

## Hybrid Antibody-Induced Topographical Redistribution of Surface Immunoglobulins, Alloantigens, and Concanavalin A Receptors on Mouse Lymphoid Cells

(cell surface/immunofluorescence/cap formation/ferritin)

CHRISTOPHER W. STACKPOLE, LAWRENCE T. DE MILIO, ULRICH HÄMMERLING, JANET B. JACOBSON, AND MICHAEL P. LARDIS

Memorial Sloan-Kettering Cancer Center, New York, N.Y. 10021

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**ABSTRACT** Redistribution of surface immunoglobulins, H-2<sup>b</sup>, Thy-1.2, and TL.1,2,3 alloantigens, and concanavalin A receptors on mouse lymphoid cells induced by hybrid rabbit F(ab')<sub>2</sub> antibody (anti-mouse immunoglobulin/anti-visual marker or anti-concanavalin A/anti-visual marker) was studied by immunofluorescence. When used directly to label surface immunoglobulin, and indirectly to label alloantigens and concanavalin A receptors, hybrid antibodies induced similar displacement of all surface components from a uniform distribution into "patches" and "caps" at 37°. One hybrid antibody preparation, anti-mouse immunoglobulin/anti-ferritin, contained negligible amounts of bivalent anti-mouse immunoglobulin antibody, and was therefore "monovalent" for the anti-mouse immunoglobulin specificity. This observation suggests that factors other than multivalent crosslinking are responsible for hybrid antibody-induced redistribution of cell-surface components. Cap formation induced by hybrid antibody was enhanced markedly by attachment of the visual marker, either ferritin or southern bean mosaic virus, at 37°. At -5°, hybrid antibody does not displace uniformly distributed H-2<sup>b</sup> alloantigen-alloantibody complexes, but patches of label develop when ferritin attaches to the hybrid antibody. These results explain the patchy distribution of cell-surface components, which is a temperature-independent characteristic of labeling with hybrid antibodies and visual markers for electron microscopy.

When mouse cell-surface alloantigens are labeled indirectly for electron microscopy with mouse alloantibody, then hybrid rabbit F(ab')<sub>2</sub> antibody directed against both mouse immunoglobulin G (IgG) and a visual marker such as ferritin, and finally the visual marker, they are typically represented as "patches" separated by unlabeled regions of the cell surface (1-3). A more uniform distribution of label is observed when H-2 alloantigen is visualized directly with ferritin-conjugated alloantibody (4). This uniform label can be displaced into patches by subsequent attachment of bivalent antibody against IgG (5). Aggregation and redistribution of surface IgG molecules and alloantigen-alloantibody com-

plexes on mouse lymphocytes induced by antibody against IgG has also been demonstrated by immunofluorescence (6). A uniform label, obtained at low temperature, transforms rapidly at higher temperatures into patches of label that coalesce into a crescent-shaped "cap" over the Golgi region of the cell. The apparent requirement of bivalent antibody to induce redistribution suggested that molecular crosslinking of individual components was involved (5, 7).

Since immunoferritin labeling with hybrid antibody results in a distribution of label similar to the initial stage of bivalent antibody-induced redistribution, hybrid antibody may also cause topographical displacement of cell-surface components. We have examined the effectiveness of hybrid antibody in inducing redistribution of five different surface components [surface IgG, receptors for the plant agglutinin concanavalin A (Con A), and H-2, Thy-1 (formerly  $\theta$ ), and TL alloantigens] on mouse thymus, lymph node, or spleen cells, by immunofluorescence. Redistribution of all components similar to that induced by bivalent antibody (6) was observed. We have also attempted to explain the following apparent discrepancies between labeling characteristics of hybrid antibody and a molecular crosslinking interpretation of induced redistribution: (i) Monovalent (Fab') fragments of antibody against IgG are ineffective in displacing surface components into either patches or caps, apparently because of an inability to crosslink these molecules (6, 8, 9). Yet, redistribution can be induced by hybrid antibody, which consists of Fab' fragments of two different specificities combined into a single molecule and should behave as a monovalent antibody (1). (ii) "Weak" antigens (i.e., antigen determinants are sparsely represented on the cell surface), such as TL or H-2 alloantigens on thymus cells, appear as several small patches widely dispersed over the cell surface when labeled with hybrid antibody and ferritin at low temperatures (2, 3). The extensive lateral movements of surface components that would seem to be required to aggregate more uniformly distributed molecules into the observed patches should be restricted by the high viscosity of the cell-surface membrane at these temperatures (10, 11).

