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T-lymphocyte heterogeneity: Wheat germ agglutinin labeling of transmembrane glycoproteins

(cytochemistry/fracture-label/freeze-fracture/mitogen/lymphocyte subpopulation)

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ABSTRACT **We have recently described "fracture-label" techniques that permit direct cytochemical labeling of freeze-fractured cells. We report here the use of fracture-labeling to investigate the distribution and partition of wheat germ agglutinin (WGA) receptor sites over the protoplasmic and exoplasmic plasma membrane faces of freeze-fractured human thymus-derived (T) lymphocytes. All exoplasmic faces are strongly labeled by WGA. In contrast, the protoplasmic faces exhibit remarkable variation, ranging from virtual absence of label in some faces to very high densities in other faces. We interpret the presence of WGA receptor sites over the protoplasmic faces to reflect the presence of transmembrane WGA-binding sialoglycoproteins that, during freeze-fracture, partition with the inner half of the plasma membrane. Our results, therefore, indicate heterogeneous expression of integral membrane proteins within populations of human T cells. Fracture-label techniques thus represent an additional tool in the definition of lymphocyte subpopulations.**

The plasma membranes of human T cells reflect in their composition and structure the complexity and functional heterogeneity intrinsic to the immune system. Current studies of the heterogeneity of human T lymphocytes are leading to the identification of cell subpopulations as defined by differences in the compositions of their plasma membranes, frequently through the identification of surface antigens and lectin binding sites, as well as through the analysis of ligand-evoked cellular responses (1–3). Wheat germ agglutinin (WGA), a plant lectin, can be used to fractionate human T lymphocytes on the basis of the presence of high-affinity or low-affinity receptors for the lectin (4, 5). In addition, microfluorimetric analysis of fluorescent-lectin binding appears to be able to distinguish subpopulations of lymphocytes (6, 7).

Recently, we developed cytochemical techniques—"fracture-label"—that permit direct labeling of the membrane components partitioned by freeze-fracture with each membrane half. In fracture labeled preparations, labeled membrane fracture faces are observed in thin sections or in platinum/carbon (Pt/C) replicas of critical point-dried preparations (8–12). Initial results show that, in human erythrocytes, most concanavalin A (Con A) receptor sites (associated with band 3, a transmembrane protein) are labeled over the protoplasmic fracture face (P face) (8). In human lymphocytes, in contrast, fracture-labeling shows that Con A binding sites are almost exclusively confined to the exoplasmic faces (E faces) (10). We report here the application of fracture-labeling techniques to the observation of the partition and distribution of WGA binding sites on the faces of freeze-fractured plasma membranes of human T lymphocytes. Our results show that WGA binding sites can be labeled over the P and E faces. Over the P faces, we observe remarkable variations in labeling intensity from cell to cell. These sites reflect the heterogeneous expression of a WGA-binding transmembrane protein that partitions with the inner half of the plasma membrane on fracture. The results suggest fracture-labeling as an additional method in the definition of lymphocyte subpopulations.

MATERIALS AND METHODS

For thin-section fracture-labeling, human peripheral lymphocytes were isolated from fresh blood on Ficoll-Hypaque gradients and separated from adherent cells by incubation at 37°C for 30 min in plastic Petri dishes. The nonadherent cells were incubated in RPMI 1640 culture medium (37°C, 30 min) to remove Fc receptor-bound Ig (see ref. 13) and fixed in 0.5% glutaraldehyde (4°C, 30 min) to avoid antibody-mediated capping in subsequent steps. The cells were treated with an IgG fraction of goat anti-human Ig (5 mg/ml, 25°C, 30 min) and then with protein A-coated colloidal gold (25°C, 3 hr) (14, 15). This procedure labeled the cell surface of B lymphocytes and Fc receptor-positive T cells and permitted their subsequent identification in thin sections. Thus, any cell that was labeled on the unfractured surface of the plasma membrane could be excluded from the analysis of fracture surfaces. The cells were then mixed 1:1 with washed glutaraldehyde-fixed human erythrocytes used here as a control for positive identification of fracture faces (see below; see also refs. 8, 10, and 12), embedded in 25% bovine serum albumin, and crosslinked by 1% glutaraldehyde, and the resulting gels were sliced, impregnated in 30% glycerol, and frozen in Freon 22 cooled by liquid nitrogen (8, 10). Frozen gels were fractured in liquid nitrogen by crushing with a glass pestle as described (8, 10). Gel fragments were thawed, deglycerinated, washed, incubated with WGA (0.25 mg/ml, 37°C, 1 hr in 0.1 M Sorensen's phosphate buffer/4% polyvinylpyrrolidone, pH 7.4), and labeled with ovomucoid-coated colloidal gold (25°C, 3 hr; see refs. 16, 17). Before being embedded, controls were incubated with 0.4 M *N*-acetyl-D-glucosamine (GlcNAc) (37°C, 15 min), treated with WGA (0.25 mg/ml) in the presence of the competing sugar, and labeled with ovomucoid-coated colloidal gold as above. The gel fragments were then dehydrated, embedded in Epon 812, and thick sectioned; favorable fracture edges were selected; the blocks were trimmed accordingly and thin sectioned (8, 10–12). Because of the small size of fracture gel fragments relative to the original gel slabs, cut faces were rare. They were distinguished by their jagged outline

