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Hapten–Conjugated Antibodies and Visual Markers Used to Label Cell-Surface Antigens for Electron Microscopy: An Approach to Double Labeling

(cell membranes/histocompatibility antigens/hybrid antibodies)

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ABSTRACT **Haptens can be coupled to mouse immunoglobulin with retention of antibody specificity. Such hapten-coupled alloantibody, when bound to cells, can be bridged to an electron microscopic marker in two ways: (*i*) by a hybrid antibody, i.e., anti-hapten:anti-marker, and (*ii*) by untreated bivalent anti-hapten antibody if the marker is also haptenized. Through the use of immunologically non-crossreactive haptens and markers it should be possible to localize multiple, different antigens on the surface of an individual cell. Double labeling has been achieved with the first method.**

Several different markers have been used to locate antigens of cell surfaces by electron microscopy. Initially, ferritin was directly conjugated to antibody (1). Subsequently, ferritin (2), southern bean mosaic virus (SBM virus) (3), and tobacco mosaic virus (4) were bridged to specific antibody on the surface of cells by hybrid $F(ab')_2$-fragments of antibody in which one valence was directed to the cell-bound antibody and the other valence to the marker. The availability of morphologically distinct markers for electron microscopy offers a challenge to develop methods for labeling different antigens on the surface of the same cell. This result is not readily possible with the original hybrid antibody method because the anti-immunoglobulin portion of the hybrid does not discriminate between antisera of different specificities.

We present here two methods, using haptens, by which this goal may be achieved. In the first method (*hybrid antibody bridge method*), a hapten is coupled to a specific antibody, which is then allowed to bind to cells. The cell-bound antibodies are, in turn, bridged to visual markers by hybrid $F(ab')_2$-fragments, of which one valence is directed to the hapten and the other valence to the marker. In the second method (*untreated anti-hapten antibody bridge method*), the hapten is coupled to both the specific antibody and the visual marker. Cells are reacted with hapten-coupled antibody, which is then bridged to the hapten-coupled marker by bivalent anti-hapten antibody. We have used both methods for locating alloantigens on the surfaces of mouse lymphoid cells. Schematic diagrams indicating how these two methods could be applied to labeling two different alloantigens on the same cell are shown in Figs. 1 and 2.

Abbreviations; SBM virus, southern bean mosaic virus; B6, C57BL/6; BzPen, benzylpenicilloyl hapten; NIP, 4-hydroxy-3-iodo-5-nitro-phenylacetyl hapten; PO_4–NaCl, phosphate-buffered saline, pH as specified.

MATERIALS AND METHODS

Mouse Alloantisera. Anti-H-2^b antiserum was prepared by immunization of (B6/H-2^k‡ x AKR)F_1 mice with the C57BL ascites leukemia EL4. Anti-H-2^k antiserum was prepared by immunization of AKR/H-2^b§ mice with the AKR strain leukemia K36, or normal lymphoid cells from AKR mice. These antisera were chosen for double labeling because they should not have specificities in common.

Rabbit Antisera. Rabbit antibody to the benzylpenicilloyl (BzPen) hapten was purified (5) from the serum of rabbits immunized with 1 mg of BzPen–bovine IgG in complete Freund's adjuvant, boosted intravenously (3 mg) at 4 weeks, and bled during the fifth and sixth weeks after initial immunization. Rabbit antibody to the 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP) hapten (6) was extracted from the serum of rabbits immunized with NIP–human IgG by an immunoadsorbant of insolubilized NIP–bovine-serum albumin. Preparation and purification of rabbit antibody against ferritin and SBM virus have been described (2, 3).

Visual Markers. Horse-spleen ferritin (Nutritional Biochemicals; twice crystallized, cadmium-free) was diluted 10-fold with 0.1 M sodium phosphate (pH 7.5) and centrifuged at 40,000 rpm in a Spinco Ti50 rotor for 1 hr at 4°. The pellet was redissolved in buffer at a concentration of 15 mg/ml and stored at 4°. Before use, it was dialyzed against 0.15 M NaCl. SBM virus was prepared as described (3).

