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Monoclonal Antibody Analysis of Complex Biological Systems

COMBINATION OF CELL HYBRIDIZATION AND IMMUNOADSORBENTS IN A NOVEL CASCADE PROCEDURE AND ITS APPLICATION TO THE MACROPHAGE CELL SURFACE*

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To simplify the construction of monoclonal antibody (MAb) libraries to complex biological systems, the use of a cascade procedure has been investigated. The procedure continually restricts MAb production to those antigens previously so unidentified. Murine macrophage membranes were detergent-solubilized and purified by *Lens culinaris* lectin affinity chromatography. Prior to immunization of rats, the previously recognized heat-stable antigen and common leukocyte antigen were removed with MAb immunoadsorbents. Analysis of serum antibody concentrations to common leukocyte antigen, heat-stable antigen, and Mac-1 (a previously recognized antigen which served as a positive control for nondepleted antigens) demonstrated the efficacy of the procedure. The ratios of anti-Mac-1 to anti-common leukocyte antigen and anti-Mac-1 to anti-heat-stable antigen antibodies were over 100-fold higher when purified, MAb immunoadsorbent-depleted material rather than whole peritoneal exudate cells were used for immunization. P3-NSI/1-Ag4-1 γ-chain nonsecretor myeloma cells were fused with spleen cells from rats immunized to immunodepleted antigen and hybrid culture supernatants were screened for immunoprecipitation of ^{125}I (lactoperoxidase)-labeled material. The common leukocyte antigen was among the most immunodominant antigens on whole peritoneal exudate cells. However, immunization with immunoadsorbent-depleted antigen did not elicit any hybrid cultures precipitating the 200,000 M_r common leukocyte antigen antigen. Eighteen cultures immunoprecipitated four different types of macrophage cell surface antigens. Clones identifying previously undescribed macrophage surface polypeptides of 32,000 M_r (Mac-2) and 110,000 M_r (Mac-3) have been isolated.

The myeloma-hybrid technique of Kohler and Milstein (1975) has recently given great impetus to the analysis of complex biological systems. For example, whole cells of one species such as the mouse may be injected into another species such as the rat (Springer *et al.*, 1978), or vice versa (Williams *et al.*, 1977). The resultant multispecific response to a large array of different cell surface molecules may then be resolved by cloning into a set of hybrid lines each secreting a monoclonal antibody recognizing a single antigenic determinant on a single cell surface molecule. The technique is so powerful that it frequently leads to the identification and study of previously unknown surface structures. One such antigen described by this laboratory is Mac-1, a granulocyte- and monocyte-specific antigen containing 105,000 and 190,000 M_r subunits which is particularly richly expressed on macrophages (Springer *et al.*, 1979).

One disadvantage of this approach is that a vast array of cell surface antigens elicit responses, while the experimenter may be interested in only one or a few of these. This necessitates laborious and often complex screening of hundreds, thousands, or even more hybrid cultures. Furthermore, clones recognizing certain (immunodominant) antigens are found with much higher frequencies than others. Thus, screening procedures which distinguish between uninteresting clones occurring at high frequency and interesting clones occurring at low frequency are often required. Of ten previously described rat anti-mouse clones, five recognized a heat-stable antigen while two reacted with a 200,000 M_r common leukocyte antigen (Springer *et al.*, 1978). In three subsequent hybridization experiments,[1] these clones have also outnumbered others, and thus appear to be immunodominant.

If the clonal response to previously identified antigens could be eliminated, 1) screening procedures could be greatly simplified, 2) the frequency of clones responding to nonimmunodominant antigens should be increased (Pross and Eidinger, 1974), and 3) novel cell surface antigens could be identified in an efficient and orderly manner. Therefore, in this report, a cascade procedure for accomplishing this has been explored in connection with the identification of further macrophage-specific antigens. Peritoneal exudate cell membranes were detergent-solublized, and the previously identified common leukocyte antigen and heat-stable antigen which are shared with peritoneal exudate cells and lymphocytes were then removed with MAb[2] immunoadsorbents before immunization for the hybridization experiment. Removal of the antigens was confirmed by radioimmunoassay and by the serological response to immunization. Serum antibodies to specific antigens were also measured to compare the efficacy of this procedure to immunization with either whole cells or MAb-coated cells. Two previously unknown macrophage-specific antigens of 32,000 and 110,000 M_r have been identified in this study. The procedure can be extended by arranging further immunoadsorbent depletions and cell fusions in a cascade series and is readily applicable to the monoclonal antibody analysis of many other multicomponent biological complexes.

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[1] T. Springer, T. Reynolds, and K. Kurzinger, unpublished work.

[2] The abbreviations used are: Mab, monoclonal antibody; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; i.v., intravenous; NSI, P3-NSI/1-Ag4-1 γ-chain nonsecretor myeloma; PAGE, polyacrylamide gel electrophoresis; PBS, 0.14 M NaCl, 0.01 M $NaPO_4$, pH 7.0; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Cell Lines—M1/69, M1/9.3, M1/89.18, and M1/70 have been deposited with and may be obtained from the Cell Distribution Center, Salk Institute, P.O. Box 1809, San Diego, Ca. Their properties are described in Table I. The NSI myeloma line was obtained from the same center. All hybrid lines were subcloned at least once. Clones are designated numerically, *i.e.* M1/70.15 and M1/70.15.1 are a clone and a subclone, respectively, of the M1/70 culture, but such designations have been omitted in most cases for brevity. Spent culture supernatants containing the antibodies (50–200 μg/ml) were obtained after growth to maximum density in 5% fetal calf serum, Dulbecco's modified Eagle's medium.

