Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3019–3023, April 1991
Medical Sciences

Intracellular sites for storage and recycling of C3b receptors in human neutrophils

(complement receptor type 1/vesicles/multivesicular bodies/endocytosis/exocytosis)

MELVIN BERGER*†, ERICA M. WETZLER*, ELISABETH WELTER*, JERROLD R. TURNER‡, AND ALAN M. TARTAKOFF‡

Departments of *Pediatrics and ‡Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106

Communicated by Frederick C. Robbins, January 2, 1991

ABSTRACT **Upon activation of human neutrophils, C3b receptors (type 1 complement receptors, CR1) are rapidly translocated from an intracellular pool to the surface, increasing plasma membrane expression 6- to 10-fold. This is followed by reinternalization and degradation of the receptors, even in the absence of ligand. Upregulation of surface CR1 may occur without exocytosis of primary or secondary granules, and intracellular CR1 in resting neutrophils does not sediment with these granules on density gradient fractionation. To directly localize the intracellular pools of CR1, we used immunoelectron microscopy of fixed, permeabilized cells. In resting neutrophils, CR1 is associated with the membranes of smooth-surfaced, empty-appearing vesicles whose irregular borders clearly distinguish them from primary and secondary granules. After activation by fMet-Leu-Phe, the intracellular pool is primarily found in large, conspicuous, multivesicular bodies. These bodies also incorporate colloidal gold from the extracellular fluid, suggesting that they are formed by endocytosis. Thus, the sites in which CR1 is stored in resting cells, and recycled in activated cells, are structurally unique and morphologically distinguishable.**

During the activation of human neutrophils (polymorphonuclear leukocytes), a number of functionally important proteins are translocated from intracellular pools to the cell surface. These include the type 1 complement receptor (CR1), which binds complement components C3b and C4b and plays an important role in phagocytosis (1); the CD11b/CD18 cell adhesion molecule (2, 3), which also serves as the type 3 complement receptor (CR3); complement decay-accelerating factor (4); additional chemoattractant receptors (5); and cytochrome b_{245}, a key element of the microbicidal oxidase (6). Simultaneously, protein kinase C and arachidonate 5-lipoxygenase also become associated with the plasma membrane (7, 8). Although the latter enzymes are cytosolic proteins whose affinity for membrane lipids increases when the cell is activated, the former are integral membrane proteins. It is likely that they are bound to membranous structures inside resting cells and that their movement to the surface results from the selective addition of intracellular membrane pools to the plasma membrane during cell activation. We previously showed (9) that internalization and degradation of CR1 accompanied its increased surface expression during neutrophil activation; however, neither the sites of intracellular storage of CR1 in resting cells nor the sites of internalization in activated cells were described.

Neutrophils are rich in granules, and since stimuli that induce secretion also increase adherence and chemotactic responses (5), it has been proposed that exocytosis of granules might account for the increased surface expression of adherence proteins and chemoattractant receptors that were stored in the membranes of the granules (10). Indeed, isopycnic fractionation experiments have shown that intracellular pools of CR3, fMet-Leu-Phe receptors, and cytochrome b_{245} cosediment with specific granules (6, 11, 12). However, available evidence suggests that the intracellular pool of CR1 is not associated with primary or secondary granules, since maximal upregulation of CR1 (and CR3) expression may occur with only minimal release of primary or specific granule constituents (13, 14), and CR1 does not cosediment with granules on density gradients (15). Although CR3 has been identified in peroxidase-negative granules by electron microscopy (16), additional evidence suggests that the intracellular pool of CR1 is distinct from that of CR3: CR3 cosediments with specific granules but not with CR1 on density gradients (15), and the Ca^{2+} requirements for CR1 and CR3 expression differ (17).

Because these observations suggested that intracellular CR1 might be stored in unique structures, we used immunoelectron microscopy to directly localize CR1 in fixed, permeabilized human neutrophils. In resting cells, we have identified CR1 in membranes of small vesicles that are clearly distinguishable from conventional granules. In activated cells, there is increased staining of CR1 on the plasma membrane, and the intracellular pool is found in large, conspicuous, multivesicular bodies. We postulate that these are sites of recycling of internalized receptor. Thus, the sites of intracellular storage of CR1 in resting neutrophils, and of its recycling in activated cells, are structurally unique and morphologically distinguishable.