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Abbreviations: WGA, wheat germ agglutinin; P face, protoplasmic membrane face; E face, exoplasmic membrane face; Con A, concanavalin A; GlcNAc, *N*-acetyl-D-glucosamine.

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and by the absence of cross-fractured erythrocytes (see refs. 8 and 10 for discussion).

Because bovine serum albumin-embedded unfractured surfaces cannot be visualized in Pt/C replicas of critical point-dried fracture-labeled preparations, a surface label could not be used to distinguish B and T cells. Therefore, we used purified T cells obtained by nylon wool filtration of Ficoll-Hypaque-isolated lymphocytes (18). These cells were more than 95% surface Ig negative as judged by staining with fluorescein isothiocyanate-labeled anti-human IgG. The T-cell suspensions were then fixed in glutaraldehyde and embedded in bovine serum albumin, and the resulting gels were sliced, glycerinated, and frozen as above. Frozen gels were transferred to a Petri dish filled with liquid nitrogen and fractured with a precooled scalpel (9). The fractured gels were thawed, deglycerinated, treated with WGA, and labeled with ovomucoid-coated colloidal gold as described above. Controls were labeled in the presence of GlcNAc as described. Labeled fragments were then dehydrated in acetone, critical point-dried, and replicated by Pt/C evaporation. The gels were digested in sodium hypoclorite (5%), and the replicas were cleaned, washed with water, and observed by transmission electron microscopy. In some replicas, numerous P faces were photographed (×13,000) and the colloidal gold particles were counted over 1-μm^2 squares chosen as representative of the label in that face and perpendicular to the electron beam.

RESULTS

Observation of the results of WGA fracture-labeling in thin sections was confined to the plasma membranes of human T lymphocytes. These were distinguished from B cells, as well as from a minor fraction of T cells bearing Fc receptors for IgG, which showed protein A-coated colloidal gold labeling (see above) over their bovine serum albumin-embedded surfaces (Fig. 1*a*). Over the P fracture faces, the WGA label appeared uniformly distributed but the intensity ranged from virtual absence in some cells to very dense in others (Fig. 2 *a–d*). Over the E fracture faces (Fig. 2 *e* and *f*), labeling was always uniform, heavy,‡ and comparable with that of the surface of unfractured cells as seen on surfaces of cells exposed by cutting the gels at room temperature (Fig. 1*b*) (8). Cross-fractured gels and cytoplasm were not labeled (Fig. 2 *a–e*). Labeling was also absent from fractured nuclear envelope membranes (Fig. 2*d*), in agreement with previous observations (11). In control cells (i.e., incubated with WGA in the presence of GlcNAc), labeling of both P faces and E faces was reduced by more than 90%. The heterogeneity of labeling observed over the P faces was unrelated to inequality of access of the lectin or colloidal gold: differently labeled faces could be observed in closely positioned cells over the same edge of the fractured gel.

