

HAPTEN-SANDWICH LABELING

I. A GENERAL PROCEDURE FOR SIMULTANEOUS LABELING OF MULTIPLE CELL SURFACE ANTIGENS FOR FLUORESCENCE AND ELECTRON MICROSCOPY*

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Recently, an antihapten antibody bridge method was proposed for specific labeling of cell surface antigens for electron microscopy (1). Hapten groups are coupled to specific antibodies and to certain markers, which are linked at the site of the designated cellular antigen with antihapten antibody. The idea of a hapten-sandwich labeling technique had also occurred to us, and we have developed a similar approach which can be applied simply to the amplification of any immunoglobulin fraction used for detecting surface antigens, including alloantigens. We report here hapten-sandwich labeling of Ig, thymus associated, or *H-2* antigens on mouse lymphocytes, visualized by fluorescence and transmission electron microscopy. The use of the same technique for specific labeling of cells observed with scanning electron microscopy is reported in another paper.¹

Our procedure succeeds in discriminating with high specificity between different cellular antigens in double-labeling experiments, an application suggested, but not realized, when the antihapten bridging method was projected (1).

Materials and Methods

Antisera and Purified Antibodies.—Antibodies to the haptens *p*-azophenyl β -D-lactoside (anti-lac)² and *p*-azophenyl arsonate (anti-ars) were isolated from pooled, high-titer antisera

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² *Abbreviations used in this paper:* anti-ars, *p*-azophenyl arsonate; anti-lac, *p*-azophenyl β -D-lactoside; anti-MBr, rabbit antimouse brain; anti-MIg, polyvalent antimouse IgG; BSA, bovine serum albumin; fer, ferritin; Fl, fluorescein; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; PHA-P, phytohemagglutinin P; Rh, rhodamine; SAS, saturated ammonium sulfate; TMV, tobacco mosaic virus; VBS, Veronal-buffered saline.

from rabbits hyperimmunized by standard procedures against hapten conjugates of keyhole limpet hemocyanin (KLH), prepared as previously described (2), and were purified by sepharose affinity chromatography (3). [¹²⁵I]anti-lac antibodies were prepared by reaction with carrier-free [¹²⁵I]iodide (New England Nuclear, Boston, Mass.) and chloramine-T (Eastman Organic Chemicals, Rochester, N. Y.) (4).

We obtained polyvalent antimouse immunoglobulin (anti-MIg) from a goat which received two i.m. injections in complete Freund's adjuvant of 100 μg protein from a 50% saturated ammonium sulfate (SAS) cut of BALB/c serum. Goat antirabbit Ig was prepared in the same manner, and antigoat Ig was made in rabbits by a similar procedure.

Rabbit antimouse brain (anti-MBr) was prepared essentially as described by Golub (5). It was then heat inactivated at 56°C for 30 min and absorbed at room temperature for 45 min with 1/5 vol of washed, packed mouse red cells. The specificity of the antiserum for T cells was established by its cytotoxicity, in the presence of complement, for various cell suspensions, its effect on the proliferative response of spleen cells to the mitogens phytohemagglutinin P (PHA-P) and lipopolysaccharide (LPS), and its effect on the in vitro antibody response to lac-KLH. At a final dilution range of 1:10-1:40, it killed 38% spleen cells, 70% lymph node cells and 98% thymocytes. This range also reduced the PHA induced [³H]thymidine incorporation by 95% without affecting the response to LPS. It was also shown to ablate the in vitro response to lac-KLH by selective killing of carrier T cells (6).

Anti-*H-2K* serum, contributed by Mr. D. Carter, Univ. of Calif., Berkeley, was prepared in BALB/c (*H-2D*) mice by seven i.p. injections, each of approximately 10⁸ lymphocytes obtained from CBA spleens and mesenteric lymph nodes. Hapten modification of the anti-MIg, anti-MBr and the anti-*H-2* antisera was done on the γ-globulin fractions which precipitated with 40% SAS.

Electron Microscope Markers.—KLH was obtained from giant keyhole limpets, *Megathura crenulata* (Pacific Bio-Marine Supply Co., Venice, Calif.) by described methods (7). Purified tobacco mosaic virus (TMV) was a gift from Dr. A. Knight, Univ. of Calif., Berkeley. Ferritin (fer), six times crystallized, was purchased from Miles Research, Kankakee, Ill. and purified by the procedure of Nicolson and Singer (8).

Before the markers were modified with hapten-coupling reagents, they were treated at concentrations of 5 mg/ml. for 2 h with 0.1% glutaraldehyde (EM grade, 8% solution stored under N₂) in 0.1 M phosphate buffer, pH 6.8. Reactions were terminated in 0.1% 2-aminoethanol. The preparations were dialyzed against 0.25 M borate buffer, pH 8.6, overnight at 4°C, and subsequently against the Veronal-buffered saline, pH 7.2, (VBS) used for staining.

Hapten Modification of Immunoglobulins and Markers.—Various levels of ars modification of [¹²⁵I]anti-lac antibody were tested to determine how much azocoupling could be achieved with preservation of antibody binding to a lac-sepharose column (3). The [¹²⁵I]anti-lac preparation, in portions of 0.6 ml borate buffer, 0.25 M, pH 8.5, containing 3 mg protein, was reacted with *p*-diazonium phenyl arsonate at concentrations of 5 × 10⁻⁴ M, 2.5 × 10⁻⁴ M, 1.25 × 10⁻⁴ M, and 7.5 × 10⁻⁵ M overnight at 4°C with gentle tumbling. The reactions were quenched by adding 4 mg of bovine serum albumin (BSA) and the samples were then passed over a 2 ml lac-sepharose column to test their binding activity. All subsequent coupling of either lac or ars to both immunoglobulins and markers was done overnight at 4°C with 4 × 10⁻⁴ M diazonium reagents and protein concentrations of 3 mg/ml. The markers KLH, TMV, and fer, were separated from free hapten by chromatography on Bio-Gel A-1.5 M columns (Bio-Rad Laboratories, Richmond, Calif.). All preparations were finally dialyzed against VBS, pH 7.2. The modified immunoglobulin preparations were kept frozen till use, and markers were kept at 4°C in 10⁻² M sodium azide.