MATERIALS AND METHODS

Preparation of Cells and Immunolocalization of CR1 in Permeabilized Neutrophils. Neutrophils were isolated from peripheral blood of normal donors using Percoll density gradients and hypotonic lysis of erythrocytes (3, 18). This procedure yields preparations that are >95% viable neutrophils. Cells were either maintained at 0°C (resting cells) or activated at 37°C with 10 nM fMet-Leu-Phe. They were then initially fixed with 10 mM periodate/75 mM lysine/2% paraformaldehyde, pH 7.4 (19), and plated on poly(L-lysine) (Sigma)-coated tissue culture dishes. Endogenous peroxidase was inactivated by three successive 20-min incubations with 10 mM H_2O_2 and 10 mM NaN_3 (20). The cells were then permeabilized with saponin (0.2 mg/ml) in phosphate-buffered saline containing 0.1% ovalbumin (Sigma). This solution was used to dilute the primary and secondary antibodies and for all subsequent washes and incubations. To

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CR1, type 1 complement receptor(s); CR3, type 3 complement receptor(s).

†To whom reprint requests should be addressed at: Department of Pediatrics, University Hospitals of Cleveland, 2074 Abington Road, Cleveland, OH 44106.

identify CR1, cells were incubated in optimal dilutions of anti-CR1 monoclonal antibodies for 1 hr at room temperature. A combination of two mouse monoclonal antibodies that recognize different epitopes on the receptor was used: the products of clone 3D9, provided by John O'Shea (National Institutes of Health), and clone C543, provided by Robert Schreiber (Washington University School of Medicine). Both antibodies were at 0.08–0.10 mg/ml. In control experiments, equivalent concentrations of monoclonal antibodies to casein (gift of Charlotte Kaetzel, Case Western Reserve University) were substituted for the anti-CR1. After incubation with these antibodies, the cells were washed four times with the saponin/ovalbumin buffer and then incubated with a 1:100 dilution of horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (Biosys, Compiègne, France) for 1 hr at room temperature. The cells were again washed four times with the same buffer, then fixed with 1.5% glutaraldehyde/5% sucrose/0.1 M cacodylate buffer, pH 7.4. The horseradish peroxidase reaction product was developed with diaminobenzidine and H_2O_2 (21). The cells were then osmicated and dehydrated, removed from the plastic by using propylene oxide, and embedded in Spurr's resin for electron microscopy, all by the use of standard techniques.

Detection of Cell Surface CR1 and Internalized Colloidal Gold–Albumin Conjugate. For detection of cell surface CR1, intact, fMet-Leu-Phe-stimulated neutrophils were fixed for 30 min on ice in 0.5% glutaraldehyde. They were then washed with phosphate-buffered saline and incubated for 1 hr with monoclonal antibody 3D9 (0.08 mg/ml), or monoclonal anti-casein as a control, then washed again and incubated with a 1:10 dilution of gold-conjugated goat anti-mouse IgG (Jansenn Life Sciences, Piscataway, NJ) for 30 min. The cells were washed, postfixed with osmium tetroxide, dehydrated with ethanol, and embedded in Spurr's resin. Sections were examined by transmission electron microscopy.

For use as a marker of fluid endocytosis, bovine serum albumin was conjugated with 15- to 20-nM colloidal gold as described (22). Gold–albumin was then incubated with neutrophils in Hanks' balanced salts solution (HBSS) with 0.1% gelatin during activation with 10 nM fMet-Leu-Phe. Following this 1-hr incubation, cells were washed three times with HBSS/gelatin and then prepared for transmission electron microscopy as described above. Control flow cytometric experiments showed that the gold–albumin did not interfere with fMet-Leu-Phe stimulation of CR1 surface expression.