Pt/C replicas of critical point-dried, fractured, purified T-cell preparations confirmed the thin-section results: in the P faces, labeling ranged from virtual absence to very dense (Fig. 3 *a–d*), while all E faces were densely labeled (not illustrated). Moreover, the replicas allowed the observation of large numbers of P faces as well as closer inspection of the pattern of distribution of the label. Often, particularly in heavily labeled cells, light clustering of colloidal gold particles was observed. Labeling density showed experimental variation probably due to variations in the number of fracture faces available for labeling in each experiment (a variable impossible to control). Yet, the basic finding reported here—heterogeneity of P-face labeling—was observed in each of 10 experiments in which hundreds of P faces were observed. This heterogeneity cannot be due to regional differences (e.g., "capping") as, in each face (frequently representing 1/3 to 1/2 of the total membrane area), the labeling pattern was uniform. We measured the number of colloidal gold particles over 1-μm^2 areas from 150 P faces selected from four experiments. We established three density ranges, each comprising one-third of the P faces: low label (0–20 particles per μm^2), medium label (20–55 particles per μm^2), and high label (55–200 particles per μm^2).

Labeling of P faces could also result from reorganization of putative glycolipid (or glycoprotein or both) molecules associated with the inner half of the plasma membrane with their oligosaccharides exposed at the inner surface (an unlikely possibility). To rule this out, we observed P faces of cells that had been densely coated with cationized ferritin (1 mg/ml; 4°C, 15 min; see ref. 8) prior to fracture and then post-fracture labeled with WGA and ovomucoid-coated colloidal gold. In these preparations, the P faces were not labeled, presumably because the cationized ferritin anchored the WGA receptor sites in such a way that they all partition with the outer membrane half (i.e., that the WGA sites observed over the P faces do not originate from oligosaccharides exposed at the inner surface of the plasma membrane). Labeling of the E faces was much reduced (not illustrated).

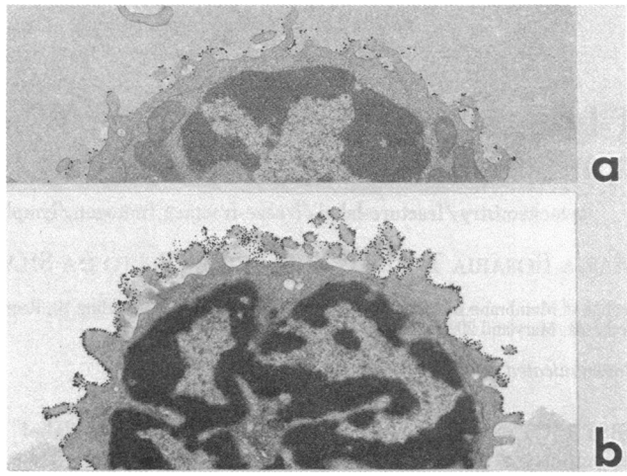

FIG. 1. Controls. (*a*) B cells and T cells bearing Fc receptors for IgG are identified in the gel by treatment with the IgG fraction of goat anti-human Ig followed by treatment with protein A-coated colloidal gold. (*b*) In gels cut at room temperature, cell surfaces are exposed and intensely labeled by WGA and ovomucoid/colloidal gold. (*a*, ×9,900; *b*, ×8,100.)

DISCUSSION

Our results show that, during freeze fracture of the plasma membranes of human T lymphocytes, various proportions of WGA-binding terminal oligosaccharides associated with trans-

‡ Labeling of E faces (Fig. 2 *e* and *f*) at first appears a paradox. It is made possible by post-fracture reorganization of membrane components, a process that occurs on thawing and exposure to a hydrophilic environment (8, 10, 12). In addition, this process, as well as the partition of some transmembrane proteins (involving dragging of the external part of these molecules across the exoplasmic half of the membrane), creates structural defects on the E face that provide access to the lectins and their markers. No attempt was made to relate the amount of surface labeling to the additive labeling of E and P faces. Cytochemical techniques are not adequate to accurately reflect the absolute number of lectin binding sites: inaccessibility to the label of the binding sites either because they are not exposed externally or because of mutual hindrance of the lectins and their markers are common problems aggravated here by fracture and post-fracture processes. Discussion of the ultrastructure, reorganization, and labeling of E faces is not necessary for the analysis of the heterogeneity of P-face labeling (the object of this paper) and is presented elsewhere (8–12).