[³H]diazonium phenyl β-lactoside, prepared as previously described (9), was added to cold reagent and reacted with rabbit gamma globulin (Pentex Biochemical, Kankakee, Ill.) to determine the number of lac-hapten groups coupled under standard reaction conditions, 4 × 10⁻⁴ M reagent and 2 × 10⁻⁵ M Ig, overnight at 4°C. The reaction was terminated by pre-

precipitation of Ig in 50% SAS. Lac-azocoupled rabbit Ig was added as cold carrier, and the protein was purified by passage through G-50 Sephadex in 0.1 M acetate, pH 7.0.

Specific activity of the tritiated lac-diazonium reagent was determined by characterization of the mono-azo resorcinol derivative (lac-resorcinol), prepared by reaction of the diazonium in > 500 times molar resorcinol excess in 0.1 M acetate at pH 4.5.

Lac-resorcinol was purified by repeated precipitation at pH 2, and the extinction determined by reference to the lac-mono-azo derivative of *N*-acetylhistidine by procedures previously reported (10). $\epsilon_M = 2.2 \times 10^4$, $\lambda_{max} = 380_m\mu$ at pH 4.5. [3H]lac-resorcinol was purified by thin-layer chromatography on Silica-gel (Brinkmann Instruments, Inc., Westbury, N.Y.) 0.25 mm, in 6:1:1 butanol-acetic-water ($R_f = 0.5$) and in 1:1 acetone-ethyl acetate ($R_f = 0.5$).

Tritiated compounds were counted in a Nuclear-Chicago scintillation counter, (Des Plaines, Ill.) ISO CAP-300; protein samples were counted in PPO-POPOP-toluene containing 5% Bio-Solv (Beckman). Counter efficiency was determined by addition of an internal standard, tritiated toluene (New England Nuclear). ^{125}I samples were counted in a Nuclear-Chicago, Automatic Gamma Well Counter.

Cell Preparations.—We used unimmunized BALB/c mice of both sexes, aged 6–12 wk. Cell suspensions were obtained by teasing in VBS, and passing through glass wool columns equilibrated with VBS containing 0.05% of BSA (obtained as a 35% solution from Pentex Biochemicals). Spleen lymphocytes were separated on a Ficoll-Hypaque gradient (11), then washed twice in VBS containing 0.05% BSA. Thymocyte and lymph node suspensions were obtained directly with two such washes of the glass wool column effluent. During all manipulations the cells were kept at 4°C and in the presence of 10^{-2} M sodium azide, unless otherwise indicated.

Fluorescent Labeling of Cells.—40% SAS cuts of the antibody preparations, anti-ars, anti-lac, goat antirabbit Ig, and rabbit antigoat Ig were conjugated with fluorescein (Fl) or tetramethyl rhodamine (Rh) isothiocyanate by dialysis at 4°C for 20 h against the dye (12). Unreacted dye was removed from the fluorescent conjugates by passage over Sephadex G-25 and fractionation on DEAE cellulose. The fluorescent antihapten antibodies were used in conjugation with the corresponding hapten-modified anti-MBr or anti-MIg to reveal the percentages of cells in thymus, spleen, and lymph node suspensions which bore T-associated or Ig surface markers. Approximately 0.1 to 0.2 ml of the cell surface antibody, at a concentration of 3–5 mg/ml, was added to an equal volume of a cell suspension containing approximately 10^7 cells/ml VBS with 0.05% BSA and 10^{-2} M azide, and incubated at 4°C for 20 min. The cells were then washed in a BSA gradient consisting of equal volumes of 5% and 10% BSA in VBS with 10^{-2} M azide, followed by one wash in azide-containing VBS. The subsequent labeling with the second layer of fluorescent antihapten antibody was done similarly.

The fractions of MIg- or MBr-bearing cells found in lymphoid cell suspensions by this labeling method were compared with those obtained by a conventional indirect method: unmodified goat anti-MIg, followed by Rh-rabbit antigoat Ig; or unmodified rabbit anti-MBr, followed by Fl-goat antirabbit Ig. Fluorescence was examined in a Leitz Orthoplan microscope (E. Leitz, Inc., Rockleigh, N.J.) using a 150W Xenon lamp with two KP490 filters and one K510 for Fl, and a 200W mercury lamp with S546, BG36, and K610 filters for Rh.

Labeling for Electron Microscopy.—The reagents required in electron microscope labeling were applied sequentially with intervening washes similar to those used in the fluorescent staining. The sequence of additions was lac-anti-MBr, anti-lac, lac-KLH, ars-anti-MIg, anti-ars, and ars-fer. Lac-KLH and ars-fer were used at a concentration of 1 mg/ml protein rather than at the concentration of 3–5 mg/ml protein which was used for the antibody layers. Control preparations were done by omitting the haptenated anti-cell surface antibody layer and, in some cases, also the bridging layer of antihapten antibody. Unless indicated otherwise, cell labeling was done at 4°C in the presence of 10^{-2} M azide. After labeling, the cells were fixed

for 10 min at room temperature in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.35. They were then washed once with cacodylate buffer, and post-fixed for 15 min at room temperature with 1% OsO₄ in the same buffer. After centrifugation at 300 g, the cell pellet was embedded in 2% agarose in 0.1 M phosphate buffer, pH 7.30. This gel was then cut into two to four pieces, dehydrated, and embedded in epon. Sections of 400–500 Å in thickness, cut from experimental and control blocks, were stained with uranyl acetate and lead citrate and examined on a Phillips 300 (Phillips Electronic Instruments, Mount Vernon, N.Y.) or an RCA EMU-3F electron microscope (RCA Electronic Components, Harrison, N.Y.). Untreated and glutaraldehyde-treated markers were examined by negative-stain techniques before and after hapten conjugation. The markers were allowed to settle on a carbon film-coated grid and were negative-stained with a 1% sodium phosphotungstate solution.