### MATERIALS AND METHODS

Mice were obtained from our colonies and included strains A, C57BL/6, (B6), and BALB/c; and the congenic stock, B6/TL+.

Abbreviations: IgG, immunoglobulin G; anti-M IgG/anti-F, hybrid antibody against mouse IgG and ferritin; B6, C57BL/6; Con A, concanavalin A; F, ferritin; FI, fluorescein isothiocyanate; G anti-R IgG/FI, fluoresceinated antibody against rabbit IgG prepared in goat; R anti-F/FI, fluoresceinated rabbit antibody against ferritin; R anti-M IgG, antibody against mouse IgG prepared in rabbit; SBMV, southern bean mosaic virus.

**Mouse Alloantisera.** Anti-H-2<sup>b</sup> alloantiserum was prepared by immunizing (B6/H-2<sup>k</sup> × A)F<sub>1</sub> mice with the C57BL ascites leukemia EL4, and had a cytotoxic titer of 1:320 on B6 lymph-node cells, and 1:8 on B6 thymus cells. Anti-Thy-1.2 alloantiserum was prepared by immunizing (A/Thy-1.1 × AKR/H-2<sup>b</sup>)F<sub>1</sub> mice with the A-strain spontaneous leukemia ASL1. Anti-TL1,2,3 alloantiserum was prepared by immunizing (A/TL- × B6)F<sub>1</sub> mice with ASL1, and had a titer of 1:64,000 on B6/TL+ mice.

**Hybrid Antibodies.** Rabbit F(ab')<sub>2</sub> hybrid recombinants, anti-mouse immunoglobulin G/anti-ferritin (anti-M IgG/anti-F), anti-immunoglobulin G/anti-SBMV (southern bean mosaic virus), anti-mouse Fc fragment/anti-ferritin, anti-mouse Fc fragment/anti-SBMV, and anti-Con A/anti-ferritin were prepared according to Hämmerling *et al.* (1, 12, 13). Anti-M IgG/anti-F hybrid was purified exhaustively with a ferritin-cellulose immunoabsorbent (13) to remove anti-M IgG/anti-M IgG homologous recombinants (1) for use in some experiments.

**Preparation of Reagents.** Fluoresceinated anti-rabbit IgG prepared in goat (G anti-R IgG/FI) specific for rabbit IgG by immunoelectrophoresis was obtained from Hyland Laboratories, Costa Mesa, Calif., and was used at a protein concentration of 300 µg/ml. Con A (three times crystallized; Miles Laboratories, Kankakee, Ill.) was purified by elution from a Sephadex G-50 column with D-glucose (14). Purified IgG of anti-H-2<sup>b</sup> alloantiserum was fluoresceinated by the method of Hämmerling *et al.* (in preparation).

**Immunofluorescence.** Thymus, lymph node, or spleen tissue was minced finely in Earle's balanced salt solution containing 5% gamma globulin-free fetal-bovine serum (Grand Island Biological Co., Grand Island, N.Y.). Cells liberated into the medium were collected and washed. Viability was determined by trypan blue exclusion. Cell suspensions used were generally 90–95% viable.

Direct or indirect membrane fluorescence staining was done with 10<sup>6</sup> cells in 50-µl volumes. Immune reagents were diluted in, and cells washed with, Earle's balanced salt solution containing 5% fetal-bovine serum, with the addition of sodium azide where appropriate. Incubations were for 30 min, and cells were maintained in suspension by periodic agitation. After each incubation, cells were washed twice by centrifugation (at 4°) at 600 rpm for 5 min and 1000 rpm for 5 min. For experiments at -5°, all procedures were done in a cold room maintained at that temperature.

Cells were resuspended in 1–2 drops of 50% glycerol in phosphate-buffered saline, pH 7, to which 100 mM sodium azide was added, and examined with a Leitz Orthoplan microscope equipped with an Osram HBO 200W mercury lamp, and BG38 excitor, KP490 interference, and K530 barrier filters. For each sample 100–200 cells were examined, generally at room temperature, although for experiments at -5°, examination and photography were done in a cold room maintained at -5°.