Assay for Antigen—Antigen was measured by inhibition of the ^{125}I-anti-rat IgG indirect binding assay. Preliminary titration of the monoclonal antibodies (MAb) in this assay showed a plateau with a break at about 1:100 dilution, below which the amount of bound ^{125}I second antibody became approximately proportional to the concentration of MAb. Therefore, MAb were used at 1:100 in the inhibition assay. Serial 3-fold dilutions of antigen (10 μl, maximum of 0.5% sodium deoxycholate) in 10% BSA-PBS were incubated with 5 μl of MAb tissue culture supernatants diluted 1/100 in 30% BSA-PBS for 2 h at 4°C in V-well microtiter plates. Inclusion of 30% BSA (which contains detergent binding sites) (Springer *et al.*, 1977) in the assay prevented inhibition due to solubilization of target cells, which occurred to some extent even after glutaraldehyde fixation. BSA (30%) was sufficient to overcome inhibition by an equal volume of 1% but not 5% sodium deoxycholate. The indirect binding assay was carried out as previously described (Springer *et al.*, 1978). Briefly, 5 μl of 5 × 10^7/ml glutaraldehyde-fixed target cells (spleen for M1/9.3 and M1/69.16, or thioglycollate-induced peritoneal exudate cells for M1/70 were added, plates shaken at 4°C for 45 min, cells were washed, suspended in 5 μl of mouse IgG-absorbed ^{125}I-F(ab')2 rabbit anti-rat IgG, shaken 45 min at 4°C, washed, and transferred to tubes for γ counting. "Antigen titer" is defined as the inverse of the dilution of antigen, in the assay volume after antibody addition, giving 50% inhibition of ^{125}I anti-rat IgG binding.

Purification of Macrophage Membranes—Peritoneal exudate cells from 25 C57BL/6 mice (Jackson Laboratory, Bar Harbor, Me.) were collected 3 days after injection of 1.5 ml of Brewer's thioglycollate medium (Difco). Cells (8 × 10^8 total, 84% macrophages, 2.5% neutrophils, 7.5% eosinophils, 4.5% lymphocytes, and 1.5% red blood cells by Wright's stain) were washed and suspended in 20 ml of PBS containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF, diluted from a 50 mM solution in ethanol) and broken by N_2 cavitation after pressurization at 600 p.s.i. for 10 min. After centrifugation at 1,500 × *g* for 5 min, the pellet was homogenized using a Teflon pestle in 30 ml of the same buffer and centrifuged at 1,500 × *g* for 5 min. The two 1,500 × *g* supernatants were combined, and the 48,000 × *g* for 30 min pellet (crude membranes, 34.8 A_{280}–A_{310} units) was suspended in 2 ml of PBS + PMSF.

Purification of MAb, L. culinaris lectin, and coupling to Sepharose—All MAb purification steps were at 4°C. Rat IgG was determined at all purification steps by Mancini radial immunodiffusion using rabbit ant-rat Fab (Ouchterlony and Nilsson, 1978). Solid $(NH_4)_2SO_4$ was added to the spent culture medium (0.5–1 liter) to bring to 2.2 M, the 10,000 × *g* pellet dialyzed *versus* 0.1 M Tris-HCl (pH 7.8 at 20°), applied to a DEAE-cellulose column (DE-52, Reeve Angell) at 8 mg of protein/ml DE-52, and immediately eluted with 6 column volumes of 0.1 M Tris-HCl, pH 7.8, containing a linear gradient of 0–0.05 M NaCl. Most MAb eluted in the flow-through volume. Peak fractions were pooled, concentrated, and applied to a 4 × 120 cm Sephadex G-200 column. Preparations were typically 90–100% pure as shown by SDS-PAGE. IgG in 0.1 M NaCl, 0.1 M $NaHCO_3$ was coupled to Sepharose CL-4B activated with 2 g of CNBr/100 ml packed Sepharose, and *Lens culinaris* lectin purified and coupled to Sepharose CL-4B as previously described (Hayman and Crumpton, 1972).

Glycoprotein Purification and Cascade Immunoadsorbent Procedure—All steps were carried out at 4°C. Step 1, membranes were solubilized with 2 volumes of 1.25% sodium deoxycholate in 0.01 M Tris-HCl, pH 8.2, and centrifuged at 100,000 × *g* for 45 min. Step 2, the supernatant was applied to 2.4 ml of 4.6 mg of *L. culinaris* lectin/ml of Sepharose CL-4B (Pharmacia) packed in a 3-ml syringe and washed with 21 ml of 0.1% sodium deoxycholate, 0.01 M Tris-HCl, pH 8.2. Glycoproteins were eluted with 9 ml of 5% methyl-D-mannoside in the same buffer at 3 ml/h and with 6 ml at 1 ml/h. The three peak fractions (Fig. 2) were pooled, dialyzed against 0.1% sodium deoxycholate, 0.01 M Tris-HCl, pH 8.2, concentrated *versus* Sephadex G-200 (Pharmacia), and 5% sodium deoxycholate added to 0.5%. Step 3, the sample was passed through 0.4 ml of 0.9 mg of M1/69.16/ml Sepharose CL-4B in a column made from a 1-ml tuberculin syringe, and washed with 1 ml of 0.1% sodium deoxycholate, 0.01 M Tris-HCl, pH 8.2. Step 4, the sample was passed through 0.5 ml of 0.9 mg of M1/89.23/ml Sepharose CL-4B as above. Step 5, the sample was passed through 0.8 ml of fresh M1/89.23 Sepharose as above.

