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Cell-Surface Differentiation in the Mouse

Characterization of "Jumping" and "Lineage" Antigens Using Xenogeneic Rat Monoclonal Antibodies

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I. Introduction

Differentiation is the process whereby indifferent cells give rise to specialized tissues and cellular subpopulations with distinctive characteristics. This involves the coordinated control of many different genes, some of which affect the expression of molecules at the cell surface. Those molecules that can be identified with antibodies and are expressed on some but not all tissues are called differentiation antigens (Boyse and Old, 1969). Both qualitative and quantitative variations occur in the expression of differentiation antigens.

A salient advantage of probing surface, as opposed to intracellular differentiation antigens, is that various methodologies involving antibodies bound to the surface allow intact cells to be separated on the basis of their state of differentiation. Functional capacity may then be related to cell-surface phenotype. Furthermore, antibodies are versatile probes that may also be used to inhibit or modify the activity of molecules in their native cell-surface environment or for the isolation and biochemical characterization of surface molecules.

The richness of the cell surface in terms of the great variety of different antigens displayed, even on a single type of cell, has in the past been a considerable obstacle to analysis. The Köhler and Milstein (1976) hybrid technique has therefore been of tremendous importance for developments in this field. We

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have explored the use of the myeloma-hybrid technique for the xenogeneic analysis of differentiation antigens in the mouse, with particular emphasis on leukocyte antigens (Springer *et al.*, 1978a,b; see Milstein *et al.*, 1979, and Milstein and Lennox, 1980, for a review of studies in both the rat and mouse). Probably more surface antigens have been defined in the mouse than in any other species, and thus one question was with what frequency the myeloma-hybrid method would allow discovery of novel surface antigens. Most previously defined surface antigens in the mouse had been identified by alloimmunization of one strain with another, in which congenic strains were used to restrict the immunizing stimulus to the polymorphic products of a short chromosomal segment. In contrast, our object was to study differentiation in the broadest sense and thus to elicit strong antibodies to as wide a range of surface molecules as possible. Xenoimmunization, of rats with mouse spleen cells, was therefore chosen. The resultant multispecific response to a large array of different cell-surface molecules was then resolved by cloning into a set of hybrid lines each recognizing an individual determinant on an individual cell-surface molecule.

II. *Techniques for Obtaining and Characterizing Monoclonal Antibodies to Differentiation Antigens*

A. *Immunization and Hybridization*

Most procedures have previously been described in detail (Springer *et al.*, 1978b). Briefly, rats were primed two times with mouse spleen cells enriched for T lymphocytes by nylon-wool filtration or by concanavalin A (Con A) stimulation and depleted of red blood cells (RBC) by Isopaque–Ficoll sedimentation. An intravenous boost was given 3 days prior to the hybridization experiment. Fusion with polyethyleneglycol (PEG) was as described (Galfré *et al.*, 1977), except the PEG solution (~50% w/w) was made by autoclaving 10 g PEG, allowing it to cool partially, and while still liquid, adding 10 ml Dulbecco's Modified Eagle's Medium (DMEM). After fusion of 2×10^8 rat spleen cells with 2×10^7 P3-NSI/1-Ag4-1 (NSI) nonsecretor myeloma cells, the cells were aliquoted into 96× 2-ml wells and grown in hypoxanthine–aminopterin–thymidine (HAT) medium (Littlefield, 1964). These cultures were designated M1/1-M1/96. Clones derived from these cultures by agar cloning are designated with a second number, e.g., M1/9.3, and if recloned, a third, e.g., M1/9.3.4. All lines described here have been subcloned at least once. For brevity, clone designations have been omitted except to distinguish clones recognizing different antigens isolated from the same culture.

B. *Identification of Positive Cultures*

After 2 weeks of growth positive clones were tested in an indirect ^{125}I-anti-rat immunoglobulin (Ig) cell binding assay as previously described (Springer *et al.*, 1978b). In this assay rat antibodies secreted into tissue-culture supernatants