RESULTS

Effect of Hapten-Coupling on Antibody-Binding Activity.—Table I shows the effect of reacting [¹²⁵I]anti-lac antibody with various concentrations of ars-

TABLE I
Effect of Diazonium Reaction on [¹²⁵I]Anti-Lac Binding to Lac-Sepharose

Molar ratio reagent: Ab	CPM unbound
	%
0	5
3	12
5	16
10	24
19	32

diazonium reagent on specific adsorption to a lac-sepharose column. About 70% of the antibody which had been reacted at a molar ratio of 19:1, reagent to antibody, was retained by the column.

Using [³H]diazoniumphenyl β-lactoside, it was determined that reaction of 4×10^{-4} M reagent with 2×10^{-5} M rabbit gamma globulin resulted in coupling 5.3 lac groups/mol Ig. No determination was made on the extent of azocoupling with ars-diazonium reagent under the same conditions, although spectral observation showed no major difference in azo-absorbance between the lac- and ars-modified Ig conjugates.

Lac-anti-MBr, prepared by our standard procedure, was compared with unmodified anti-MBr in complement-mediated cytotoxicity against lymph node and thymic lymphocytes and was found to be 60% less effective (Fig. 1).

Fluorescent Labeling of Cells that bear MIg or MBr Surface Antigens.—Mouse spleen and thymus lymphocyte preparations were each treated with ars-anti-MIg and Lac-anti-MBr sera, followed by Rh-anti-ars and Fl-anti-lac antibodies. In each preparation, cells showed either rhodamine or fluorescein staining, in patchy rings; no significant fraction of doubly staining cells was observed. Table II shows the percentage of cells positive for MBr and MIg in one series of fluorescent assays.

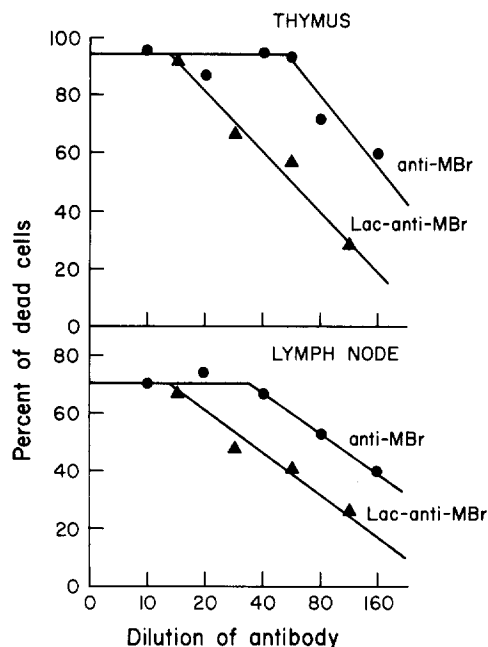


FIG. 1. A comparison of the complement-dependent cytotoxicity of rabbit antimouse brain antibody before (anti-MBr, ●) and after lac-modification (lac-anti-MBr, ▲). Dead cells were scored by trypan blue uptake.

TABLE II
Fluorescent Labeling of Cells that Bind Hp-Coupled Antibodies

Lymphocytes	ars-anti-MIg, Rh-anti-ars	lac-anti-MBr, Fl-anti-lac
	% Rh	% Fl
Spleen	60	30
Thymus	<2	>95

In several experiments, the fraction of Ig-bearing lymphocytes in a spleen cell suspension was determined after labeling aliquots in two different ways: either as above, with ars-anti-MIg and Rh-anti-ars, or with unmodified anti-MIg and Rh-rabbit antigoat Ig. Both labeling procedures yielded the same fraction of rhodamine stained cells. Parallel experiments compared the labeling of thymus-associated antigens in spleen, lymph node, and thymus by two procedures: either with lac-anti-MBr and Fl-anti-lac, or with unmodified anti-MBr and Fl-goat antirabbit Ig. The results in Table III show that both procedures yielded Fl-staining in virtually all thymus cells; but small fractions of the splenic and lymph node T cells which were detected with the conventional indirect method were not scored as positive with the Fl-antihapten

TABLE III
Fluorescent Labeling of MBr Bearing Cells by Two Procedures

Lymphocytes	lac-anti-MBr, Fl-anti-lac	anti-MBr, Fl-anti-rabbit Ig
	% Fl	% Fl
Spleen	32	38
Thymus	>95	>95
Lymph node	70	85

technique. While most T cells stained strongly with both procedures, somewhat lower fluorescence intensity was achieved with the Fl-anti-lac reagent.

In all assays where fluorescent antihapten antibody was used, controls showed that no significant staining occurred unless the cells were first treated with the appropriately hapten-coupled anti-MIg or anti-MBr reagent. The hapten-sandwich technique was also applied to fluorescent labeling of *H-2* antigens. Lac-anti-*H-2K*, followed by Fl-anti-lac antibody, stained all cells in CBA spleen cell suspensions, but did not label BALB/c (*H-2D*) spleen cells.

