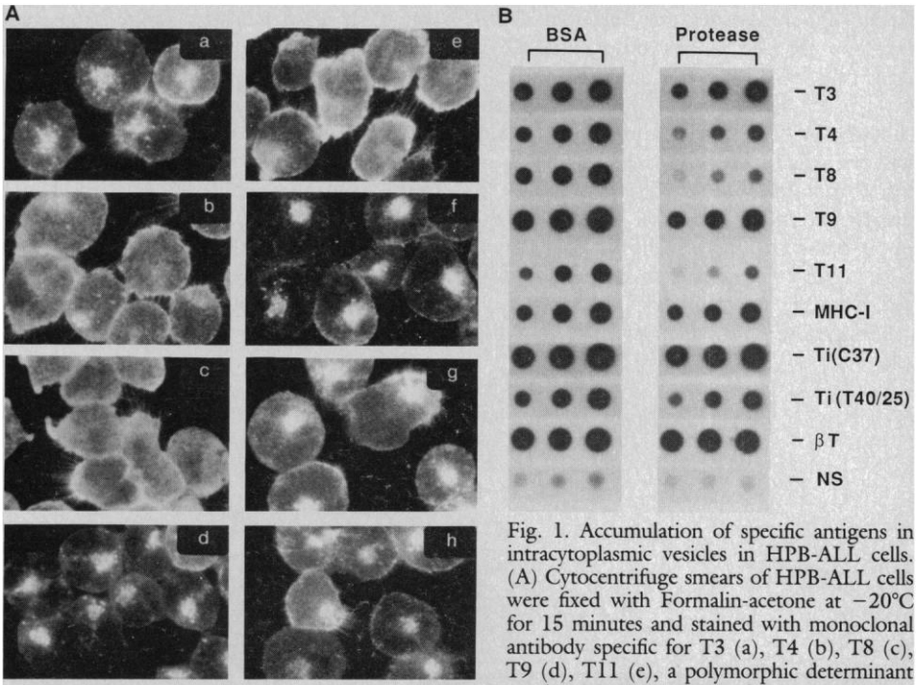

Fig. 1. Accumulation of specific antigens in intracytoplasmic vesicles in HPB-ALL cells. (A) Cytocentrifuge smears of HPB-ALL cells were fixed with Formalin-acetone at −20°C for 15 minutes and stained with monoclonal antibody specific for T3 (a), T4 (b), T8 (c), T9 (d), T11 (e), a polymorphic determinant on class I MHC antigens (f), and two distinct determinants on Ti (g and h) of HPB-ALL cells. Fluorescein isothiocyanate (FITC)–conjugated goat antibody to mouse Ig was then applied. The primary monoclonal antibodies used were, respectively, Leu4 (Becton Dickinson), OKT4, OKT8, OKT9 (*18, 30, 31*), OKT11a (Ortho Diagnostics), GA2 (*32*), C37 (*6*), and T40/25 (*5*). Immunofluorescence microscopy was performed as described (*19*). (B) HPB-ALL cells were treated with 20 mg of subtilisin per milliliter or with bovine serum albumin (BSA) as indicated, spotted onto 0.45-micrometer nitrocellulose, and lysed (*33*), then blocked with 20% fetal calf serum (FCS) in Hanks basic salt solution and blotted with monoclonal antibodies specific for the antigens indicated or with a nonspecific (NS) monoclonal antibody. Antibodies used were the same as those described in Figs. 1A and 2A. Blots were then developed as described (*34*). Number of cells spotted were (from left to right) 0.5×10^5, 1.0×10^5, and 2.0×10^5 for both protease and BSA-treated samples. The number of cells recovered after protease treatment was more than 70% that recovered after BSA treatment, and viability was >95% as determined by trypan blue exclusion. This experiment was representative of two others.