RESULTS

In order to directly localize the intracellular pool of CR1 in resting neutrophils, we selected an immunoelectron microscopic procedure in which lightly fixed cells were stained before embedding, so that we could obtain a large amplification factor through the use of enzyme-coupled reagents. We considered that this would be necessary since there are only 50,000–100,000 CR1 molecules per cell (1, 3, 9). In resting neutrophils, which express little CR1 on their surface, we found the intracellular pool primarily associated with membranes of smooth-surfaced, empty-appearing vesicles (Figs. 1 and 2). The irregular borders of these structures and their small size (mean diameters = 0.11 × 0.15 μm) clearly distinguish them from the readily identifiable primary and secondary granules. There were relatively few (from 3 to 10) positively staining vesicles per section (Table 1). This distribution is clearly distinct from that reported for CR3 in neutrophils, although different techniques were used to localize that protein (16). The vesicular structures that contained CR1 were not necessarily found only near the periphery of the cell; they were also found in more central locations, including occasional Golgi cisternae (Fig. 2*a*). The CR1-positive vesicles comprised 4–15% of the total number of granules visible in the same sections (Table 1).

Upon activation of the neutrophils with 10 nM fMet-Leu-Phe, the amount of CR1 on the surface increased 6- to 10-fold as indicated by monoclonal antibodies and flow cytometry (3). This surface CR1 was not well visualized by the peroxidase technique employed above, perhaps because of diffusion of the enzyme reaction product and/or some loss of surface receptors due to the saponin. Therefore, we avoided saponin and peroxidase and used gold-conjugated anti-mouse antibodies to detect the receptors on the plasma membrane. The surface CR1 was detected in a clustered distribution (Fig. 3*d*), in agreement with a previous report (23). Equivalent preparations in which anti-casein was substituted for anti-CR1 had no gold particles associated with the membrane (data not shown). However, as we previously showed (9) by quantitative immunofluorescence of fixed, permeabilized

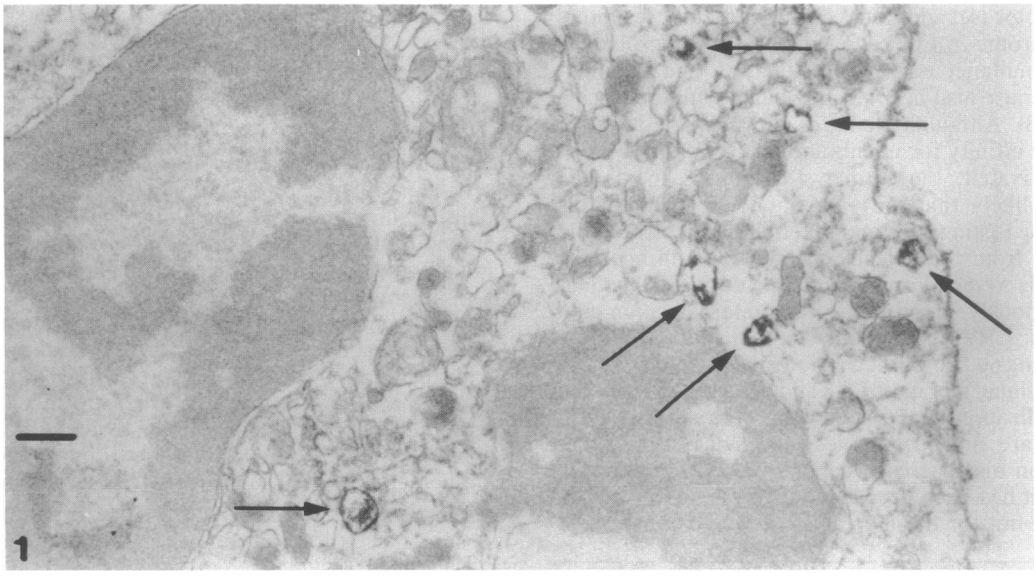

FIG. 1. Localization of CR1 in fixed, permeabilized resting neutrophils. Horseradish peroxidase reaction product is associated with the membranes of empty-appearing vesicles (arrows). (Bar = 0.28 μm.)