Electron Microscopic Visualization of Cell Surface Labeling.—Fig. 2 shows electron micrographs of two preparations of ars-KLH, one of which was treated with glutaraldehyde before conjugation with hapten. The conjugate prepared without prefixing appears amorphous, whereas the other shows the discrete structures typical of KLH as a marker in electron microscopy. Stabilization of KLH and other markers with glutaraldehyde did not significantly increase the number of intermolecular aggregates. To verify that glutaraldehyde pre-treated hapten conjugates are suitable cell surface markers, we labeled aliquots of a spleen cell suspension with ars-anti-MIg and anti-ars antibody followed either by prefixed ars-KLH, ars-fer, or ars-TMV. Fig. 3 shows examples of cells labeled with the prefixed ars-KLH (*a*) and ars-TMV (*b*) markers; cells labeled with prefixed ars-fer are exhibited in Figs. 4 and 5.

In a series of experiments, mouse spleen, lymph node, or thymus preparations were labeled for electron microscopic visualization of both MIg and MBr surface antigens. Each cell suspension was treated with lac-anti-MBr, anti-lac antibody, and lac-KLH, as well as with ars-anti-MIg, anti-ars antibody, and ars-fer. Cells in each of the lymphoid populations showed either exclusive labeling with the MIg (ars-fer) marker, a predominance of the MBr (ars-KLH) marker, or the absence of either. Figs. 4–7 show lymphocytes from the double-labeling experiments. Fig. 4 shows a field of splenic lymphocytes containing one cell heavily labeled with the lac-KLH MBr marker, one exclusively with the ars-fer MIg marker, one completely unlabeled cell, and one with only a trace of ars-fer. (The identity of the markers on the labeled cells was ascertained at higher magnification.) Fig. 5 shows an MIg-bearing spleen lymphocyte which is labeled with ars-fer on or near the base of microvilli. Fig. 6 shows a capped thymus lymphocyte labeled with the lac-KLH MBr marker; in this experiment, labeled cells were incubated at room temperature for 20 min, without azide,

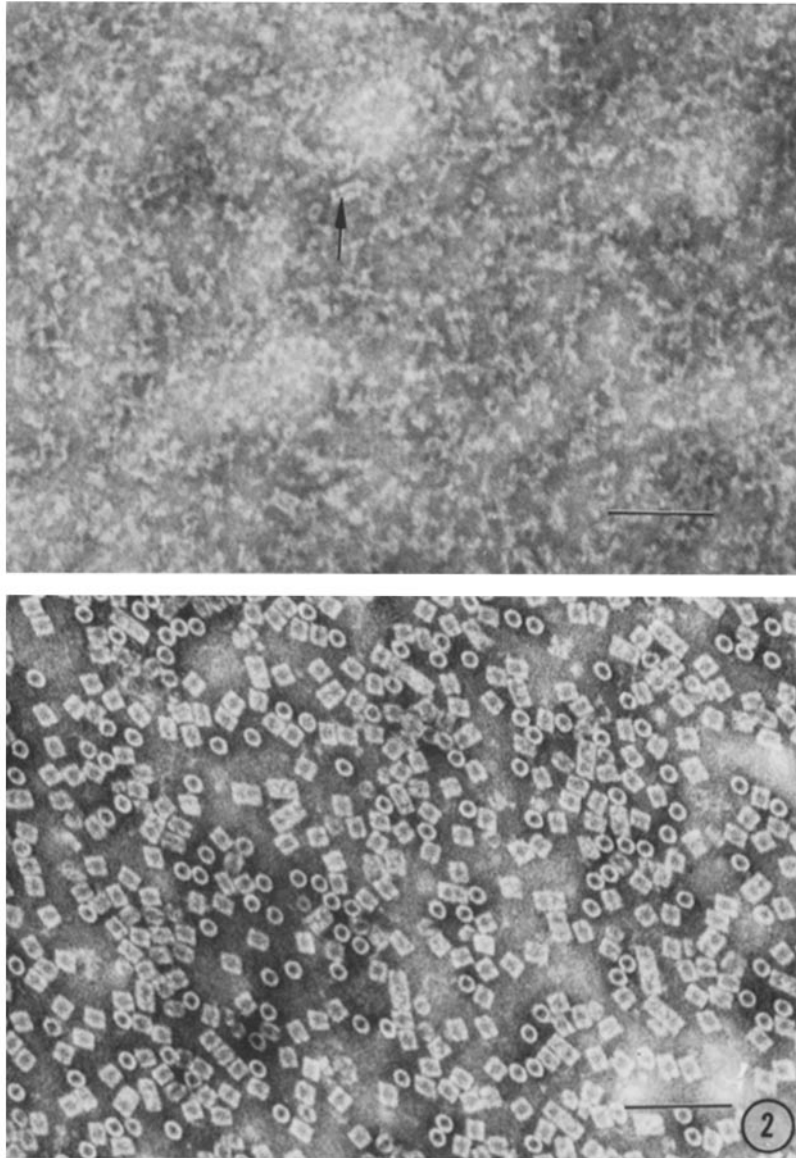


FIG. 2. Upper photo: an azocoupled ars-KLH preparation. Lower photo: an ars-KLH preparation azocoupled after stabilization of the KLH with glutaraldehyde (see Materials and Methods). The black line indicates 0.2μ . $\times 69,000$.

before glutaraldehyde fixation. Fig. 7 shows a lac-KLH labeled splenic T lymphocyte at high magnification. It can be seen that some ars-fer is interspersed in the lac-KLH aggregates. Most ars-fer-labeled cells, presumably B lymphocytes, exhibited virtually no lac-KLH, and unlabeled lymphocytes