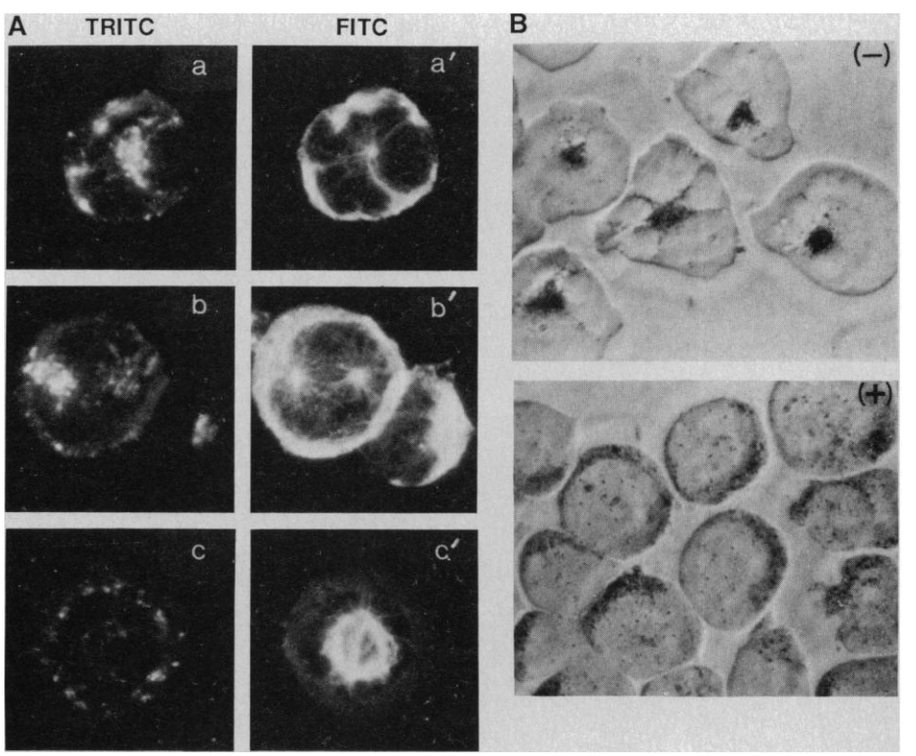

Fig. 2. Association of intracytoplasmic vesicles with the microtubule organizing center in HPB-ALL. (A) Cytocentrifuge smears of HPB-ALL were fixed with ethanol at −20°C for 15 minutes, stained first with monoclonal antibody to Ti (T40/25, IgG_{2a}) followed by tetramethyl rhodamine isothiocyanate (TRITC)–conjugated goat antibody to mouse IgG_{2a}, then restained with monoclonal antibodies to α,β-tubulin (Amersham, both IgG_1) followed by FITC-conjugated goat antibody to mouse IgG_1. Left (a to c) and right (a′ to c′) frames are the same fields viewed with optics specific for TRITC and FITC. Cells in different stages of the cell cycle are shown: top panels, interphase cell; middle panels, a cell in cytokinesis; bottom panels, a cell in mitosis. Experimental details are as described (*19*). (B) Phase-contrast microscopy of HPB-ALL cells treated for 10 minutes with 100 μg of colchicine per milliliter (+) or an equivalent volume of ethanol (−) at 37°C for 10 minutes and stained for acid phosphatase by enzyme cytochemistry (*10*).

lation of polypeptides that form the T-cell receptor complex is not unique to HPB-ALL cells. We have also found T3- and Ti-positive intracytoplasmic vesicles in certain subcultures of Jurkat, a human T-cell line, and we have observed the expression of intracytoplasmic vesicles containing T3 in primary cultures of normal human T lymphoblasts from peripheral blood after activation by mitogen or in a mixed lymphocyte reaction (*20, 23*).

Thus, there is increasing evidence that the accumulation of specific proteins in intracytoplasmic vesicles is related to the activation of T lymphocytes: such proteins include acid hydrolases (*8–10*), recycling cell-surface glycoproteins (*18–22*) and, as reported here, molecules that form the antigen-recognition complex of these cells. We do not know whether the specific proteins that accumulate in one area of HPB-ALL cells reside in the same or different vesicles or whether these vesicles are Golgi elements and a compartment for the uncoupling of recycling receptor and ligand or lysosomes and multivesicular bodies. However, vesicles containing these proteins are tightly associated with the CMTN. One aspect of this association is related to endocytosis. It does not, however, appear to determine the capacity of HPB-ALL to internalize an extracellular ligand such as transferrin, but seems to be a means by which these cells can collect endocytic vesicles to a specific region, around the MTOC, within the cell's cytoplasmic space (*14, 24*).