Coupling of Haptens to Antibodies and Visual Markers. A crude immunoglobulin fraction of alloantiserum was prepared by precipitation with 40% ammonium sulfate and dialysis at 10 mg/ml against 0.5 M $NaHCO_3$ (pH 8.6). A solution of 40 mg of NIP-azide (6) in 1 ml of dimethylformamide was prepared, and 0.5 mg of NIP-azide (13 μl) per 10 mg of protein was added at 4°. The mixture was stirred overnight at 4° and dialyzed exhaustively against phosphate-buffered saline (PO_4–NaCl) (pH 7.2). The degree of substitution was determined accord-

‡ Congenic stock, differing genetically from the C57BL/6 (H-2^b) strain (abbreviated B6) only at the *H-2* locus, by substitution of an *H-2^k* allele (from an AKR strain) for the H-2^b allele.

§ Congenic stock with an *H-2^b* allele (from a B6 strain) substituted for an *H-2^k* allele.

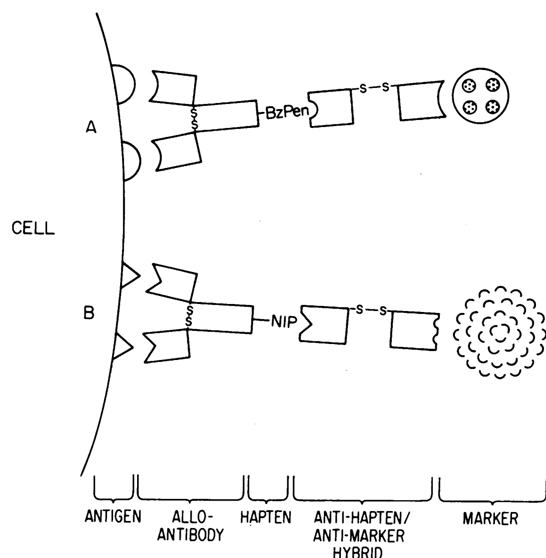

FIG. 1. Schematic diagram of double-labeling of cell-surface antigens by the hybrid antibody bridge method. Antigen A is labeled with ferritin, bridged to BzPen-coupled anti-A alloantibody by an anti-BzPen: anti-ferritin hybrid antibody. Antigen B is labeled with SBM virus, bridged to NIP-coupled anti-B alloantibody by an anti-NIP: anti-SBM virus hybrid. Double labeling has been achieved with this system.

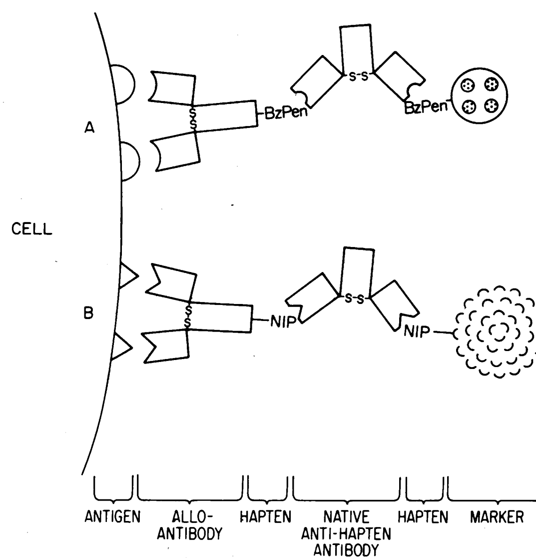

FIG. 2. Double-labeling of cell surface antigens by the untreated anti-hapten antibody bridge method. Labeling with SBM virus in this manner has not yet been achieved due to disruption of the virus during haptenization.

ing to Brownstone *et al.* (6); a ratio of about 10 NIP groups per molecule is obtained under these conditions.