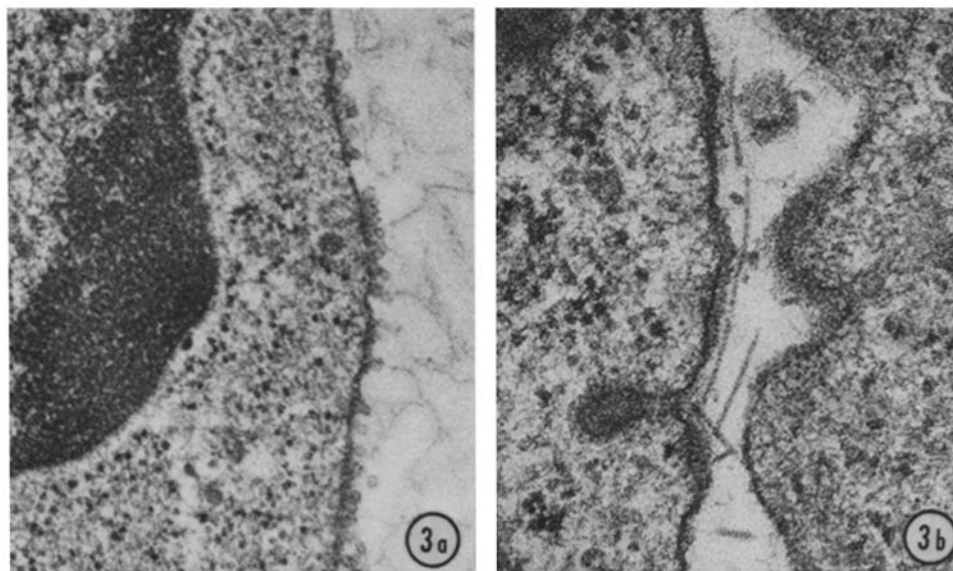


FIG. 3. Spleen lymphocytes labeled with ars-anti-MIg, anti-ars antibody, followed by a prefixed ars-azoconjugate: (a) ars-KLH and (b) ars-TMV. (a) $\times 48,000$, (b) $\times 64,000$.

and reticulocytes were remarkably free of either marker. In contrast, cells with the lac-KLH marker usually showed some (10% or less) inclusion of ars-fer in the aggregates of attached label; exclusive areas of ars-fer labeling were not observed on these cells.

In double-labeling experiment controls, where the antihapten antibodies and conjugated markers were added without prior treatment with anti-cell surface antibodies, cells were always free of label.

DISCUSSION

By the hapten-sandwich method, cell surface antigens can be labeled specifically for visualization with fluorescence and electron microscopy. Our procedure uses azocoupling of haptens to modify both antibodies and markers, which can then be linked by the proper antihapten antibodies.

The simple azocoupling procedure permits the addition of amplifying hapten groups to immunoglobulins with a high degree of retention of antibody-binding activity (Table I). Thus, the immunoglobulin fraction of any specific antiserum can be converted easily to a reagent that can be used conveniently and economically in an indirect (sandwich) labeling scheme. This method is particularly suited to labeling with alloantisera, which generally cannot be amplified by antibodies made in another species.

We have been able to overcome one limitation encountered in earlier attempts to use hapten-markers, namely, that chemical modification appears to