## RESULTS

Surface IgG was visualized on 45–60% of BALB/c spleen cells by incubating cell suspensions with hybrid anti-M IgG/anti-F antibody, then with G anti-R IgG/FI. Control experiments verified that when applied at 0° in the presence

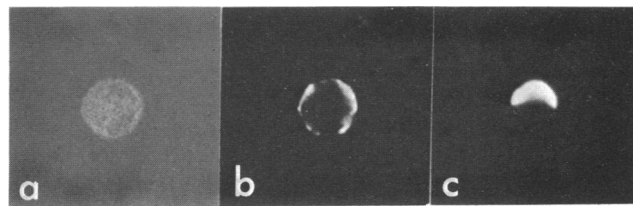


FIG. 1. Immunofluorescence of surface IgG on BALB/c spleen cells. (a) Uniform labeling obtained using 10 µg/ml of hybrid anti-M IgG/anti-F antibody, then G anti-R IgG/FI, at 0°; (b and c) patchy and capped labeling patterns, respectively, obtained with 100 µg/ml of anti-M IgG/anti-F antibody at 37°, and G anti-R IgG/FI at 0°; ×1100.

of 100 mM sodium azide, G anti-R IgG/FI does not cause redistribution of surface-bound molecules that it labels. Therefore, in all experiments, G anti-R IgG/FI was applied to cells in this manner. When cells were incubated with a low concentration of hybrid antibody (1–10 µg/ml) at 0°, a weak fluorescence, uniformly distributed over the cell surface, was observed (Fig. 1a); increasing the temperature to 37° for 30 min after incubation with hybrid antibody at 0° did not alter this labeling pattern. Higher concentrations of hybrid (>50 µg/ml) resulted in a uniform fluorescence on all cells only at -5°, with label coalescing into discontinuous "patches" (Fig. 1b) on many cells within 30 min at 0° (or faster at higher temperatures). If the temperature was raised to 37° after labeling at -5°, a rapid transformation of the patchy pattern to a crescent-shaped "cap" (Fig. 1c) was observed on 50–70% of the cells, the process being completed within 1–2 min.

H-2<sup>b</sup>, Thy-1.2, and TL1,2,3 surface alloantigens (hereafter referred to simply as H-2, Thy-1, and TL antigens) were visualized on B6 lymph node or thymus cells, A thymus cells, and B6/TL+ thymus cells, respectively, by incubating cell suspensions first with the appropriate mouse alloantiserum, then hybrid anti-M IgG/anti-F or anti-mouse Fc fragment/anti-ferritin antibody (100 µg/ml), and finally G anti-R IgG/FI. Hybrid antibody directed against the Fc fragment of mouse IgG was used to discern H-2 alloantibody applied to alloantigen from naturally occurring surface IgG on lymph-node cells, since anti-Fc antibody does not attach to surface IgG(15). The percentage of cells labeled was 75–85% for H-2 (on both thymus and lymph-node cells), 80–90% for Thy-1 on thymus cells, and 40–60% for TL on thymus cells. When cells were incubated at 37° with the appropriate mouse alloantiserum at a very low antibody concentration (1:10,000 for anti-H-2, 1:1000 for anti-Thy-1, and 1:100,000 for anti-TL) and then with hybrid antibody at -5°, uniform labeling was observed; patches developed within 10 min after the temperature was raised to 0°, and caps formed rapidly at 37°. Greater concentrations of mouse alloantiserum, applied at 37°, resulted in the appearance of patches on all cells when hybrid antibody was added at any temperature, even -5°. At 37°, capping occurred in the same manner as for surface IgG, to a maximum extent of 70–80% for TL, 20–30% for H-2 on lymph-node and thymus cells, and 15–20% for Thy-1 antigen. These three antigens were not capped during incubation with mouse alloantiserum alone for 30 min at 37° followed by hybrid antibody and G anti-R IgG/FI at 0°.