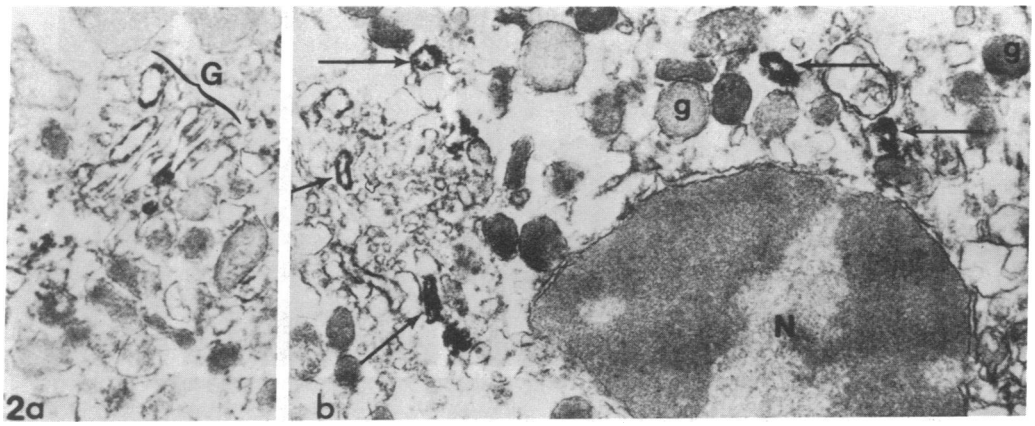

FIG. 2. Other examples of localization of CR1 in fixed, permeabilized resting neutrophils show horseradish peroxidase reaction product indicating the presence of CR1 in the membranes of some Golgi cisternae (G) (*a*) and emphasize the distinction between the CR1-containing vesicles (arrows) and conventional granules (g) (*b*). The cell nucleus (N) is identified for reference.

neutrophils, there was still a substantial pool of CR1 inside the cell.

Immunoperoxidase staining of activated cells that had been fixed and permeabilized showed a marked decrease in the number of small, irregular vesicles staining positive for CR1 as compared with the resting cells (Table 1), and most of the intracellular CR1 was found in large (mean diameters = 14.7 × 10.5 μm), conspicuous, multivesicular bodies (Fig. 3 *a* and *e*) that were not seen in resting cells. At most, one or two of these structures were seen per section. CR1 was identified in both the limiting membrane and the membranes of the smaller vesicles contained in these structures. Controls with irrelevant monoclonal antibody in place of the anti-CR1 (Fig. 3*b*) did not show corresponding staining of the multivesicular bodies, verifying that the staining was due to the presence of CR1 in these structures. Because they were not frequently seen in resting cells, it seemed likely that these multivesicular bodies might be formed during endocytosis of CR1 that had been expressed on the surface. To confirm the endocytic origin of these structures, we included colloidal gold–albumin as a fluid-phase marker during activation of the neutrophils with fMet-Leu-Phe. Gold was internalized by the neutrophils and was conspicuous in similar-appearing multivesicular bodies (Fig. 3*c*). A similar appearance also resulted when colloidal gold–ovalbumin was used as the fluid-phase marker (data not shown). All of the multivesicular bodies were stained with both gold and anti-CR1.

Table 1. Quantitation of CR1-positive vesicles in resting and activated human neutrophils

	CR1-positive vesicles			
	No. per cell profile*			% of total
Experiment	0°C	37°C	fMLP	granules
a	10	—	3	12
b	6	6	—	6
c	3	—	1	4
d	8	8	—	15

In four separate experiments, using cells of different donors, CR1 was localized by immunoelectron microscopy. The tabulated peroxidase-positive vesicles were characteristically stained along their membrane perimeter but had an apparently empty interior. Prints of 40 cell cross sections photographed at ×10,000 and including at least one nuclear profile were evaluated. In the last column, the number of CR1-positive vesicles at 0°C is expressed as a fraction of the total number of granules, including those with obvious content (primary and secondary) plus the CR1-positive vesicles.

*Before fixation, cells were incubated 1 hr on ice (0°C), or at 37°C in buffer alone, or at 37°C in the presence of 10 nM fMet-Leu-Phe (fMLP). —, Not studied.