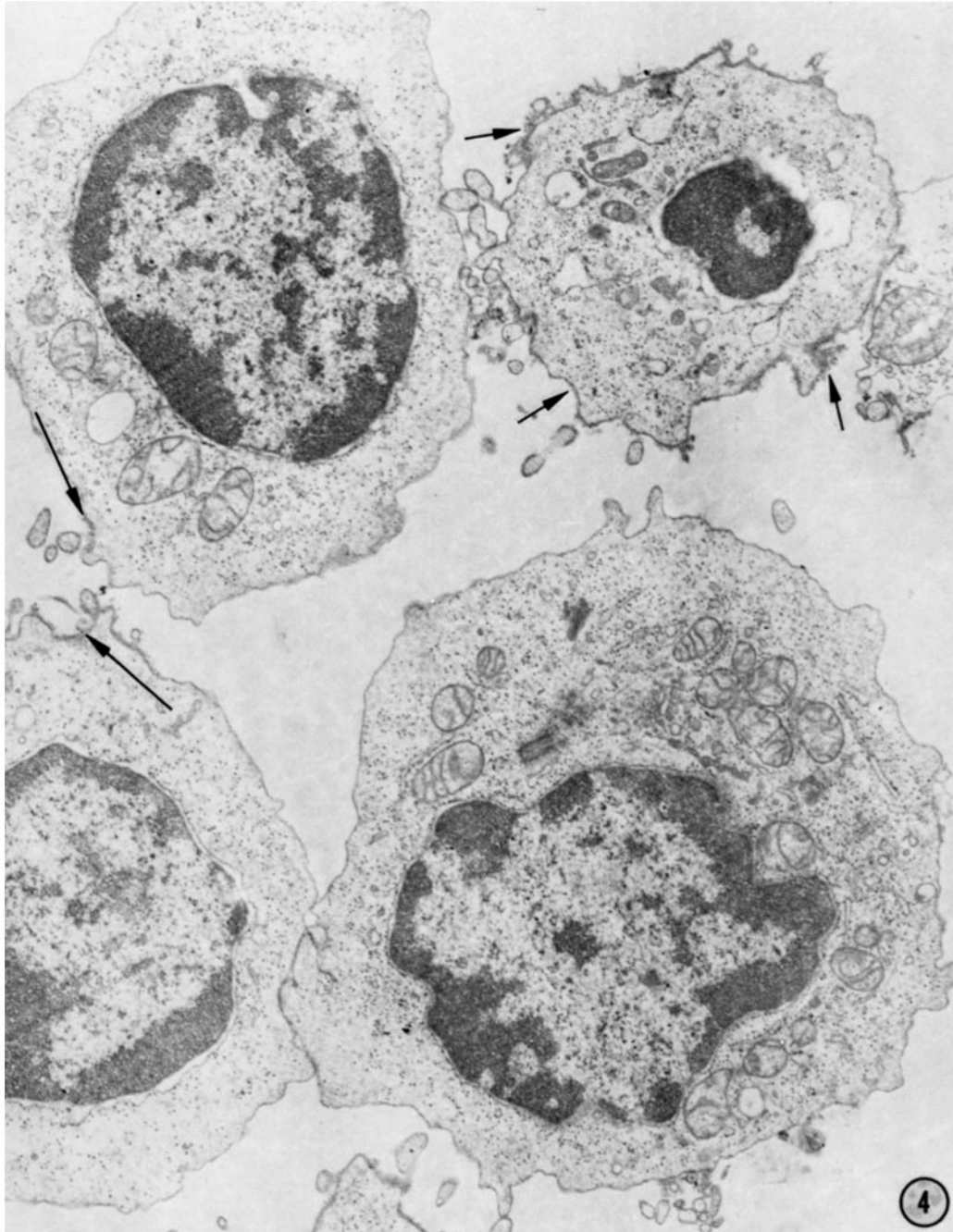


FIG. 4. Cells from a double-labeling experiment to identify MBr (lac-KLH) and MIg (ars-fer) antigens on mouse spleen lymphocytes. The cell in the upper right corner is extensively labeled with lac-KLH; the one at lower left is labeled with ars-fer; at lower right is a completely unlabeled cell section; there may be a trace of ars-fer on the cell at upper left. The identity of the markers was established at higher magnification.

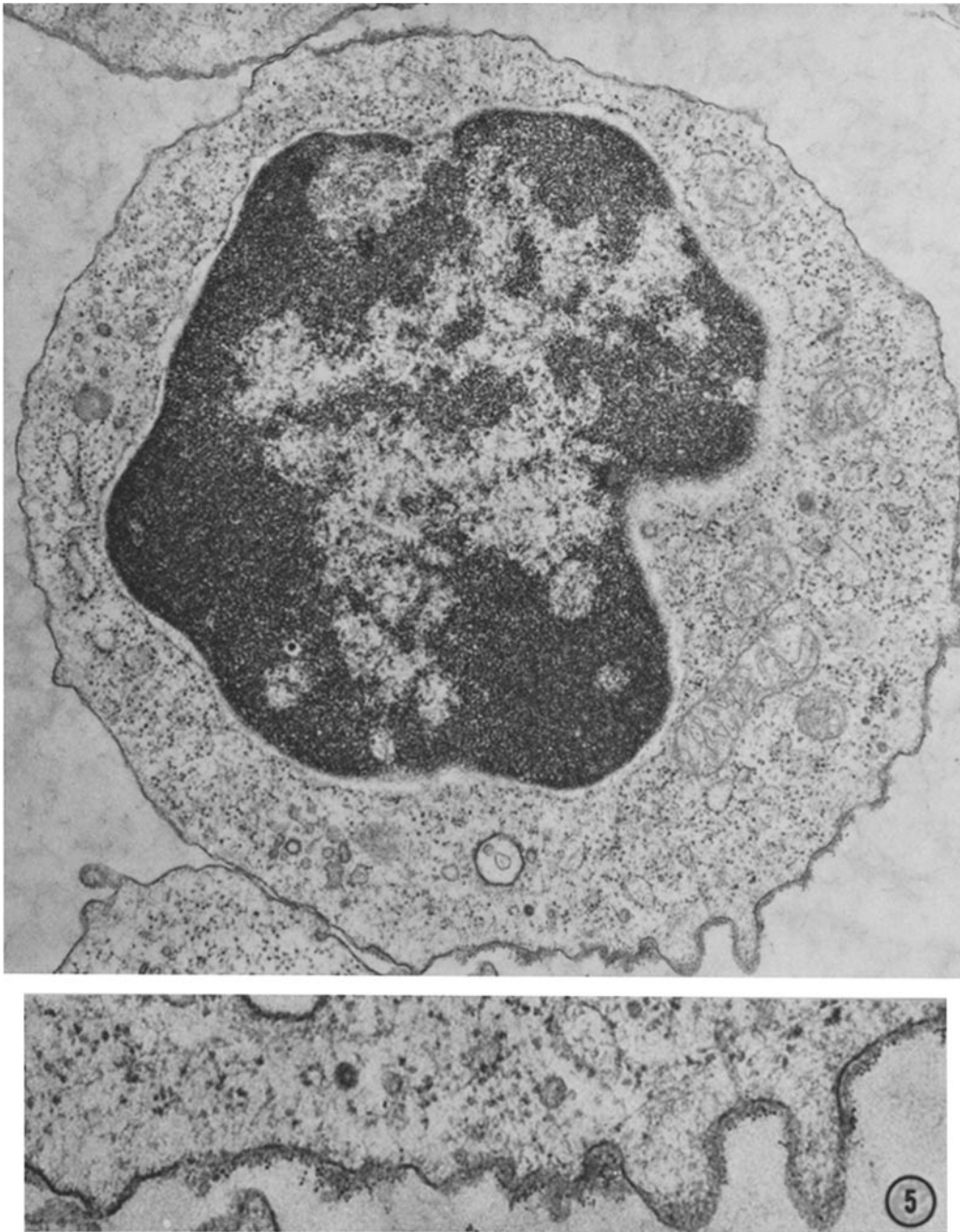


FIG. 5. From a double-labeling experiment, a spleen lymphocyte labeled exclusively with the ars-fer Mlg marker. The lower photo shows concentrations of label at the base of microvilli. Upper, $\times 20,000$; Lower, $\times 50,000$.