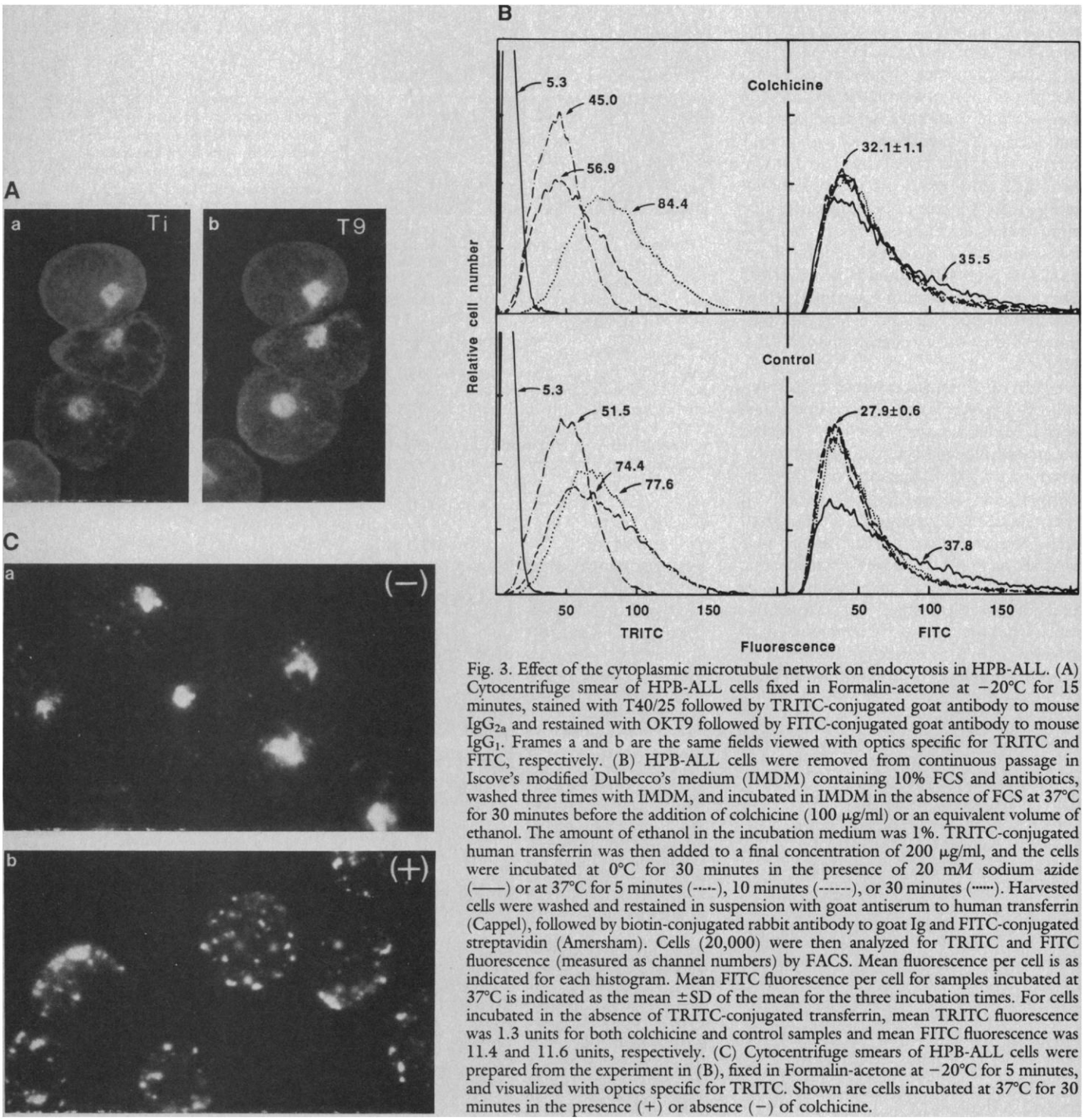

Fig. 3. Effect of the cytoplasmic microtubule network on endocytosis in HPB-ALL. (A) Cytocentrifuge smear of HPB-ALL cells fixed in Formalin-acetone at −20°C for 15 minutes, stained with T40/25 followed by TRITC-conjugated goat antibody to mouse IgG_{2a} and restained with OKT9 followed by FITC-conjugated goat antibody to mouse IgG_1. Frames a and b are the same fields viewed with optics specific for TRITC and FITC, respectively. (B) HPB-ALL cells were removed from continuous passage in Iscove's modified Dulbecco's medium (IMDM) containing 10% FCS and antibiotics, washed three times with IMDM, and incubated in IMDM in the absence of FCS at 37°C for 30 minutes before the addition of colchicine (100 μg/ml) or an equivalent volume of ethanol. The amount of ethanol in the incubation medium was 1%. TRITC-conjugated human transferrin was then added to a final concentration of 200 μg/ml, and the cells were incubated at 0°C for 30 minutes in the presence of 20 m*M* sodium azide (——) or at 37°C for 5 minutes (·····), 10 minutes (------), or 30 minutes (·····). Harvested cells were washed and restained in suspension with goat antiserum to human transferrin (Cappel), followed by biotin-conjugated rabbit antibody to goat Ig and FITC-conjugated streptavidin (Amersham). Cells (20,000) were then analyzed for TRITC and FITC fluorescence (measured as channel numbers) by FACS. Mean fluorescence per cell is as indicated for each histogram. Mean FITC fluorescence per cell for samples incubated at 37°C is indicated as the mean ±SD of the mean for the three incubation times. For cells incubated in the absence of TRITC-conjugated transferrin, mean TRITC fluorescence was 1.3 units for both colchicine and control samples and mean FITC fluorescence was 11.4 and 11.6 units, respectively. (C) Cytocentrifuge smears of HPB-ALL cells were prepared from the experiment in (B), fixed in Formalin-acetone at −20°C for 5 minutes, and visualized with optics specific for TRITC. Shown are cells incubated at 37°C for 30 minutes in the presence (+) or absence (−) of colchicine.