BzPen groups were coupled to whole antiserum (diluted 1:2 with saline) and to ferritin (3 mg/ml) by reaction with potassium penicillin G (70 mg/ml of antiserum and 0.8 mg/mg of ferritin) at pH 11.5 and 10.5, respectively, in a pH-stat (5). After incubation for 1 hr at room temperature, the hapten-coupled reagents were dialyzed against PO_4–NaCl (pH 8.0). The BzPen–ferritin was centrifuged for 10 min at 10,000 rpm ($12,000 \times g$) and the precipitate (about 20% of the total) was discarded. The supernatant, which specifically precipitated rabbit anti-BzPen antibody in immunodiffusion tests, was centrifuged for 10 min at 1000 rpm ($100 \times g$) and filtered through a 0.65-μm Millipore filter immediately before use. BzPen–ferritin tends to aggregate; for best results it should be prepared shortly before use.

All hapten-coupled antisera retained their specificities and titers, as measured by cytotoxicity and hemagglutination.

Hybrid Antibodies. The hybrid antibody, rabbit anti-NIP/rabbit anti-SBM virus, was prepared by the method of Nisonoff (7), as described by Hämmerling *et al.* (2, 3). The hybrid, rabbit anti-BzPen/rabbit anti-ferritin, was prepared similarly, except that the $F(ab')_2$-fragments were purified by precipitation with 18% Na_2SO_4 (8) instead of gel filtration.

Labeling of Cells. Test cells, obtained from lymph nodes of mouse strains B6(H-2^b), AKR/H-2^b, AKR (H-2^k), B6/H-2^k, and (B6 x AKR)F_1 (H-2^b/H-2^k), were kept viable at 4° throughout the labeling procedure. After they were washed with Earle's balanced salt solution, 5 to 7×10^6 cells, in volumes of 0.2 ml, were incubated in one of the following sequences of reagents:

Hybrid Bridge (Single Label). (*i*) NIP- or BzPen-coupled alloantiserum diluted 1:10; (*ii*) hybrid antibody, 0.04 mg; (*iii*) ferritin (0.2 mg) or SBM virus (0.1 mg).

Hybrid Bridge (Double Label). (*i*) NIP- and BzPen-coupled alloantisera, both diluted 1:5; (*ii*) anti-BzPen:anti-ferritin and anti-NIP:anti-SBM virus, 0.04 mg of each; (*iii*) ferritin and SBM virus, 0.1 mg of each.

Bivalent Anti-Hapten Bridge. (*i*) BzPen-coupled alloantiserum, diluted 1:5; (*ii*) rabbit anti-BzPen antibody, 0.6 mg; (*iii*) BzPen-coupled ferritin, 0.5 mg.

In all cases, incubations were for 30 min, followed by two washings with Earle's salt solution.

TABLE 1. *Analysis of labeling by the hybrid antibody bridge method*

Specificity of lymph node cells	Hapten-coupled alloantiserum	Hybrid antibody	Percent of cell surface labeled by marker*
H-2^b	Anti-H-2^b–NIP	aNIP: aSBM virus	23
H-2^k	Anti-H-2^b–NIP	aNIP: aSBM virus	<1
H-2^k	Anti-H-2^k–BzPen	aBzPen: aFerritin	12
H-2^b	Anti-H-2^k–BzPen	aBzPen: aFerritin	<1
H-2^b	Anti-H-2^b–NIP	aNIP: aSBM virus	22
	plus	plus	
	Anti-H-2^k–BzPen	aBzPen: aFerritin	<1
H-2^k	Anti-H-2^b–NIP	aNIP: aSBM virus	<1
	plus	plus	
	Anti-H-2^k–BzPen	aBzPen: aFerritin	15
H-2^b/H-2^k	Anti-H-2^b–NIP	aNIP: aSBM virus	14
	plus	plus	
	Anti-H-2^k–BzPen	aBzPen: aFerritin	6

* Percent of the total perimeter labeled (map measuring device) in sections from 25 cells.