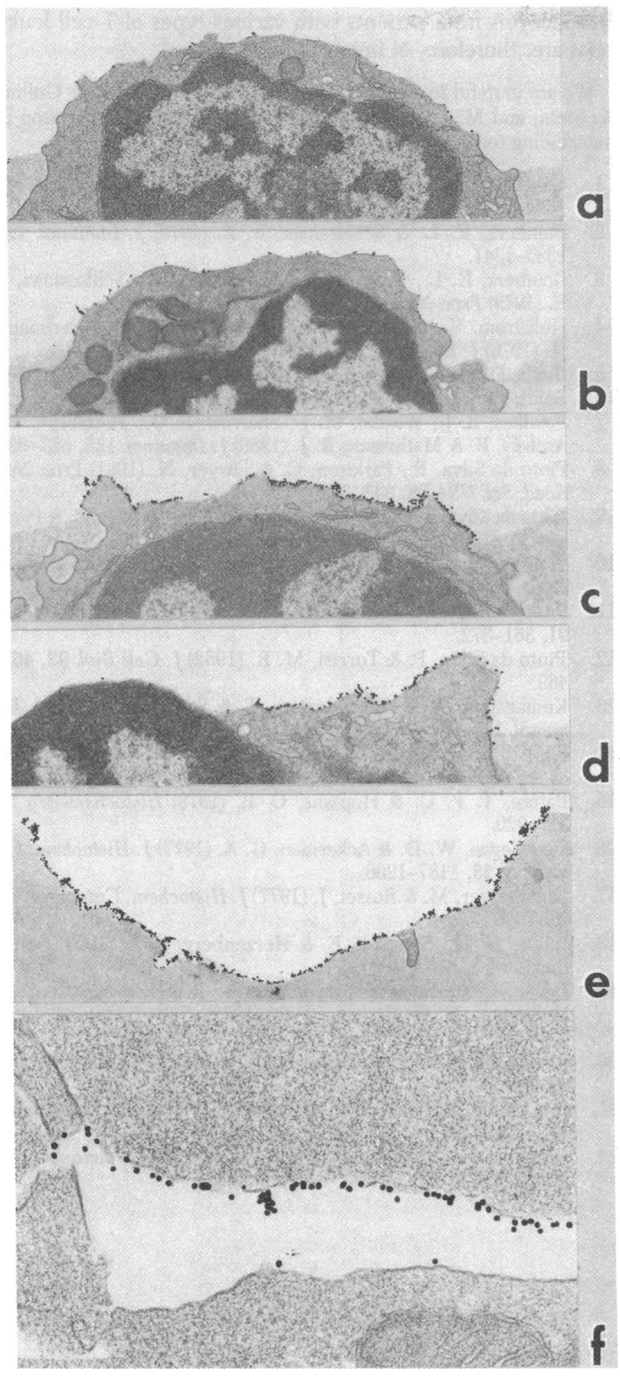

FIG. 2. Thin-section views of human T cells fracture-labeled by WGA and ovomucoid/colloidal gold. Over the P faces, labeling intensity is variable (*a–c*); over the E faces, it is always intense (*e* and *f*). Even when the WGA label is intense over the P face, it is absent over the fractured nuclear envelope membrane or cross-fractured cytoplasm (*d*). (*a*, ×10,800; *b*, ×9,900; *c*, ×13,500; *d*, ×19,800; *e*, ×11,700; *f*, ×53,100.)