The accumulation of T-cell receptor complex molecules in vesicles associated with the MTOC in HPB-ALL may be interpreted in several ways. (i) The vesicles around the MTOC may be *trans*-Golgi elements and may be the site of assembly for the oligomeric complex that makes up the T-cell receptor. Since these vesicles stained positively for wheat germ agglutinin, the T3 and Ti polypeptides that they contained were probably sialylated (*25*). However, it is not known if these terminally glycosylated polypeptides were tightly complexed to each other (*1, 3*) or whether this is indeed the site for such assembly. (ii) Vesicles containing the T-cell receptor complex may represent a latent pool of the assembled molecules. The selective accumulation of proteins in vesicles (*26*) and the association of vesicles with the CMTN (*27*) have been observed in other cell types. Likewise, latent intracellular cell-surface receptors have recently been identified (*28*). (iii) Vesicles containing the T-cell receptor complex may be involved in recycling. However, ligands of the T-cell receptor consist of integral components of the plasma membrane of another cell (*1, 2*). Therefore, if the T-cell receptor indeed recycles, it is unlikely that this process mediates the uptake of soluble extracellular moieties. Nevertheless, our observation of a highly ordered cellular pathway in HPB-ALL cells whereby endocytosed molecules can be brought into the proximity of an area rich in processing enzymes suggests various possibilities for their postinternalization modification at an intracellular site. If the T-cell receptor complex is internalized, processed, and re-expressed, it may be a cellular pathway involved in the recognition and regulation of one T cell by another. This hypothesis could also account for the presence of class I MHC antigens in intracytoplasmic vesicles (Fig. 1A). Molecules that are necessary to provide context for presentation of the T cell's clonotype are also collected in the same or interconnected vesicular compartments of these cells (*19, 22, 29*).