TABLE 1. Immunofluorescence analysis of purified and processed\* hybrid antibody

Incubation sequence (with BALB/c spleen cells)†	R anti-M IgG‡ or anti-M IgG/anti-F protein conc. used (µg/ml)	Label
<b>Unprocessed hybrid antibody</b>		
Anti-M IgG/anti-F + F + R anti-F/FI	1	+++
	0.1	++
	0.01	+
	0.001	-
<b>Bivalent antibody</b>		
R anti-M IgG + G anti-R IgG/FI	10	+++
	1	++
	0.1	+
	0.01	-
<b>Controls</b>		
F + R anti-F/FI		-
G anti-R IgG/FI		-
<b>Processed hybrid antibody</b>		
Anti-M IgG/anti-F + F + R anti-F/FI	100	-
Anti-M IgG/anti-F + G anti-R IgG/FI	100	+

\* Hybrid anti-M IgG/anti-F and homologous anti-F/anti-F recombinants were removed by treatment with ferritin.

† All incubations were at 37°. Ferritin (F) was used at a concentration of 100 µg/ml; fluoresceinated rabbit anti-ferritin (R anti-F/FI) and fluoresceinated goat anti-rabbit IgG (G anti-R IgG/FI) were used at 300 µg/ml.

‡ The purified IgG fraction of rabbit anti-mouse IgG (R anti-M IgG) was used.

Con A receptors on BALB/c spleen cells were labeled by incubating cells initially with Con A and then with hybrid anti-Con A/anti-ferritin antibody (100 µg/ml), followed by G anti-R IgG/FI. Uniform labeling resulted from incubating cells with low concentrations (<25 µg/ml) of Con A at 37°, then hybrid antibody and G anti-R IgG/FI, both applied at -5°. Patches and caps developed rapidly at 37°, while patches formed even at -5° when higher concentrations of Con A (>50 µg/ml) were used. After incubation with 100 µg/ml of Con A and hybrid antibody at 37°, and G anti-R IgG/FI at 0°, 20-30% of cells were capped. Cap formation did not occur when cells were incubated with Con A alone at 37° and then with hybrid antibody and G anti-R IgG/FI at 0°.

The following experiment was designed to ascertain whether hybrid antibody-induced redistribution is due to hybrid antibody molecules or to contaminating homologous (bivalent anti-M IgG/anti-M IgG) recombinants (1). Although no bivalent anti-M IgG activity could be demonstrated by Ouchterlony immunodiffusion analysis in a hybrid anti-M IgG/anti-F antibody preparation purified to remove these recombinants (see *Methods*), a significant capacity to induce capping of surface IgG and TL alloantigen remained. A more sensitive assay for the presence of extremely low concentrations of anti-M IgG antibody was found to be a reaction with surface IgG on BALB/c spleen cells, visualized by indirect immunofluorescence (Table 1). With this procedure, as little as 0.01 µg/ml of anti-M IgG/anti-F antibody or 0.1 µg/ml of bivalent rabbit anti-mouse IgG antibody (R anti-M

TABLE 2. Effect of ferritin on hybrid antibody-induced cap formation

	Marker*	°C	% Cells capped
<b>Surface IgG on BALB/c spleen</b>			
Hybrid anti-M IgG/ anti-F antibody	0°	F	0
	37°	-†	3
	37°	F	50
	37°	F	54
	37°	SBMV	37
	37°	SBMV	42
<b>TL.1,2,3 alloantigen on B6/TL+ thymus</b>			
Hybrid anti-M IgG/ anti-F antibody	0°	F	0
	37°	-†	12
	37°	F	70
	37°	F	58
	37°	F	37
	37°	F	92

\* F = ferritin; SBMV = southern bean mosaic virus.

† Cells were incubated with hybrid antibody alone for 60 min at 37°; all other incubations were for 30 min.

IgG) can be detected. Antibody valency cannot be determined, however, since bivalent anti-M IgG antibody must be present in a concentration greater than 10 µg/ml in order to induce capping.

To determine the approximate amount of residual anti-M IgG/anti-M IgG antibody remaining in this purified hybrid antibody preparation, hybrid anti-M IgG/anti-F and bivalent anti-F recombinants were first removed in the following manner: 100 µl of hybrid antibody (500 µg/ml) was mixed with 50 µg of ferritin (removed from a suspension in Earle's balanced salt solution by centrifugation) and incubated for 1 hr at 0°. The ferritin was then removed by centrifugation for 90 min at 40,000 rpm. This process was repeated four times, with fresh ferritin each time.