Immunization—*L. culinaris* and immunoadsorbent-purified glycoproteins were dialyzed for 7 days against two changes daily of 0.01 M Tris-HCl, pH 8.2, to remove sodium deoxycholate. Two (Lewis × BN)F_1 rats were injected with 40 μg of protein emulsified in complete Freund's adjuvant on days 0 and 18 and bleeds were taken on day 35. Four months later, one rat was injected i.v. with 100 μg of protein in saline and splenectomized for fusion 3 days later. For immunization with whole cells, (Lewis × BN)F_1 rats were injected on days 0 and 21 with 1.1 × 10^7 thioglycollate-induced peritoneal exudate cells with or without preincubation with an excess of MAb M1/70.15.2, M1/69.16, and M1/9.3 followed by washing. Animals were bled on day 28.

Other methods were as previously published (Springer *et al.*, 1978, 1979).

RESULTS

Purification and Assay of Antigens—Macrophage surface glycoproteins were purified as described under "Materials and Methods" according to the scheme of Fig. 1. Purification and/or depletion of the M1/9.3 common leukocyte antigen, the M1/69 heat-stable antigen, and M1/70 Mac-1 antigen was monitored by inhibition of the indirect binding assay. (For brevity, these antigens will be named according to their defining MAb. See Table I.) Inhibition curves obtained with antigens at different stages of purification were parallel, and showed excellent dose-response characteristics (Fig. 2), suggesting no change in antigen avidity during purification.

A glycoprotein-enriched fraction was obtained by *L. culinaris* lectin affinity chromatography (Fig. 3). The majority of the M1/9.3 antigen and the M1/70 antigen material was bound to the lectin column; the remainder was retarded relative to the protein peak. After elution with methyl mannoside, the M1/9.3 and M1/70 glycoproteins were obtained in 75 and 81% yield, respectively, while only 5% of the total protein was bound and eluted (Table II). This suggests that other macrophage surface glycoproteins were similarly purified (also see below). About two-thirds of the M1/69 putative glycolipid heat-stable antigen was unretarded by the lectin column, while one-third was bound and eluted (Fig. 3). M1/69 antigen is expressed in very low amounts on macrophages (Springer *et al.*, 1979), and it is likely that much of the M1/69 antigen in these preparations was derived from small percentages of granulocytes, lymphocytes, and red blood cells in the peritoneal exudate cells (see "Materials and Methods").

Immunoadsorbent Removal of M1/9.3 and M1/69 Antigens—In order to increase the likelihood of obtaining macrophage-specific MAb, it was desired to remove antigens shared with other types of cells. Therefore, the widely distributed 200,000 M_r M1/9.3 antigen and the putative glycolipid M1/69 antigen were removed by immunoadsorbent procedures. The lectin-purified material was first passed through an M1/69 immunoadsorbent column. This resulted in complete removal of M1/69 antigen but had no effect on M1/9.3 or M1/70 antigen (Table II). The M1/9.3 antigen was removed using M1/89.23 MAb-Sepharose. This confirmed the previous suggestion (Springer *et al.*, 1978) that M1/9.3 and M1/89.23 MAb recognize determinants on the same antigen. Passes through two M1/89.23 columns were required to completely remove M1/9.3 antigen (Table II), presumably because the capacity of the first column was exceeded. The specificity of M1/69 and M1/9.3 antigen removal was demonstrated by comparison to M1/70 antigen, which was only slightly affected by these procedures (Table II).