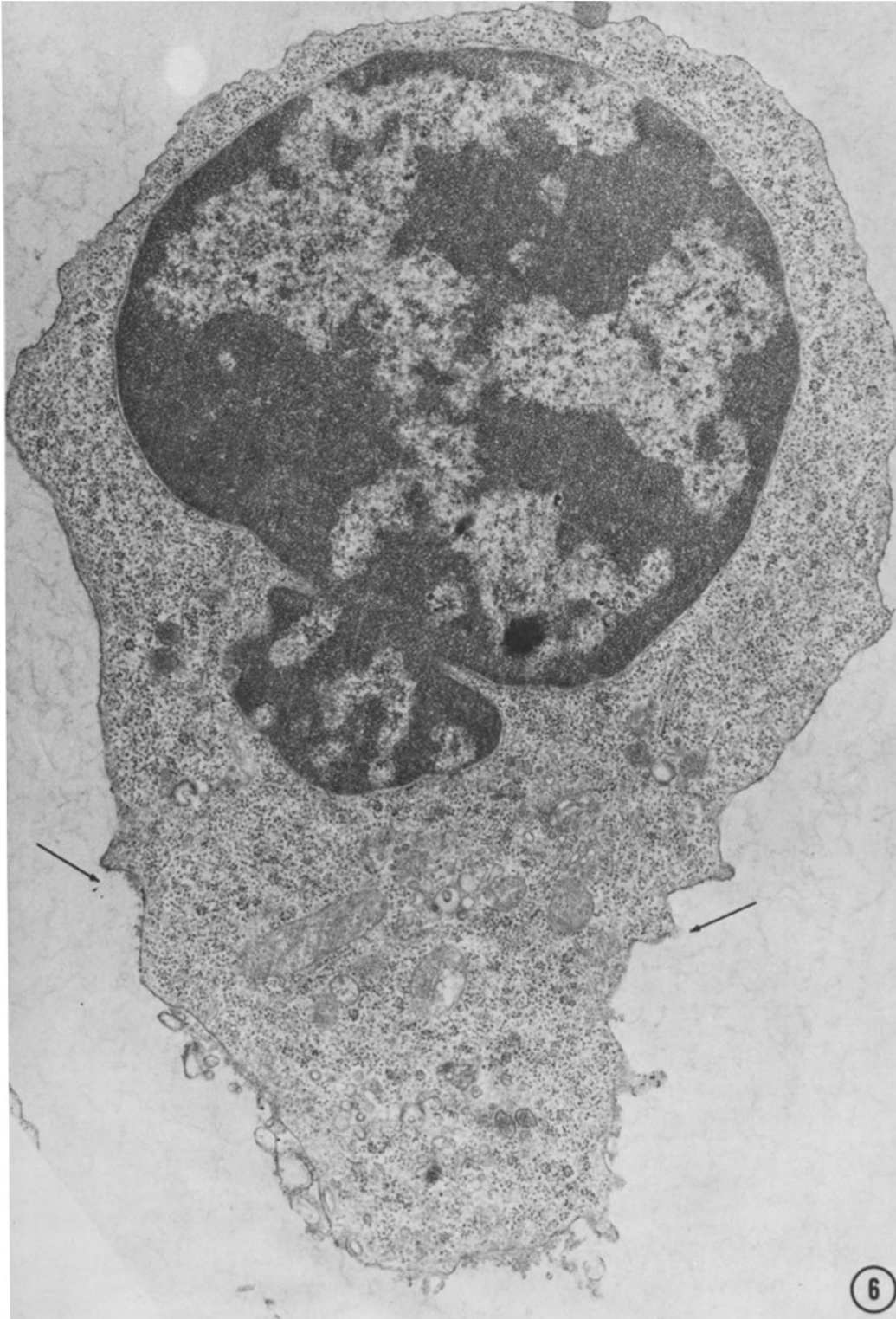


FIG. 6. From a double-labeling experiment, a thymocyte capped with the lac-KLH MBr marker. $\times 16,000$.