The amount of anti-M IgG/anti-F antibody remaining in this processed preparation (Table 1) was below the level of visual detection of specific fluorescence (about 0.01 µg/ml), indicating that most anti-ferritin recombinants had been removed by treatment with ferritin. A barely detectable amount (about 0.1 µg/ml) of anti-M IgG activity remained. Even if all of this residual anti-M IgG antibody was bivalent, it would have to be present in at least 100 times higher concentration (>10 µg/ml) in order to induce capping of surface IgG. The hybrid antibody molecule must, therefore, be capable of inducing redistribution of cell-surface components.

For immunoelectron microscopy, hybrid antibodies must be used in conjunction with visual markers such as ferritin or SBMV for visualization. Immunofluorescence allows a separation to be made between the effects of hybrid antibody alone, and hybrid antibody coupled to a marker, on induction of redistribution of cell-surface components.

Table 2 summarizes the effects of hybrid antibody, alone or coupled to ferritin, on capping of surface IgG and TL antigen. Addition of ferritin for 30 min at 0° after incubation with hybrid antibody for 30 min at 37° resulted in levels of capping comparable to those achieved by incubation

with hybrid alone for 60 min at 37°. When ferritin was added at 37°, however, the percentage of cells labeled for surface IgG or TL antigen that formed caps increased considerably. An inappropriate marker (SBMV) does not increase capping of surface IgG, indicating that specific attachment of the marker is required to obtain this enhancing effect. SBMV specifically attached to cell-bound hybrid antibody (anti-mouse IgG/anti-SBMV) similarly enhances capping.

The following experiment was designed to clarify the nature of the interactions among hybrid antibody, ferritin, and cell-bound mouse alloantigen. B6 thymus cells were incubated with 200 µg/ml of fluoresceinated H-2<sup>b</sup> alloantiserum, then with hybrid anti-M IgG/anti-F antibody (purified to remove anti-M IgG/anti-M IgG recombinants), and finally with ferritin, all at -5° in the continuous presence of 100 mM sodium azide (which completely suppresses endocytosis of label). Samples were examined at -5° by fluorescence microscopy after each incubation. Uniform labeling was observed after incubation with fluoresceinated alloantiserum (Fig. 2a). Subsequent incubation with hybrid antibody (100 µg/ml) had no effect on this labeling pattern. After incubation with ferritin (100 µg/ml), however, small patches of label appeared, while the rest of the cell surface retained a weak uniform fluorescence (Fig. 2b). When an aliquot of these cells was fixed for electron microscopy at -5°, the cell surface was labeled with ferritin only in small widely separated patches.

#### DISCUSSION

At the level of resolution of immunofluorescence, surface IgG appears to be distributed uniformly over the surfaces of mouse lymphocytes. This has been determined by labeling in a manner in which redistribution apparently does not occur, such as with fluoresceinated monovalent (Fab') fragments of anti-IgG antibody (6, 8, 9) or bivalent antibody after saturation of the cell surface with Con A (16). Con A receptors and H-2 alloantigen also appear to be uniformly distributed over the surfaces of mouse lymphoid cells when labeled with fluoresceinated Con A or H-2 alloantibody (16, 17). We have observed uniform labeling of surface IgG, alloantigens, and Con A receptors when these components were labeled with low concentrations of primary reagent, with the addition of secondary antibodies at very low temperatures. A uniform distribution by immunofluorescence implies only that components are randomly distributed in a long-range sense, rather than in gross patches (2, 3); a short-range order may exist, which is beyond the scope of immunofluorescence.

Redistribution of a uniform label into patches, and ultimately into caps, apparently occurs in the same basic manner for alloantigens and Con A receptors as for surface IgG. Except for surface IgG, however, patch and cap formation could not be induced by incubation with the primary reagent (mouse alloantiserum or Con A) alone for 30 min at 37°, but required addition of a secondary antibody at high temperature. The main difference between redistribution of surface IgG and alloantigens or Con A receptors may be the extreme ease and rapidity with which surface IgG can be displaced into patches and caps (18).