Serological Studies on Rats Immunized with Immunoad-

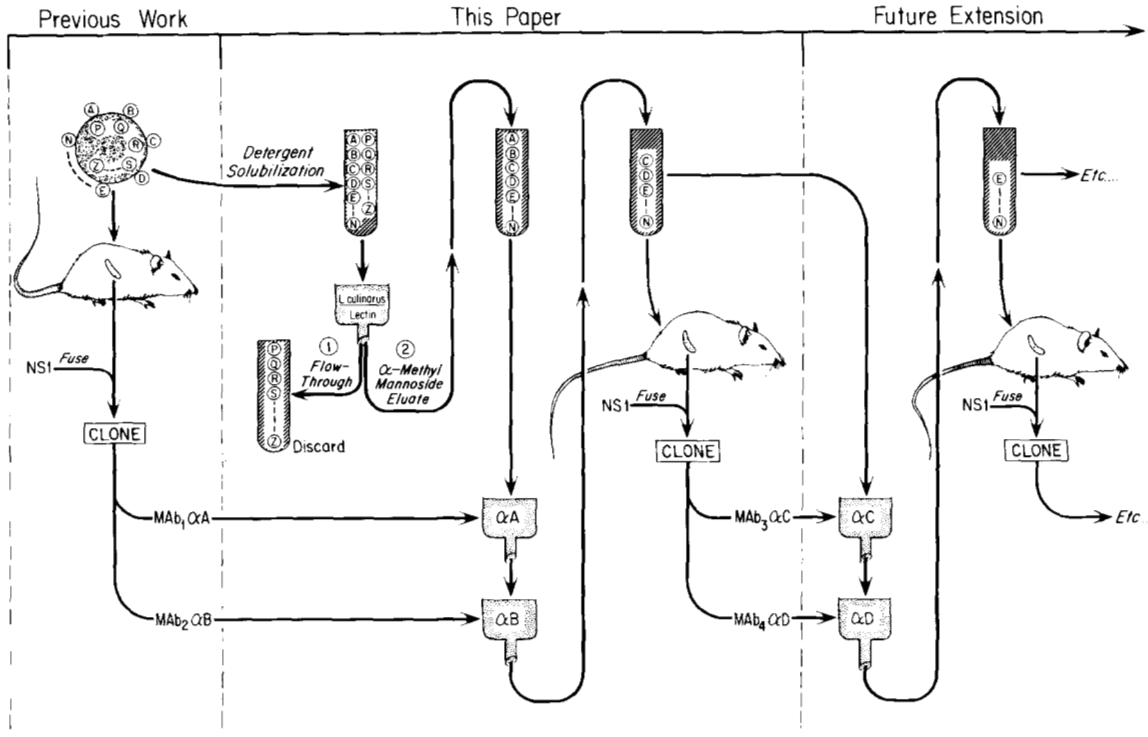

FIG. 1. **Arrangement of cell hybridization experiments and removal of previously recognized antigens using MAb-immuoadsorbents in a cascade.** MAb are obtained to different sets of antigens in each successive hybridization experiment, and the immunogenic stimulus is continually narrowed to those antigens which are yet to be recognized using MAb. Portions of the scheme are separated by *dotted lines* which correspond to previously described work utilizing whole cell immunization (Springer *et al.*, 1978), presently described work, and hypothetical extension of the procedure into the future. MAb_1 = M1/69; A = heat-stable antigen; MAb_2 = M1/89.23; B = common leukocyte antigen. MAb M1/70 was generated in the first hybridization experiment, but its corresponding antigen was not removed prior to the second hybridization. This allowed M1/70 antigen to serve as a positive control for elicitation of macrophage-specific antibodies.

TABLE I

Rat anti-mouse monoclonal antibodies used in these studies

Clones	Cellular recognition	Antigen	Designation
M1/69[a]	Mouse red blood cells, granulocytes, monocytes, B lymphocytes, thymocytes, but not peripheral T lymphocytes	Heat-stable,[c] no iodinated or [^{35}S]methionine-labeled component, putative glycolipid	Heat-stable antigen
M1/9.3[a] M1/89.18[a]	Leukocytes	~200,000 M_r[d]	Common leukocyte antigen, T200[e]
M1/70[a]	Granulocytes and mononuclear phagocytes	190,000 and 105,000 M_r	Mac-1
M3/31,[b] M3/38[b]	Mononuclear phagocytes	32,000 M_r	Mac-2
M3/84[b]	Mononuclear phagocytes	110,000 M_r	Mac-3

[a] Described in Springer *et al.*, 1978, 1979, and Springer, 1980.
[b] Described here.
[c] Stable at 120 °C for 15 min.
[d] The molecular weight of this antigen depends on the source from which it is isolated: 230,000 M_r from B lymphocytes or 200,000 and 180,000 M_r from T lymphocytes (Springer, 1980).
[e] Trowbridge, 1978.

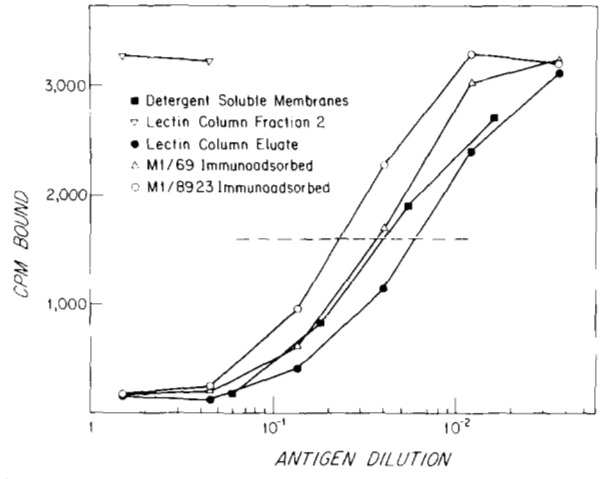

FIG. 2. **Inhibition of the indirect binding assay for M1/70 by sodium deoxycholate-solubilized membrane fractions.** Serial dilutions of fractions (10 μl) were incubated with 5 μl of 1/100 M1/70 culture supernatant in 30% BSA-PBS (5 μl) and tested in the indirect ^{125}I-anti-rat IgG-binding assay as described under "Materials and Methods."

sorbent Purified Antigen and with Whole Cells—To determine whether immunoadsorbent purification of macrophage antigens was advantageous, the responses of animals immunized to purified antigen, whole cells, or antibody-coated cells were compared (Table III). The amount of antibody reactive with a given target cell was measured as the cell-binding titer, or the inverse of the dilution giving half-maximal binding in the indirect assay. Immunization with immunoadsorbent-depleted material gave cell binding titers 18-fold higher on peritoneal exudate cells than spleen, while no specificity for peritoneal exudate cells was evident after immunization with whole peritoneal exudate cells or antibody-coated peritoneal exudate cells. Furthermore, anti-peritoneal exudate cells titers