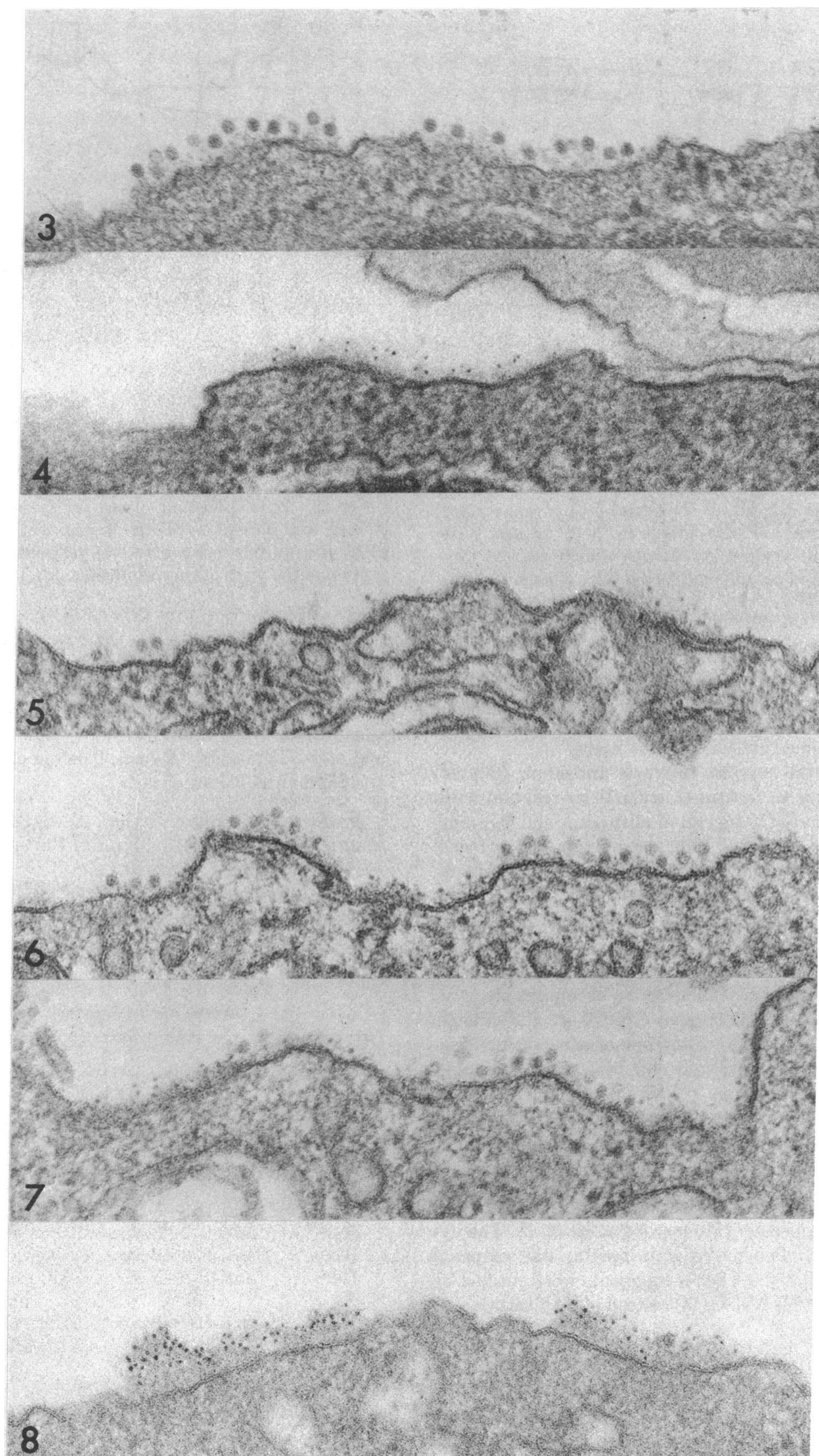

FIG. 3. H-2^b lymph-node lymphocyte reacted with anti-H-2^b alloantiserum coupled to NIP, then with anti-NIP: anti-SBM virus hybrid, and finally with SBM virus. Stained with uranyl acetate and lead citrate. $\times$100,000.