REFERENCES AND NOTES

1. S. C. Meuer, O. Acuto, T. Hercend, S. F. Schlossman, E. L. Reinherz, *Annu. Rev. Immunol.* **2**, 23 (1984).
2. K. Haskins, J. Kappler, P. Marrack, *ibid.*, p. 51.
3. J. Borst, M. A. Prendiville, C. Terhorst, *Eur. J. Immunol.* **13**, 576 (1983); J. Borst, B. Alexander, J. Elder, C. Terhorst, *J. Biol. Chem.* **258**, 5135 (1983); J. Borst *et al.*, *Nature (London)* **312**, 455 (1984); H. C. Oettgen, J. Kappler, W. J. M. Tax, C. Terhorst, *J. Biol. Chem.* **259**, 12,039 (1984).
4. H. C. Oettgen, C. L. Pettey, W. L. Maloy, C. Terhorst, *Nature (London)* **320**, 272 (1986).
5. J. Kappler *et al.*, *Cell* **35**, 295 (1983).
6. C. Y. Wang *et al.*, *Hybridoma*, **5**, 179 (1986).
7. B. R. Brinkley, *Annu. Rev. Cell Biol.* **1**, 145 (1985).
8. A. C. Allison and L. Mallucci, *Lancet* **1964-II**, 1371 (1964); J. W. Parker, W. Haruki, R. J. Lukes, *Lab. Invest.* **14**, 1736 (1965).
9. R. Hirschhorn, J. M. Kaplan, A. F. Goldberg, K. Hirschhorn, G. Weissmann, *Science* **147**, 55 (1965); D. M. Knowles II, T. Hoffman, M. Ferrarini, H. G. Kunkel, *Cell Immunol.* **35**, 112 (1978); G. A. Machin, J. P. Halper, D. M. Knowles II, *Blood* **56**, 1111 (1980).
10. D. M. Knowles II and J. P. Halper, *J. Immunol.* **125**, 2823 (1980); *Am. J. Pathol.* **106**, 187 (1982); K. Yang, R. M. Bearman, G. A. Pangalis, R. J. Zelman, H. Rappaport, *Am. J. Clin. Pathol.* **78**, 141 (1982); A. Crockard, D. Chalmers, E. Matutes, D. Catovsky, *ibid.*, p. 437.
11. M. G. Farquhar, *Annu. Rev. Cell Biol.* **1**, 447 (1985).
12. A. A. Rogalski and S. J. Singer, *J. Cell Biol.* **99**, 1092 (1984); A. A. Rogalski, J. E. Bergman, S. J. Singer, *ibid.*, p. 1101; J. J. Lin and S. A. Quealley, *ibid.* **92**, 108 (1982); G. Hiller and K. Weber, *Exp. Cell Res.* **142**, 85 (1982).
13. S. Goldfischer, *J. Histochem. Cytochem.* **7**, 717 (1982).
14. K. Miller, J. Beardmore, H. Kanety, J. Schlessinger, C. R. Hopkins, *J. Cell Biol.* **102**, 500 (1986).
15. R. G. W. Anderson and R. K. Pathak, *Cell* **40**, 635 (1985).
16. H. J. Geuze, J. W. Slot, G. J. A. M. Strous, *ibid.* **32**, 277 (1983); A. Dautry-Varsat and H. F. Lodish, *Trends Neurosci.* **6**, 484 (1983); H. J. Geuze *et al.*, *Cell* **37**, 195 (1984); J. W. Woods, M. Doriaux, M. G. Farquhar, *J. Cell Biol.* **103**, 277 (1986).
17. M. D. Snider and O. C. Rogers, *J. Cell Biol.* **100**, 826 (1985).
18. J. W. Golding and G. F. Burns, *J. Immunol.* **127**, 1256 (1981); R. Sutherland *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4515 (1981); I. S. Trowbridge and F. Lopez, *ibid.* **79**, 1175 (1982); A. Dautry-Varsat, A. Ciechanover, H. F. Lodish, *ibid.* **80**, 2258 (1983).
19. D. B. Tse and B. Pernis, *J. Exp. Med.* **159**, 193 (1984).
20. B. Pernis and D. B. Tse, in *Cell Biology of the Major Histocompatibility Complex*, H. Vogel and B. Pernis, Eds. (Physicians and Surgeons Biomedical Symposium, Academic Press, New York, 1985), p. 153.
21. L. M. Neckers and J. Cossman, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3494 (1983).
22. D. B. Tse, C. R. Cantor, J. McDowell, B. Pernis, *J. Mol. Cell. Immunol.* **2**, 315 (1986).
23. D. B. Tse, N. Agostino, Y. Thomas, B. Pernis, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 2218 (1985).
24. C. R. Hopkins, *Cell* **35**, 321 (1983); ——— and I. S. Trowbridge, *J. Cell Biol.* **97**, 508 (1983).
25. I. Virtanen, P. Ekblom, P. Laurila, *J. Cell Biol.* **85**, 429 (1980).
26. L. G. Paavola, *ibid.* **79**, 45 (1978); H.-P. Moore and R. B. Kelly, *ibid.* **101**, 1773 (1985); J. S. Bonifacino, P. Perez, R. D. Klausner, I. V. Sandoval, *ibid.* **102**, 516 (1986).
27. R. D. Allen, *ibid.* **64**, 497 (1975); D. G. Pipeleers, M. A. Pipeleers-Marichal, D. M. Kipnis, *Science* **191**, 88 (1976); S. T. Brady, R. J. Lasek, R. D. Allen, *ibid.* **218**, 1129 (1982); W. G. Kelly, A. Passaniti, J. W. Woods, J. L. Daiss, T. F. Roth, *J. Cell Biol.* **97**, 1191 (1983); A. J. Baines and V. Bennet, *Nature (London)* **319**, 145 (1986).
28. W. A. Dunn, T. P. Connolly, A. L. Hubbard, *J. Cell Biol.* **102**, 24 (1986).
29. J. L. Goldstein, M. S. Brown, R. G. W. Anderson, D. W. Russel, W. J. Schneider, *Annu. Rev. Cell Biol.* **1**, 1 (1985).
30. P. C. Kung *et al.*, *Science* **206**, 347 (1979); E. L. Reinherz, P. C. Kung, G. Goldstein, S. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4061 (1979).
31. E. L. Reinherz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1588 (1980).
32. V. Quaranta, M. A. Pellegrino, S. Ferrone, *Immunogenetics* **14**, 403 (1981).
33. M. G. Reth, P. Ammirati, S. Jackson, F. W. Alt, *Nature (London)* **317**, 353 (1985).
34. L. T. Feldman, M. J. Imperiale, J. R. Nevins, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4952 (1982).

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