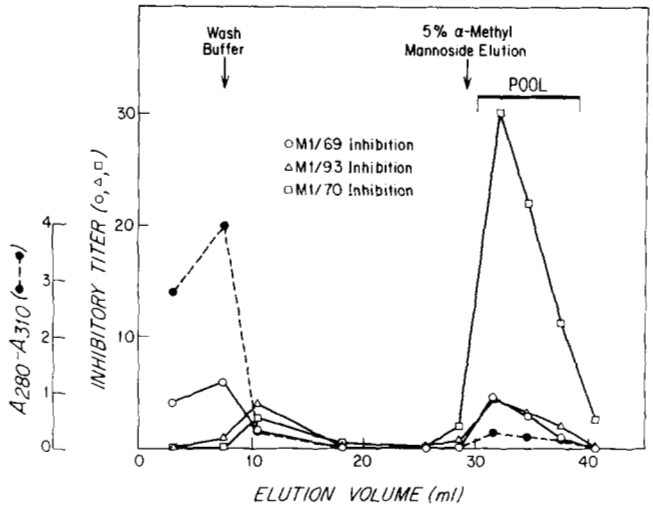

FIG. 3. ***L. culinaris* lectin affinity chromatography of sodium deoxycholate-solubilized macrophage membranes.** The 100,000 × *g* supernatant from sodium deoxycholate-solubilized peritoneal exudate cell membranes was applied to an *L. culinaris* lectin-Sepharose CL-4B column and washed and eluted as described under "Materials and Methods." Indicated fractions (7–9) were pooled and concentrated for immunoadsorbent chromatography.

were 6-fold higher in cascade-immunized animals than in animals receiving whole cells. The serum concentrations of antibodies competing for the same cell surface sites as the M1/70 and M1/9.3 MAb were measured using inhibition of binding of the corresponding ^{3}H-labeled monoclonal antibodies (Table III). Antibodies to the M1/9.3 binding site were elicited by whole cells but not by cascade purified antigen. Antibodies capable of precipitating M1/9.3 antigen were also elicited by whole cells but not by purified antigen (Table III). This suggests that not only the M1/9.3 antigenic determinant but also other determinants on the same molecule were removed by the immunoadsorbent procedure. Antibodies to the M1/69 antigen agglutinate mouse red blood cells, and therefore M1/69-like antibodies were measured by indirect hemagglutination. Immunization with cascade-purified material resulted in much lower anti-red blood cells titers than the other methods (Table III). In contrast, antibodies to the M1/70 site were elicited in 20-fold greater quantities by purified antigen than by whole cells. Thus, the titer of M1/70-like antibodies relative to M1/9.3-like and M1/69-like antibodies was more than 100-fold greater in sera from cascade-purified antigen-immunized animals than in sera from animals immunized to whole cells (Table III). Coating cells with M1/9.3, M1/69, and M1/70 monoclonal antibodies had only a slight effect on the responses to the respective antigens (Table III).

TABLE II

Lectin and cascade immunoadsorbent purification of macrophage glycoproteins

Purification step	M1/69 antigen		M1/9.3 antigen		M1/70 antigen		Protein
	Units[a]	Yield	Units[a]	Yield	Units[a]	Yield	
		%		%		%	A_{280} *units*
1. Detergent-soluble supernatant	53	(100)	40	(100)	234	(100)	34.8
2a. Lectin flow-through	48	91	15	38	18	8	29.4
2b. Lectin eluate	27	51	30	75	189	81	1.8
3. M1/69 Immunoadsorbent flow-through	0	0	32	80	171	73	ND[b]
4. 1st M1/89.23 Immunoadsorbent flow-through	0	0	13	33	152	65	ND[b]
5. 2nd M1/89.23 Immunoadsorbent flow-through	0	0	0	0	151	65	ND[b]

[a] Units = inhibitory titer × volume (ml).
[b] Not done.

TABLE III

Serum antibody specificities after immunization with cascade-purified antigen or whole cells

Rats were immunized and bled as described under "Materials and Methods." Sera from 2–3 animals/group were either pooled or assayed individually.

Immunogen	Cell-binding titer[a]		M1/70 titer		M1/9.3 titer		M1/69-like titer, RBC[b] agglutination[c]	M1/70 inhib. titer/M1/9.3 inhib. titer	M1/70 inhib. titer/M1/69-like titer
	PEC	Spleen cells	Inhib.[d]	Precip.[e] titer	Inhib.[d]	Precip.[e] titer			
			μg/ml		*μg/ml*				
Cascade purified PEC ag.	275	15	186	50	<0.5	<0.2	1.3	>370	140
PEC	47	49	9.6	2	3.3	2	19	2.9	0.5
PEC (antibody-coated)	44	38	6.6	1.5	2.4	1	19	2.8	0.35
Spleen cells[f]	ND	ND	6	ND	2.3	2	110	2.6	0.05
None (preimmune serum)	<1	<1	<0.1	<0.2	<0.5	<0.2	<0.5		