Basic similarities between induction of patch and cap formation by hybrid and bivalent antibodies (18) indicate that

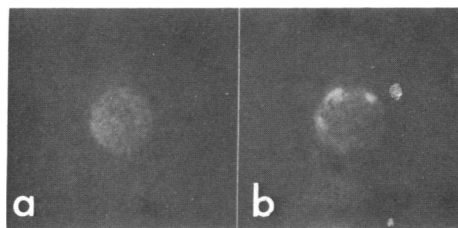


FIG. 2. (a) H-2<sup>b</sup> alloantigen on a B6 thymus cell, labeled with fluoresceinated H-2<sup>b</sup> alloantiserum at -5° in the presence of 100 mM sodium azide, and photographed at -5°; (b) a cell from the same preparation, but labeled additionally with hybrid anti-M IgG/anti-F antibody and ferritin, in the presence of azide; labeling and photography were done at -5°;  $\times 1200$ .

similar mechanisms are involved. Since hybrid antibody containing negligible amounts of bivalent anti-M IgG/anti-M IgG recombinants is capable of inducing typical redistribution of surface components, bivalency may not be a requirement for a reagent to induce topographical displacement of cell surface components. The situation is confusing, however, unless hybrid F(ab')<sub>2</sub> recombinants are shown to possess characteristics quite different from monovalent Fab' antibody fragments, which lack the capacity to induce redistribution (6, 8, 9).

Hybrid antibody might effect redistribution and aggregation of labeled cell-surface components in several ways without necessitating multivalent crosslinking: (i) hybrid antibody molecules may tend to aggregate once they become bound to the cell surface; (ii) attachment of hybrid antibody might perturb cell-surface components in such a way as to result in selective aggregation of all molecules of the labeled component; and (iii) attachment of hybrid antibody may result in thermodynamically favored aggregation of surface components by altering the electrostatic charge characteristics of the labeled molecules.

The first possibility appears most unlikely, since hybrid antibody-antibody complexes do not aggregate in solution. The second possibility would be unlikely if it required that each molecule of every surface component possess receptors specific for other molecules of that component. However, attachment of hybrid antibody to surface components may induce their aggregation by altering the transmembrane relationship between component molecules and a cytoplasmic assembly that may interconnect cell-surface receptors and account for certain membrane receptor-cytoplasmic interactions (19). This is an intriguing possibility for which we have no evidence, but which warrants further investigation.

At present, the third possibility may be the most likely explanation for hybrid antibody-induced redistribution, although it does not necessarily account for the persistence of aggregates once they have formed. Electrostatic repulsion may be responsible for maintaining a random distribution of a particular component over the cell surface (11), and aggregation may occur as a result of altering the electrostatic charge of component molecules by attachment of a ligand (20). Perturbation of the electrostatic charge characteristics of the surfaces of human erythrocyte ghosts by alteration of the pH or by exposure to proteolytic enzymes can result in aggregation of intramembranous particles demonstrable by freeze-etching (21, 22).

Although multivalent crosslinking may not be required for patch and cap formation, it may accentuate the rate and extent of redistribution. Ferritin and SBMV attached specifically to cell-bound hybrid antibody significantly increased the level of capping of surface IgG and TL alloantigen. Each individual marker is multivalent and could potentially bind several hybrid antibodies already attached to cell-surface components (13). In this manner, crosslinking of labeled molecules could be achieved, resulting in increased aggregation and cap formation.

Even at extremely low temperatures, ferritin can sequester uniformly distributed H-2 alloantigen-alloantibody-hybrid antibody complexes into patches. It is surprising that aggregation of surface components can occur at such low temperatures, since lateral movement within the surface membrane should be restricted by the high viscosity of the membrane lipids (10, 11). However, aggregation of intramembranous particles of human erythrocyte ghosts has been obtained at temperatures as low as  $-16^{\circ}$  (21).

The capacity of hybrid antibodies and visual markers to induce topographical displacement of surface components on mouse lymphoid cells, even at low temperatures, explains the consistent electron microscopic observations of patches of alloantigens labeled with hybrid antibodies (2, 3).

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