Electron Microscopy. Cell pellets were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 15 min, post-fixed with 1% cacodylate-buffered OsO_4 for 1 hr, treated overnight with 0.5% aqueous uranyl acetate (pH 4.6) (9), dehydrated with ethanol, and embedded in Epon. Thin sections were stained with 5% uranyl acetate in methanol and lead citrate (10), or examined unstained with a Zeiss EM 9S-2 electron microscope.

RESULTS

Hybrid bridge

H-2^b cells incubated with anti-H-2^b coupled to NIP, anti-NIP: anti-SBM virus hybrid, and SBM virus, and H-2^k cells incubated with anti-H-2^k coupled to BzPen, anti-BzPen: anti-ferritin hybrid, and ferritin, are labeled in discontinuous sectors typical of the hybrid antibody method (Figs. 3 and 4) (2, 3). The significantly smaller percentage of the cell surface labeled in the H-2^k system than in the H-2^b system (Table 1) may reflect different labeling efficiencies, a potentially important factor in any attempt at double labeling.

As indicated in Table 1, both haptenized alloantibody–hybrid antibody systems are specific for the corresponding antigens, whether reacted separately or in combination. In a double-labeling experiment, lymph node cells from H-2^b/H-2^k heterozygous mice were reacted with both labeling systems simultaneously. The extent of labeling with each marker on these cells is considerably reduced compared to homozygous cells (Table 1), an expected finding if the total cell surface complement of H-2 antigen remains constant, whether composed of the products of the same or different alleles. In most cases, ferritin and SBM virus label mutually exclusive sectors of the cell surface, whether these sectors are distant or adjacent (Figs. 5 and 6).

To determine whether or not this segregation of SBM virus and ferritin markers into separate sectors indicates regional segregation of H-2^b and H-2^k alloantigens on the cell surface, the following control was performed: homozygous H-2^b cells were reacted simultaneously with two samples of the same anti-H-2^b alloantiserum, one sample coupled to BzPen, the other coupled to NIP; cells were subsequently incubated with anti-NIP: anti-SBM virus and anti-BzPen: anti-ferritin hybrids simultaneously, and were finally incubated with SBM virus and ferritin. Ferritin and SBM virus again appeared in separate sectors (Fig. 7), although the ferritin and SBM virus sectors were generally closely apposed. Replacement of the anti-NIP: anti-SBM virus and anti-BzPen: anti-ferritin hybrids with anti-mouse immunoglobulin: anti-SBM virus and anti-mouse immunoglobulin: anti-ferritin resulted in complete intermixing of ferritin and SBM virus within the same sectors, a result that indicates that steric interference or related phenomena between the different markers are not responsible for their segregation. It is possible that the segregation of markers is a secondary phenomenon, perhaps reflecting the extent of conjugation of alloantibody with hapten and/or the presence of some homologous anti-hapten: anti-hapten recombinants.

Bivalent anti-hapten bridge

H-2^k lymph node cells incubated with BzPen coupled anti-H-2^k alloantiserum, rabbit anti-BzPen, and BzPen–ferritin, are labeled in a discontinuous manner similar to that observed with other indirect procedures (2, 11, 12). The marked clustering of ferritin within labeled sectors (Fig. 8) is similar to that obtained with ferritin chemically coupled to anti-mouse IgG from rabbit (2). The extent of clustering varies somewhat with different preparations of BzPen–ferritin. The dense material interspersed with ferritin on the labeled cells is probably rabbit anti-BzPen antibody. Labeling is specific, as indicated by the absence of ferritin on H-2^b cells reacted as above or on H-2^k cells reacted in the usual manner, but with omission of BzPen-coupled alloantiserum and/or rabbit anti-BzPen.

DISCUSSION

Indirect methods that use anti-globulin reagents cannot readily be used to discriminate between alloantisera of differing specificity on the same cell; for this reason, it has not previously been possible to achieve double labeling. The present approach is based on the supposition that alloantibodies of differing specificities could be distinguished by coupling them to different, non-crossreacting haptens. We have found that hapten-coupled alloantisera retain their specificities and can be linked in two different ways to visual markers. In both cases, various control experiments indicate that the labeling systems are reproducible and specific. With the hybrid bridge method, double labeling was achieved. In the case of the untreated anti-hapten bridge method, we have not yet succeeded in coupling haptens to SBM virus without damaging the virus. We intend to pursue this goal because for certain purposes labeling antigens with an untreated anti-hapten bridge offers the advantage of ease of preparation. The two methods could also be used conjointly.