[a] Sera were tested in the indirect binding assay on 10^5 3-day-thioglycollate-induced PEC or spleen cells. Titer = (dilution giving half-maximal binding)$^{-1}$.
[b] The abbreviations used are: RBC, red blood cells; PEC, peritoneal exudate cells; ND, not done.
[c] Serially diluted sera were tested for indirect RBC agglutination as previously described (Springer *et al.*, 1978).

$$\text{Agglutination titer} = \left(\frac{\text{serum agglutination titer}}{\text{M1/69 agglutination titer}}\right) \times \text{M1/69 concentration } (\mu\text{g/ml})$$

[d] Inhibition of binding of ^{3}H-labeled antibodies to target cells was as previously described (Springer *et al.*, 1978). The concentration of serum antibody competing for the same site as monoclonal antibody (MAb) = (serum inhibitory titer/homologous MAb inhibitory titer) × MAb concentration. MAb concentration was determined by Mancini radial immunodiffusion (Ouchterlony and Nilsson 1978).
[e] Indirect immunoprecipitates using 0.2, 1, or 5 μl of sera were subjected to SDS-PAGE, autoradiography, and densitometry (Laskey and Mills, 1977). Precipitation titer = 10 × (volume (μl) giving maximal precipitation)$^{-1}$. Bands measured were the 190,000-M_r M1/70 polypeptide from PEC or the 230,000-M_r M1/9.3 polypeptide from spleen cells.
[f] Sera from rats immunized for the M1 fusion (Springer *et al.*, 1978).

Monoclonal Antibodies to Immunoadsorbent-depleted, Purified Antigens—After priming with purified antigen in complete Freund's adjuvant as described under "Materials and Methods," one (Lewis × BN)F1 rat received 100 μg of antigen in saline i.v. and its spleen was removed for fusion 3 days later. Fusion with NSI was carried out as previously described (Springer *et al.*, 1978) and the cells were aliquoted into 5 × 96 well microculture plates. The 86 cultures giving the highest binding in the indirect binding assay were saved. However, since concentration-dependent background binding was given by normal rat IgG, the 86 cultures may have included some with high concentrations of irrelevant antibodies. Culture supernatants were next screened for immunoprecipitation of ^{125}I (lactoperoxidase)-labeled peritoneal exudate cell surface antigens (Fig. 4). Supernatants from five of the M3 cultures precipitated 190,000 M_r and 105,000 M_r polypeptides and thus appeared to have the same specificity as M1/70. Other supernatants showed specificity for quite different polypeptides. Antibodies from 11 cultures precipitated a 32,000-M_r polypeptide, and one (M3/37) a 180,000-M_r polypeptide. Another culture (M3/84) identified a 110,000-M_r polypeptide (not shown).

Some but not all cultures were successfully cloned as determined by the indirect binding assay and were retested for immunoprecipitation (Fig. 5). Supernatants from the M3/37 culture precipitated a single polypeptide of 180,000 M_r, but lost antibody activity before cloning (Fig. 5, *A–E*). The M3/84 culture defining a 110,000-M_r polypeptide was more stable (Fig. 5, *F–H*). Successful clones (Fig. 5*I*) and subclones (Fig. 5*J*) were isolated. M3/37, M3/84, and M1/70 all precipitated distinctive high molecular weight polypeptides as seen by side-by-side comparison (Fig. 5 *A–K*). Two different cloned lines (M3/31 and M3/38) were obtained which precipitate 32,000-M_r polypeptides (Fig. 5, *M* and *N*). M3/31 is an IgM while M3/38 is an IgG2a as determined by double immunodiffusion with subclass-specific antibodies.