DISCUSSION

In this study we used an enzyme-amplified immunoelectron microscopic technique to directly localize CR1 in fixed, permeabilized neutrophils. The results demonstrate that the sites of intracellular CR1 in resting vs. activated neutrophils are structurally unique and morphologically distinguishable. In resting neutrophils, the intracellular CR1 is stored in a type of membranous structure that is clearly distinguishable from the classically described granules. There are relatively few of these vesicles as compared to the total number of granules visualized in any given cell section. With the permeabilization technique we have employed, they are irregularly shaped and devoid of matrix or dense constituents. This "empty" appearance may correlate with the previously reported inability to separate the intracellular pool of CR1 from plasma membranes by centrifugation (15).

Fusion of the vesicular structures in which CR1 is stored with the plasma membrane may thus account for the observations that this receptor can be expressed on the surface without extensive secretion of primary or secondary granule constituents (13). It is possible that translocation of these vesicles accompanies exocytosis of the gelatinase-containing "tertiary granules," which also occurs in response to mild stimuli that do not cause secretion of classical granule constituents (14, 24). However, the storage sites of that enzyme appear morphologically distinct (25) from the vesicles in which we have identified CR1, although different techniques have been used to localize these different proteins. Similarly, the vesicles in which we have identified CR1 appear distinct from the structures in which CR3 has been identified in neutrophils (16). The latter structures, like the gelatinase-containing structures, appear to be a subset of peroxidase-negative specific granules and contain a dense matrix that stains positively for lactoferrin (16, 25). Interestingly, when the same technique used for localizing CR3 in neutrophils was applied to monocytes, that protein was also found in the membranes of clear vacuoles and smaller vesicles, in addition to peroxidase-negative granules (26). It is possible that the clear vesicles in which CR3 has been found in monocytes are similar to the structures in which we have found CR1 in neutrophils.

The structures in which we have identified CR1 resemble the vesicular structures that contain the intracellular pools of two other proteins that are readily translocated to the surface upon cell activation: alkaline phosphatase in neutrophils (27) and glucose transporters in 3T3-L1 adipocytes (28). Thus, the membranes of these vesicles may also contain other proteins whose surface expression can be upregulated with kinetics similar to CR1, such as alkaline phosphatase (29) and com-