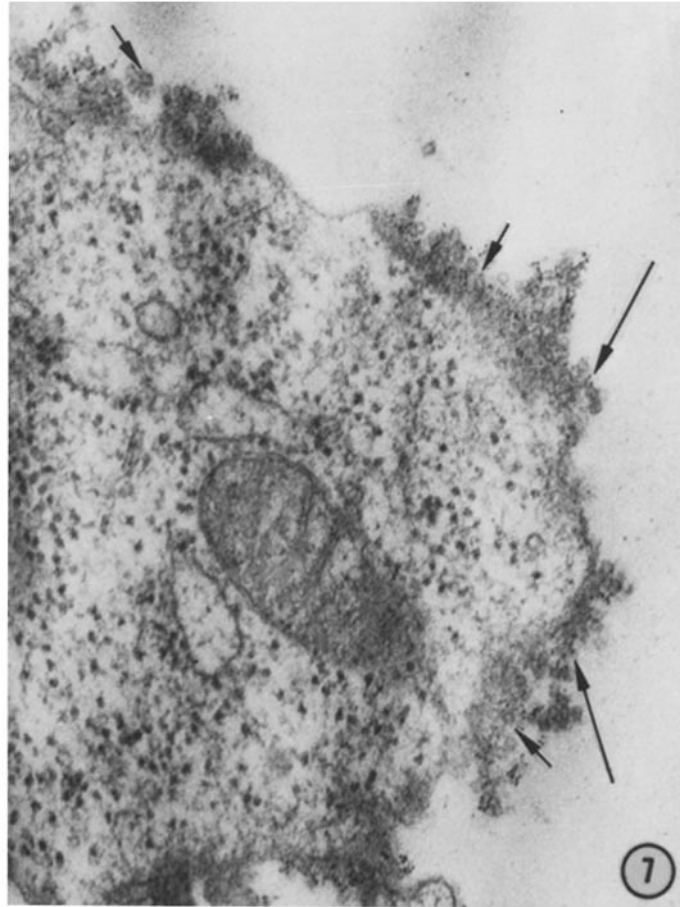


FIG. 7. From a double-labeling experiment, a spleen cell labeled predominantly with the lac-KLH MBr marker. Ars-fer appears interspersed in the lac-KLH aggregates. $\times 48,000$.

disrupt some larger molecular weight markers that have multiple subunits (1). Mild glutaraldehyde treatment before conjugation with hapten protects the proposed marker (Fig. 2), while leaving tyrosine and histidine residues free for azocoupling. As a result, we have been successful in using hapten-modified hemocyanin (Fig. 3 *a*) and tobacco mosaic virus (Fig. 3 *b*) as markers not only for transmission, but also for scanning electron microscopy.¹ The prefixing of markers before Hp-modification makes available a wide selection of distinct labeling combinations. This, in turn, makes feasible the simultaneous labeling of more than one surface antigen, which could not be done with the earlier antihapten bridge procedure (1).

The double-labeling experiments to detect cells with MIg or MBr antigens

have illustrated the high degree of specificity attainable with our hapten-sandwich procedures (Table II, Figs. 4-7). In populations of cells bearing immunoglobulin or thymus-associated antigens, the appropriate Rh- and Fl-antihapten antibodies clearly discriminated B from T cells. Similarly, examination of lymphocyte sections in the electron microscope revealed that the hapten-markers (ars-fer for MIg and lac-KLH for MBr) segregated on different cell surfaces. Backgrounds of nonspecific labeling appear to be extremely low, since most ars-fer-labeled cells showed virtually no lac-KLH, and some lymphocytes and all reticulocytes were completely free of either marker. MBr bearing cells, which labeled with lac-KLH, generally showed some of the ars-fer (MIg) marker. This may not be due primarily to the presence of Ig on the T cells. It may be that some ars-fer tends to adhere nonspecifically to cell surface-bound lac-KLH, since the ars-fer was usually included in the lac-KLH patches and appeared most often not to be directly attached to membrane. Controversy about Ig on or in T cells is sufficiently complicated so that we hesitate to assess the relevance of our results in this regard without further experiments, including attempts to correlate labeling with a determination of whether the observed Ig is intrinsic to the labeled cells (13).

The advantages of using readily prepared Hp-conjugated antibodies for fluorescent labeling of alloantigens, as well as for electron microscopic visualization of any cell surface antigen, are obvious. This method is clearly more effective for fluorescence visualization than direct staining with Fl-anti-cell surface antibodies, where no more than 1-2 fluorescent groups can be attached without significant inactivation of antibody. For example, detection of T cells by direct staining with Fl-anti- θ is not practical (14). Table III shows, however, that the Fl-anti-Hp labeling procedure is somewhat less sensitive than a conventional indirect method using unmodified antimouse antibodies from one species and amplifying with anti-Ig from another. Both procedures readily detected B cells and both detected an equal number of MBr-bearing thymus cells. However, a small fraction of the cells scored for MBr in lymph node and spleen by conventional indirect staining escaped detection with the Fl-anti-Hp reagent (Table III). A difference in labeling efficiency would be expected simply from a difference in the amplification factor in the compared procedures: a maximum of 5.3 with the Hp-modified antibody, whereas the effective antigenic valence for unmodified Ig may be over ten (15). Of course, fewer Hp-modified anticell surface antibodies must be bound to begin with, as indicated by somewhat reduced binding of ars-anti-lac to lac-sepharose (Table I) and diminished cytotoxic activity of lac-anti-MBr (Fig. 1).

Since a large variety of antibodies against azophenyl haptens can be prepared and purified easily, the procedure described here lends considerable versatility to the hapten-sandwich method. We believe it should be possible to increase the sensitivity of the hapten-sandwich method still further by using other reactions for hapten conjugation that permit maximal retention of anti-

body activity (e.g., amidation [16]). This is desirable not only to permit detection of sparsely occurring antigens (T cells with little thymus-dependent antigen may be of special interest), but to clarify whether certain antigens are systematically associated on a lymphocyte surface. Active antibodies with many attached hapten (or radioactive) determinants may also be very useful reagents in a range of biological studies.

SUMMARY

A hapten-sandwich procedure has been developed for specific labeling of cell surface antigens for fluorescence or electron microscopy. Haptens are azo-coupled to immunoglobulins specific for a cell surface antigen; the hapten-modified cell-bound antibodies can then be visualized by adding fluorescent antihapten antibody, or by adding antihapten antibody followed by hapten-modified markers for electron microscopy. Virus or high molecular weight protein markers are lightly cross-linked before conjugation with hapten to prevent their disruption. Such stable hapten-modified markers, and the accessibility of many different purified anti-azophenyl-hapten antibodies, make it feasible to distinguish more than one membrane antigen in a given labeling experiment. When mouse lymphoid cell populations are labeled with separate markers for Ig and for thymus-associated antigens, many cells exhibit the Ig marker exclusively or the thymic marker predominantly, and some cells are completely free of label.

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