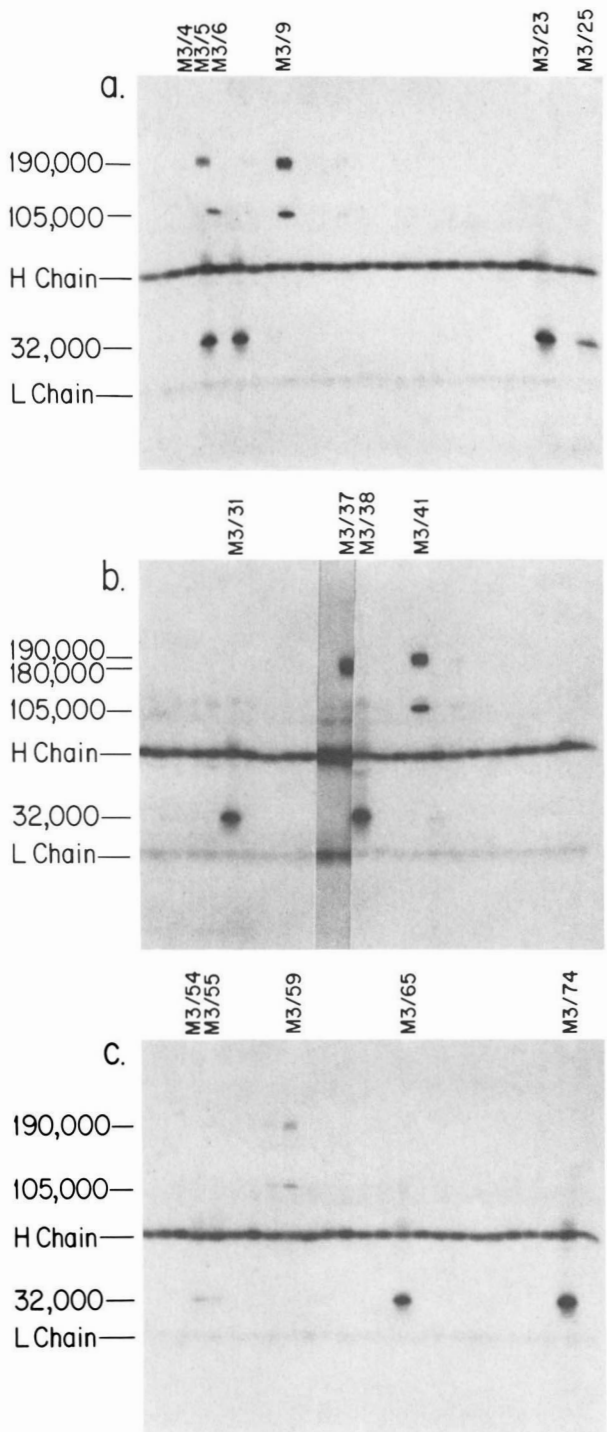

FIG. 4. **SDS-PAGE screening of M3 cultures for precipitation of macrophage surface antigens.** Culture supernatants (100 μl) were mixed with detergent-solubilized antigen from ^{125}I(lactoperoxidase)-labeled 4 d thioglycollate-induced peritoneal exudate cells, and precipitation was induced with rabbit anti-rat IgG. SDS-PAGE of reduced samples was in 5–12% polyacrylamide gradient gels, as described (Springer *et al.*, 1978). H and L chains, apparently derived from Fc receptor-bound mouse IgG, were precipitated by cross-reaction with rabbit anti-rat IgG and serve as a positive control for precipitation. The lane containing M3/37 and the adjacent lane to the left were exposed longer to emphasize the band precipitated by M3/37.

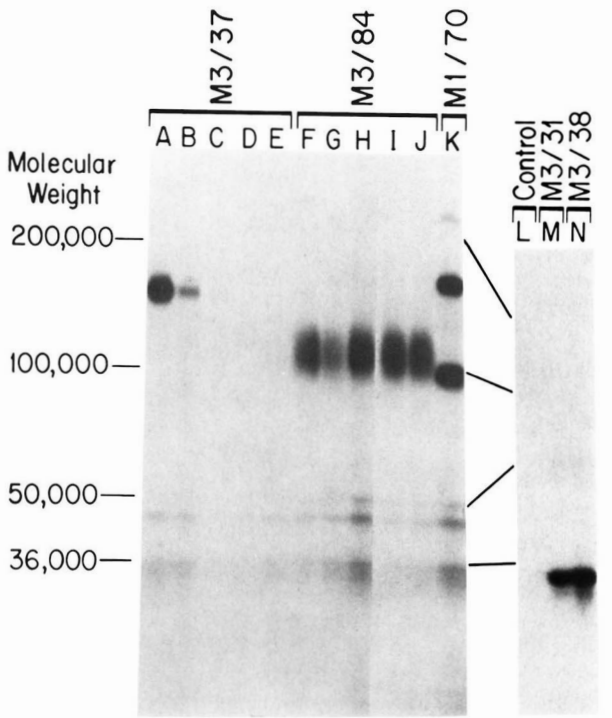

FIG. 5. **SDS-PAGE comparison of ^{125}I-labeled macrophage surface antigens.** A different preparation of ^{125}I-labeled macrophages were lysed, immunoprecipitated, and analyzed as described in Fig. 4. *Lanes A–K* and *L–N* are from different gels. *A–E*, M3/37, uncloned supernatants at weekly intervals; *F–H*, M3/84-uncloned supernatants at weekly intervals; *I*, M3/84.6 clone; *J*, M3/84.6.34 subclone; *K* M1/70.15.1 subclone; *L*, NSI + 50 μg/ml of rat IgG control; *M*, M3/31.1.1 subclone; *N*, M3/38.1.2.8 subclone.

DISCUSSION

This paper describes a procedure which greatly simplifes the collection of MAb libraries directed toward individual components of complex biological systems. Removal of previously recognized antigens with immunoadsorbent columns has been combined with cell hybridization in a cascade which restricts the immunizing stimulus to previously unrecognized antigens. The cascade of alternating cell hybridization and further immunoadsrobent depletion experiments can be indefinitely extended (Fig. 1) until the library is completed.

In this application of the method, the M1/9.3 common leukocyte and M1/69 heat-stable antigens shared with lymphocytes and peritoneal exudate cells were removed from detergent-solubilized peritoneal exudate cell membranes to help focus the immune response toward macrophage differentiation antigens. Quantitative depletion was demonstrated with a radioimmunoassay. The common leukocyte and heat-stable antigens appear to be the most immunodominant antigens in xenogeneic rat anti-mouse spleen cell immunization. In one anti-spleen (Springer *et al.*, 1978), two anti-T lymphocyte, and two anti-T lymphoblast (unpublished) fusions, clones recognizing these antigens have consistently outnumbered those recognizing H-2 and other antigens. The M1/9.3 and M1/69 MAb were derived from anti-spleen immunizations, but were used here to remove antigens from peritoneal exudate cells. Therefore, it was necessary to test whether in fact the removal of these antigens improved the quality of anti-macrophage responses. This was done by comparing antibodies elicited by immunoadsorbent purified antigens and by whole macrophages. Removal of the common leukocyte and heat-stable antigens resulted in a considerable increase in the macrophage specificity of whole antiserum. This suggests that the common leukocyte and heat-stable antigens are among the most immunodominant antigens in rat anti-mouse macrophage as well as in anti-mouse spleen cell immunization. Indeed, similar anti-common leukocyte antigen antibody levels were found after spleen cell and peritoneal exudate cell immunization. Furthermore, whole peritoneal exudate cells elicited almost as high a concentration of anti-common leukocyte antigen as anti-Mac-1 antibodies. Thus, while the cascade-purified material elicited five anti-Mac-1 and zero anti-common leukocyte antigen hybridomas, whole peritoneal exudate cells would have been expected to elicit similar number of each.