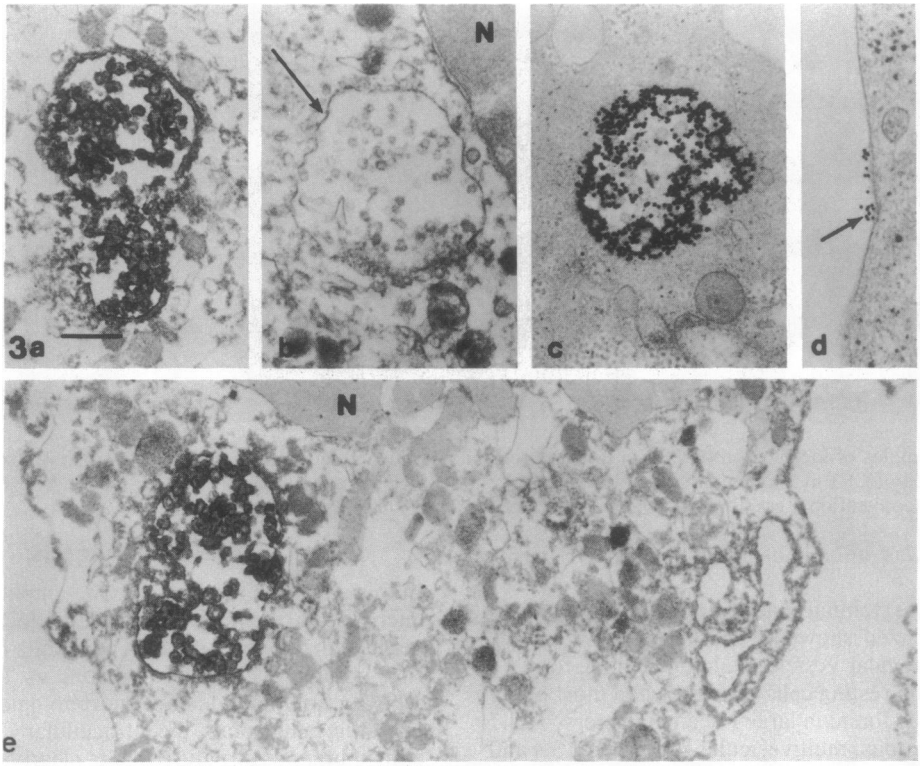

FIG. 3. CR1 and multivesicular bodies in activated neutrophils. Cells were activated with 10 nM fMet-Leu-Phe for 1 hr at 37°C. (*a*, *b*, and *e*) Fixed, permeabilized neutrophils: *a* shows detail of a positively staining multivesicular body, and *e* shows a multivesicular body relative to other cell constituents (N, nucleus). *b* differs from *a* only in that anti-casein was substituted for anti-CR1 antibodies as a negative control. (*c*) Transmission electron micrograph showing internalized albumin–gold in a multivesicular body. (*d*) Transmission electron micrograph showing surface CR1 stained with monoclonal anti-receptor antibody followed by gold-conjugated anti-mouse IgG. (Bar = 0.37 μm.)

plement decay-accelerating factor (4). The size, distribution within the cell, irregular shape, and empty appearance of the vesicles in which we have identified CR1 in resting neutrophils are also very similar to the features described for vesicles containing an intracellular pool of type III Fc receptors in neutrophils (30). It has been proposed that the intracellular pool of type III Fc receptors is also mobilized to the neutrophil surface during cell activation (31). It is tempting to speculate, therefore, that these vesicular structures represent a compartment that exists primarily to store functionally important membrane proteins whose surface expression is regulated rather than constitutive, in much the same way that the granules serve as storage sites for soluble proteins whose secretion is regulated.

The large multivesicular bodies in which we have identified CR1 in activated neutrophils have not been commonly described in resting cells, but previous results (32) and our own kinetic studies (33) indicate that these structures are formed rapidly upon activation of endocytosis. Our results with colloidal gold–albumin (Fig. 3*c*) suggest that these structures are formed by internalization of the plasma membrane in the absence of specific ligands. It seems likely that these structures correspond to the bright accumulations of CR1 identified inside permeabilized activated neutrophils by immunofluorescence (9), which we previously postulated might be involved in degradation of internalized CR1. Multivesicular bodies are believed to serve as a prelysosomal sorting compartment in other types of cells (34). Nevertheless, it has been reported that neutrophil multivesicular bodies stain positively with monoclonal antibodies to the lysosomal membrane proteins hLAMP 1 and 2 (35). These multivesicular bodies, which are formed in the absence of targets of phagocytosis, may play an important role in membrane and receptor recycling that is independent from the actual processes of phagocytosis and formation of phagolysosomes.

In summary, by the use of a direct approach employing immunoelectron microscopy, we have identified the intracellular storage pool of CR1 in resting human neutrophils in a unique type of small vesicles, and we have shown that these receptors accumulate in large multivesicular bodies in activated cells.

We are grateful to Dr. William Brown (Cornell University) for advice and helpful discussions about the immunoperoxidase procedure. This work was supported by National Institutes of Health Grants AI22687, AI21269, DK27651, and DK38181.

1. Fearon, D. T. & Collins, L. A. (1983) *J. Immunol.* **130**, 370–375.
2. Arnaout, M. A., Spits, H., Terhorst, C., Pitt, J. & Todd, R. F., III (1984) *J. Clin. Invest.* **74**, 1291–1300.
3. Berger, M., O'Shea, J., Cross, A. S., Folks, T. M., Chused, T. M., Brown, E. J. & Frank, M. M. (1984) *J. Clin. Invest.* **74**, 1566–1571.
4. Berger, M. & Medof, E. M. (1987) *J. Clin. Invest.* **79**, 214–220.
5. Fletcher, M. P., Seligman, B. E. & Gallin, J. I. (1982) *J. Immunol.* **128**, 941–948.
6. Borregaard, N., Heiple, J. M., Simons, E. R. & Clark, R. A. (1983) *J. Cell Biol.* **97**, 52–61.
7. McPhail, L. C., Clayton, C. C. & Snyderman, R. (1984) *Science* **224**, 622–625.
8. Rouzer, C. A. & Kargman, S. (1988) *J. Biol. Chem.* **263**, 10980–10988.
9. Turner, J. R., Tartakoff, A. M. & Berger, M. (1988) *J. Biol. Chem.* **263**, 4914–4920.
10. Gallin, J. I. (1985) *Clin. Res.* **33**, 320–328.
11. Todd, R. F., Arnaout, M. A., Rosin, R. E., Crowley, C. A., Peters, W. A. & Babior, B. M. (1984) *J. Clin. Invest.* **74**, 1280–1290.
12. Fletcher, M. P. & Gallin, J. I. (1983) *Blood* **62**, 792–799.
13. Berger, M., Wetzler, E. & Wallis, R. S. (1988) *Blood* **71**, 151–158.