In all cases studied the distribution of surface antigens was discontinuous, as reported (2, 3, 11, 12). Although all experiments were performed at 4° to minimize possible secondary rearrangements of cell-surface components (13, 14), we are hesitant to interpret the finding that H-2^b and H-2^k antigens on heterozygous cells became labeled in separate sectors, particularly in view of the control experiment in which there was some segregation of the two markers even though the two haptens had been coupled to different aliquots of the same alloantiserum.

The methods described here should be generally applicable to studies of macromolecules (e.g., plant agglutinins, hor-

FIG. 4. H-2^k lymphocyte reacted with anti-H-2^k alloantiserum coupled to BzPen, then with anti-BzPen: anti-ferritin hybrid, and finally with ferritin. Stained with lead citrate. ×100,000.

FIGS. 5 and 6. H-2^b/H-2^k heterozygous lymphocyte reacted with anti-H-2^b–NIP and anti-H-2^k–BzPen, then with anti-NIP: anti-SBM virus and anti-BzPen: anti-ferritin, and finally with SBM virus and ferritin. In Fig. 5, the SBM virus and ferritin sectors are widely separated, while in Fig. 6 they are adjacent. Stained with lead citrate. ×100,000.

FIG. 7. H-2^b lymphocyte reacted with anti-H-2^b-NIP and anti-H-2^b–BzPen, then with anti-NIP: anti-SBM virus and anti-BzPen: anti-ferritin, and finally with SBM virus and ferritin. Ferritin and SBM virus sectors are partially segregated. Stained with lead citrate. ×100,000.

FIG. 8. H-2^k lymphocyte reacted with anti-H-2^k–BzPen, then with bivalent rabbit anti-BzPen, and finally with BzPen–ferritin. Unstained. ×100,000.

mones, etc.) that bind specifically to constituents of cell surfaces.

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1. Singer, S. J. & Schick, A. F. (1961) *J. Biophys. Biochem. Cytol.* **9**, 519-537.
2. Hämmerling, U., Aoki, T., de Harven, E., Boyse, E. A. & Old, L. J. (1968) *J. Exp. Med.* **128**, 1461-1473.
3. Hämmerling, U., Aoki, T., Wood, H. A., Old, L. J., Boyse, E. A. & de Harven, E. (1969) *Nature* **223**, 1158-1159.
4. Aoki, T., Wood, H. A., Old, L. J., Boyse, E. A., de Harven, E., Lardis, M. P. & Stackpole, C. W. (1971) *Virology* **45**, 858-862.
5. Levine, B. B. & Levytska, V. (1969) *J. Immunol.* **102**, 647-655.
6. Brownstone, A., Mitchison, N. A. & Pitt-Rivers, R. (1966) *Immunology* **10**, 465-479.
7. Nisonoff, A. & Rivers, M. M. (1961) *Arch. Biochem. Biophys.* **93**, 460-462.
8. Nisonoff, A. (1964) *Methods Med. Res.* **10**, 134-141.
9. Farquhar, M. G. & Palade, G. E. (1965) *J. Cell Biol.* **26**, 263-291.
10. Reynolds, E. S. (1963) *J. Cell Biol.* **17**, 208-212.
11. Silvestre, D., Kourilsky, F. M., Niccolai, M. G. & Levy, J. P. (1970) *Nature* **228**, 67-68.
12. Nicolson, G. L., Hyman, R. & Singer, S. J. (1971) *J. Cell Biol.* **50**, 905-910.
13. Frye, L. D. & Edidin, M. (1970) *J. Cell Sci.* **7**, 319-335.
14. Taylor, R. G., Duffus, W. P. H., Raff, M. C. & de Petris, S. (1971) *Nature* **233**, 225-229.