The efficacy of the cascade immunization procedure was most convincingly demonstrated by comparing antibody levels to the common leukocyte and heat-stable antigens which were removed, and the Mac-1, which was not. The ratios of Mac-1 to common leukocyte antigen and Mac-1 to heat-stable antigen antibodies were more than 100-fold higher in animals receiving immunoadsorbent-purified antigens than in those receiving whole cells. Animals receiving immunoadsorbent-purified antigen also had higher absolute levels of anti-Mac-1 antibodies, but this could have been due in part to the use of complete Freund's adjuvant. In the phenomenon of antigenic competition, co-administered antigens nonspecifically suppress the immune response to each other (Pross and Eidinger, 1974). Therefore, membrane and glycoprotein purification and immunoadsorbent depletion could also have contributed to the higher absolute Mac-1 antibody response.

Other procedures have also been proposed for narrowing the range of MAb elicited in cell hybridization experiments. Tolerance induction to B lymphocytes has been used to enhance the percentage of T cell-specific hybridomas (Middleton *et al.*, 1980). Blocking surface antigens of one type of cell with whole antiserum to another type of cell has also been used to increase specificity (Kennett *et al.*, 1978). These procedures have the disadvantage that they cannot continue to narrow the response to unidentified cell surface antigens in further hybridization experiments. An alternative method of blocking cell surface sites with MAb before injection was used here with little success. MAb differ from conventional sera in that only one out of a large number of potential antigenic sites would be blocked, and this may have contributed to the observed lack of effect. Also, it is likely that a fraction of cell-surface-bound MAb would dissociate after injection and allow immune responses.

Previously, a number of investigators have prepared anti-macrophage serum by xenoimmunization with macrophages and exhaustive absorption with other tissues (Unanue, 1968; Hirsch *et al.*, 1969; Gallily and Gornostansky, 1972). Several macrophage-specific antigens have also been defined by monospecific sera. The Mac-1 antigen containing polypeptide chains of 190,000 and 105,000 M_r is present on monocytic and granulocytic but not erythroid or lymphoid lines of differentiation (Springer *et al.*, 1979). An alloantigen, Mph-1, has also been defined by cytotoxicity and by genetic mapping but has not been structurally characterized (Archer and Davies, 1971). The mouse Fc receptor II of 47,000 and 70,000 M_r has also been identified with MAb (Mellman and Unkeless, 1980), and is expressed on both macrophages and B lymphocytes. Antigens of 32,000 M_r (designated Mac-2, 110,000 M_r (designated Mac-3), and an antigen of 180,000 M_r have now additionally been described.

Further detailed studies on the cell distribution and biochemistry of these antigens[3] show they are present on stimulated macrophages but absent from erythroid, lymphoid, and granulocytic cells and thus appear restricted to the mononuclear phagocyte line of differentiation. Purified macrophages incorporate [^{35}S]methionine into the M3/38 32,000-M_r and M3/84 110,000-M_r polypeptides, suggesting these cells synthesize as well as express these antigens on their surfaces. These monoclonal antibodies should prove invaluable in studying macrophage subpopulations, differentiation, localization, and the structure and function of these antigens.

Theoretically, the hybridoma approach should allow the complete repertoire of cell surface antigens to be catalogued. The cascade procedure (Fig. 1) allows this to be done in an efficient and straightforward manner, and has three considerable advantages over whole antigen mixtures. First, repetition of the monoclonal past is avoided, thereby greatly simplifying screening procedures. Second, immunodominant antigens are removed, and therefore should not inhibit the immune response to other antigens by antigenic competition (Pross and Eidinger, 1974). Third, the procedure can be extended at will in a cascade which continually restricts MAb production to previously unidentified antigens. Before each new hybridization experiment, the antigen preparation is simply passed through immunoadsorbent columns containing the most recently isolated MAb. The procedure is not much more difficult than immunization with whole antigen mixtures. If sufficient quantity of antigen can be obtained, preliminary procedures such as membrane isolation, detergent solubilization, and lectin affinity chromatography (if desired) need be done only once. The cascade approach is also applicable to any other complex system where the antigenic components can be separated under nondenaturing conditions, such as tissue extracts, detergent-solubilized organelles, or mixtures of different viruses. When this work was in progress, the theoretical application of this method to cytoplasmic proteins was also independently proposed in a review article (Milstein and Lennox, 1980). The use of cell hybridization-MAb-immuno-

[3] M. Ho and T. Springer, manuscript in preparation.

adsorbent cascades should greatly facilitate the analysis of biological complexity using myeloma-hybrid technology.

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