14. Petrequin, P. R., Todd, R. F., III, Devall, L. J., Boxer, L. A. & Curnutte, J. T. (1987) *Blood* **69**, 605–610.
15. O'Shea, J. J., Brown, E. J., Seligman, B. E., Metcalf, J. A., Frank, M. M. & Gallin, J. I. (1984) *J. Immunol.* **134**, 2580–2587.
16. Bainton, D. F., Miller, L. J., Kishimoto, T. K. & Springer, T. A. (1987) *J. Exp. Med.* **166**, 1641–1653.
17. Berger, M., Birx, D. L., Wetzler, E. M., O'Shea, J. J., Brown, J. E. & Cross, A. S. (1985) *J. Immunol.* **135**, 1342–1348.
18. Harbeck, R. J., Hoffman, A. A., Redecker, S., Biundo, T. & Kurnick, J. (1982) *Clin. Immunol. Immunopathol.* **23**, 682–690.
19. McLean, I. W. & Nakane, P. K. (1974) *J. Histochem. Cytochem.* **22**, 1077–1083.
20. Ohno, Y. & Gallin, J. I. (1985) *J. Biol. Chem.* **260**, 8438–8446.
21. Brown, W. J. & Farquhar, M. G. (1984) *Cell* **36**, 295–307.
22. Roth, J. (1983) in *Techniques in Immunocytochemistry*, eds. Bullock, G. R. & Petrusz, P. (Academic, London), Vol. 2, pp. 217–284.
23. Petty, H. R., Smith, L. M., Fearon, D. T. & McConnell, H. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6587–6591.
24. Dewald, B., Bretz, U. & Bagiolini, M. (1982) *J. Clin. Invest.* **70**, 515–525.
25. Hibbs, M. S. & Bainton, D. F. (1989) *J. Clin. Invest.* **84**, 1395–1402.
26. Miller, L. J., Bainton, D. F., Borregaard, N. & Springer, T. A. (1987) *J. Clin. Invest.* **80**, 535–544.
27. Rustin, G. J. S., Wilson, P. D. & Peters, T. J. (1979) *J. Cell Sci.* **36**, 401–412.
28. Blok, J., Gibbs, E. M., Leinhard, G. E., Slot, J. W. & Geuze, H. J. (1988) *J. Cell Biol.* **106**, 69–76.
29. Borregaard, N., Miller, L. J. & Springer, T. A. (1987) *Science* **237**, 1204–1206.
30. Jost, C. R., Huizinga, T. W. J., de Gode, R., Fransen, J. A. M., Tetteroo, P. A. T., Dahe, M. R. & Ginsel, L. A. (1990) *Blood* **75**, 144–151.
31. Tosi, M. F. & Zakem, H. (1990) *Clin. Res.* **38**, 395A (abstr.).
32. Zabucchi, G., Menegazzi, R., Soranzo, M. R. & Patriarea, P. (1986) *Am. J. Pathol.* **124**, 510–518.
33. Berger, M., Wetzler, E. & Tartakoff, A. M. (1990) *FASEB J.* **4**, A2311 (abstr.).
34. Tougard, C., Louvard, D., Picart, R. & Tixier-Vidal, A. (1985) *J. Cell Biol.* **100**, 786–793.
35. Bainton, D. F. & August, J. T. (1988) *J. Histochem. Cytochem.* **36**, 953 (abstr.).