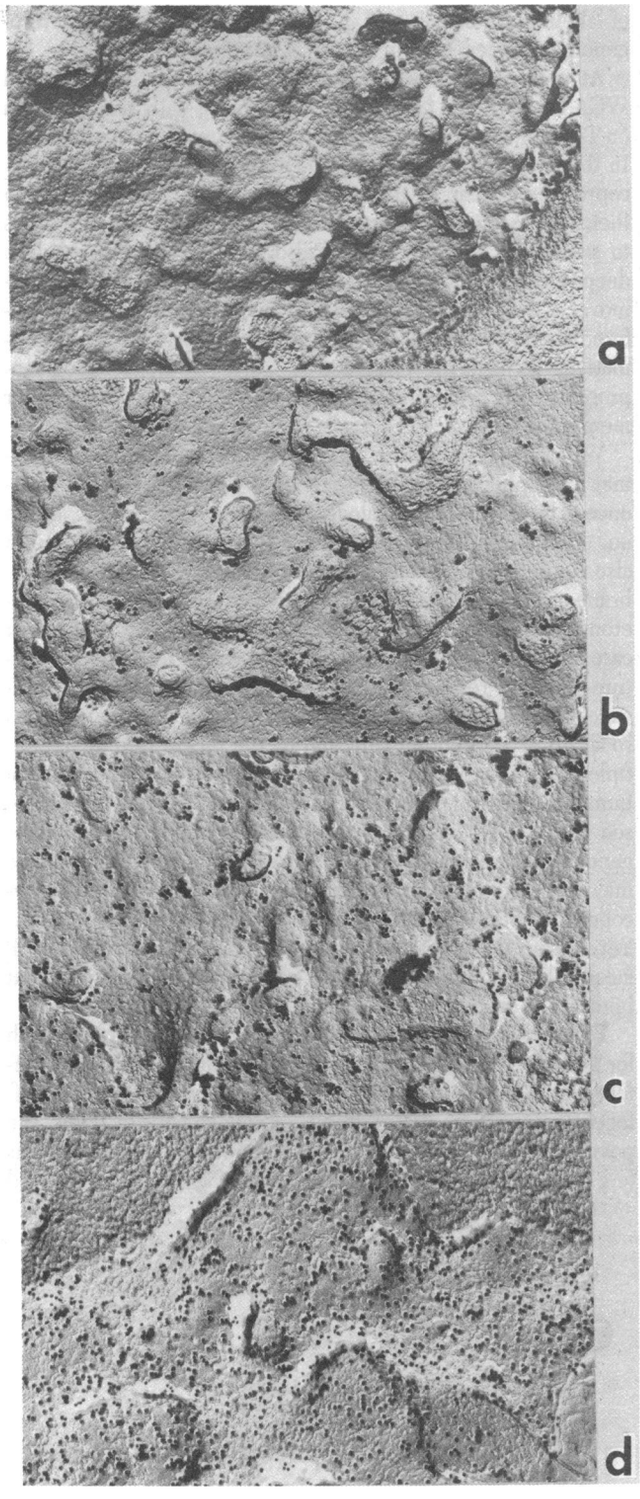

FIG. 3. Pt/C replicas of fractured and critical point-dried purified T cells. The WGA label on the P faces is uniformly distributed and ranges from virtual absence (*a*) to very high density (*d*). (×27,900.)

membrane protein(s) are dragged with the protoplasmic membrane half across the outer (exoplasmic) half. Labeling of the E face probably reflects the presence of peripheral membrane proteins, integral membrane proteins that partition with the exoplasmic face and, possibly, glycolipids. In erythrocytes, where detailed knowledge of the structure and organization of membrane proteins is available, we have shown that lectin binding sites expressed at the outer surface by the main transmembrane proteins can be dragged across the exoplasmic membrane half during fracture: most Con A binding sites associated with band 3, as well as a minority of glycophorin-associated WGA binding sites, are labeled on the P face (12). In lymphocytes and other eukaryotic cells, fracture-label experiments show that most Con A binding sites consistently partition with E faces (10, 11). In addition, for each cell type observed so far, the patterns

of labeling are invariant; i.e., they do not display the heterogeneity reported here in human T lymphocytes.

At present, opportunities for correlation of the pattern of WGA labeling of P faces, as reported here, with available data on T-lymphocyte plasma membrane glycoproteins are limited. In thymus-derived lymphocytes, most plasma membrane glycoproteins appear to contain terminal sialic acid residues on their oligosaccharide chains (6, 19). WGA recognizes and binds to sialic acid, although it can also bind to GlcNAc residues deeper in the oligosaccharide chains that are revealed after removal of the terminal sialic acid by neuraminidase (20). Therefore, labeling by WGA of P faces of freeze-fractured T-cell populations reflects the existence of transmembrane sialoglycoprotein(s) that preferentially partition on fracture with the inner membrane half.

We propose, therefore, that the plasma membranes of human T lymphocytes are heterogeneous with respect to the presence of transmembrane sialoglycoproteins. Conceivably, but in our opinion less likely, individual differences in labeling might also result from differences in the association of the transmembrane proteins with putative components of the membrane skeleton, resulting in differential partition on fracture. In either case, heterogeneity of T-cell populations, as revealed by fracture-labeling, retains possible operational value and a significance that is reinforced by the homogeneous labeling patterns so far observed in other cells and by the recent finding of multiple WGA-binding glycoproteins in the plasma membranes of human T cells (19). The effect of WGA (stimulatory/inhibitory; see refs. 21, 22) appears ambiguous and the binding heterogeneity that we report may indeed reflect this ambiguity. One may speculate on the role that these transmembrane sialoglycoproteins play in signaling the contradictory effects of WGA. Yet, as Con A—a clear mitogen—does not bind to sites over P faces (10), it is unlikely that the transmembrane proteins that bind WGA contain receptor sites for Con A.

Fracture-labeling appears to open a new approach to search for and characterize lymphocyte subpopulations based on differences in the expression of transmembrane proteins. The effects of stimulatory and inhibitory concentrations of WGA, the possible correlation with existent lymphocyte subpopulations (e.g., helper/suppressor), and the screening of fracture-labeled lymphocytes from patients with various types of T-cell leukemias are, therefore, of immediate concern.

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1. Cantor, H., Shen, F. W. & Boyse, E. A. (1976) *J. Exp. Med.* **143**, 1391–1401.
2. Reinherz, E. L. & Schlossman, S. F. (1979) *J. Immunol.* **122**, 1335–1341.
3. Reinherz, E. L., Kung, P. C., Goldstein, G. & Schlossman, S. F. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4061–4065.
4. Hellstrom, V., Dillner, M. L., Hammarstrom, S. & Perlmann, H. (1976) *J. Exp. Med.* **144**, 1381–1385.
5. Boldt, D. H. & Lyons, R. D. (1979) *J. Immunol.* **123**, 808–816.
6. Boldt, D. H. (1979) *Mol. Immunol.* **17**, 47–55.
7. Fowlkes, B. J., Waxdal, M. J., Sharrow, S. O., Thomas, C. A., Asofsky, R. & Mathieson, B. J. (1980) *J. Immunol.* **125**, 623–630.
8. Pinto da Silva, P., Parkison, C. & Dwyer, N. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 343–347.
9. Pinto da Silva, P., Kachar, B., Torrisi, M. R., Brown, C. & Parkison, C. (1981) *Science* **213**, 230–233.
10. Pinto da Silva, P., Parkison, C. & Dwyer, N. (1981) *J. Histochem. Cytochem.* **29**, 917–928.
11. Pinto da Silva, P., Torrisi, M. R. & Kachar, B. (1981) *J. Cell Biol.* **91**, 361–372.
12. Pinto da Silva, P. & Torrisi, M. R. (1982) *J. Cell Biol.* **93**, 463–469.
13. Kumagai, K., Abo, T., Sekizawa, T. & Sasaki, M. (1975) *J. Immunol.* **115**, 982–987.
14. Roth, J., Bendayan, M. & Orci, L. (1978) *J. Histochem. Cytochem.* **26**, 1074–1081.
15. Batten, T. F. C. & Hopkins, C. R. (1979) *Histochemistry* **60**, 317–320.
16. Geoghegan, W. D. & Ackerman, G. A. (1977) *J. Histochem. Cytochem.* **25**, 1187–1200.
17. Horisberger, M. & Rosset, J. (1977) *J. Histochem. Cytochem.* **25**, 295–305.
18. Julius, M. H., Simpson, E. & Herzenberg, L. A. (1973) *Eur. J. Immunol.* **3**, 645–649.
19. Dillner-Centerlind, M. L., Axelsson, B., Hammarstrom, S., Hellstrom, U. & Perlmann, P. (1980) *Eur. J. Immunol.* **10**, 434–442.
20. Bhavanandan, V. P. & Katlic, A. W. (1979) *J. Biol. Chem.* **254**, 4000–4008.
21. Gordon, L. K., Hamill, B. & Parker, C. W. (1980) *J. Immunol.* **125**, 814–819.
22. Greene, W. C. & Waldmann, T. A. (1980) *J. Immunol.* **124**, 2979–2987.