were first incubated with target cells at 4°C for 45 min in microtiter plates, washed, then incubated with ^{125}I-rabbit F(ab')$_2$ anti-rat IgG or Fab for a further 45 min at 4°C, washed, and counted in a Y spectrometer. Xenogeneic antibodies are much more convenient to assay than allogeneic ones because ^{125}I-anti-rat Ig absorbed with mouse IgG can be used, preventing cross-reaction with mouse Ig on B lymphocytes or absorbed onto Fc receptors. Of 94 cultures 92 contained specific antibody-secreting hybrids. From the Poisson distribution it was estimated that each culture contained an average of four independently fused, successful, specific antibody-secreting cells. These were in addition to an undetermined number of negative hybrids. After further growth over a 7-week period, faster-growing clones became dominant, and some cultures became negative. Then 17 of the cultures were cloned in soft agar. A total of 500 clones were transferred to liquid culture, and their culture supernatants were tested in the indirect ^{125}I-anti-rat Ig-binding assay. From 7 of the cultures 47 clones were identified as positive. The fraction positive ranged from 2/48 for M1/9 to 19/19 for M1/89. Multiple clones from the same culture were compared for reactivity with different cells, such as thymocytes, spleen white cells, and mouse RBC and sheep RBC, and by titration of clonal supernatants (Springer *et al.*, 1978b) or of spleen target cells (Springer *et al.*, 1978a) in ^{125}I-anti-rat Ig indirect binding assays. In the case of multiple identical clones, two representatives, generally with the highest titer in the binding assay, were saved. From each of three cultures, two different clones with distinct antigenic specificities were isolated, i.e., M1/9.3 and M1/9.47, M1/22.25 and M1/22.54, and M1/89.1 and M1/89.18. A single type of clone was isolated from four cultures.

Since these clones (the first 10 listed in Table I) were selected for no criteria other than reactivity in the indirect binding assay, they represent a random collection of monoclonal antibodies directed against surface antigens. They recognize five types of antigens, some of which had been previously identified by alloantisera. One, the Forssman antigen, had previously been identified by relatively monospecific xenoantisera (Boyd, 1966). The common leukocyte antigen (CLA) had previously been precipitated by one component of polyspecific antilymphocyte sera (Trowbridge and Mazauskas, 1976), but monspecific sera had not been previously obtainable, even by absorption. The M1/69, M1/75, and M1/70 antigens (Table I) had not been previously identified by any means. The small degree of overlap between these antigens and those previously identified and the ease with which further novel antigens could be identified (see Section IV) suggests that currently known antigens only represent the tip of the iceberg, and that the vast majority of antigens are still lurking on the cell surface awaiting identification.

C. *Stability*

When lines are grown continuously for long periods in culture, antibody activity is periodically monitored and recloning is carried out every 6 months. After each cloning aliquots of cells are frozen in liquid N_2. After the first subcloning the lines appear quite stable, e.g., variants with loss of either the specific

TABLE I
Mouse Differentiation Antigens Identified by Rat Monoclonal Antibodies

Clones	Cellular recognition	Antigen	Designation
M1/22.25 M1/87	Early embryos,[a] germinal tissue, erythroblasts, sheep RBC but not mouse RBC	Heat-stable,[b] Forssman glycosphingolipid	Forssman
M1/9.47, M1/22.54 M1/69, M1/89.1	Mouse RBC, granulocytes, monocytes, B lymphocytes, thymocytes but not peripheral T lymphocytes	Heat-stable, no iodinated or [^{35}S]methionine-labeled component	Heat-stable antigen (HSA)[c]
M1/75	Mouse RBC, not on thymocytes or lymphocytes	Heat-stable, no iodinated or [^{35}S]methionine-labeled component	Heat-stable antigen (HSA)[c]
M1/9.3 M1/89.18	Leukocytes	MW ~ 200,000[d]	Common leukocyte antigen (CLA)
M1/42	Almost all cells	MW 46,000 and 12,000	H-2
M1/84	Leukocytes, mast-nucleated bone marrow cells, others?	MW 46,000	?
M1/70	Granulocytes and mononuclear phagocytes[e]	MW 190,000 and 105,000	Mac-1
M3/31, M3/38	Mononuclear phagocytes	MW 32,000	Mac-2
M3/84	Mononuclear phagocytes	MW 110,000	Mac-3

[a]Stern *et al.* (1978); Willison and Stern (1978).
[b]Stable at 120°C for 15 min.
[c]This group of antibodies competes between themselves for binding to mouse red blood cells.
[d]The molecular weight of this antigen depends on the source from which it is isolated: molecular weight 230,000 from B lymphocytes or molecular weights 200,000 and 180,000 from T lymphocytes.
[e]Springer *et al.* (1979).

or myeloma light chain are found at a frequency of slightly less than 10^{-2} several months later. However, it is extremely important to clone the initial cultures, even if derived from a single hybrid cell, since chromosome losses leading to specific heavy- or light-chain losses are particularly frequent in the early stages of growth. Using the procedures described above no lines have ever been lost subsequent to obtaining the first positive clone.

D. *Immunoprecipitation*

Antigen specificity was investigated by immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of radioactively labeled cell-surface molecules (Fig. 1). An extremely heterogeneous mixture of proteins was precipitated by the serum antibodies from the rat contributing the spleen for the fusion. The monoclonal pattern was much simpler. Two mono-

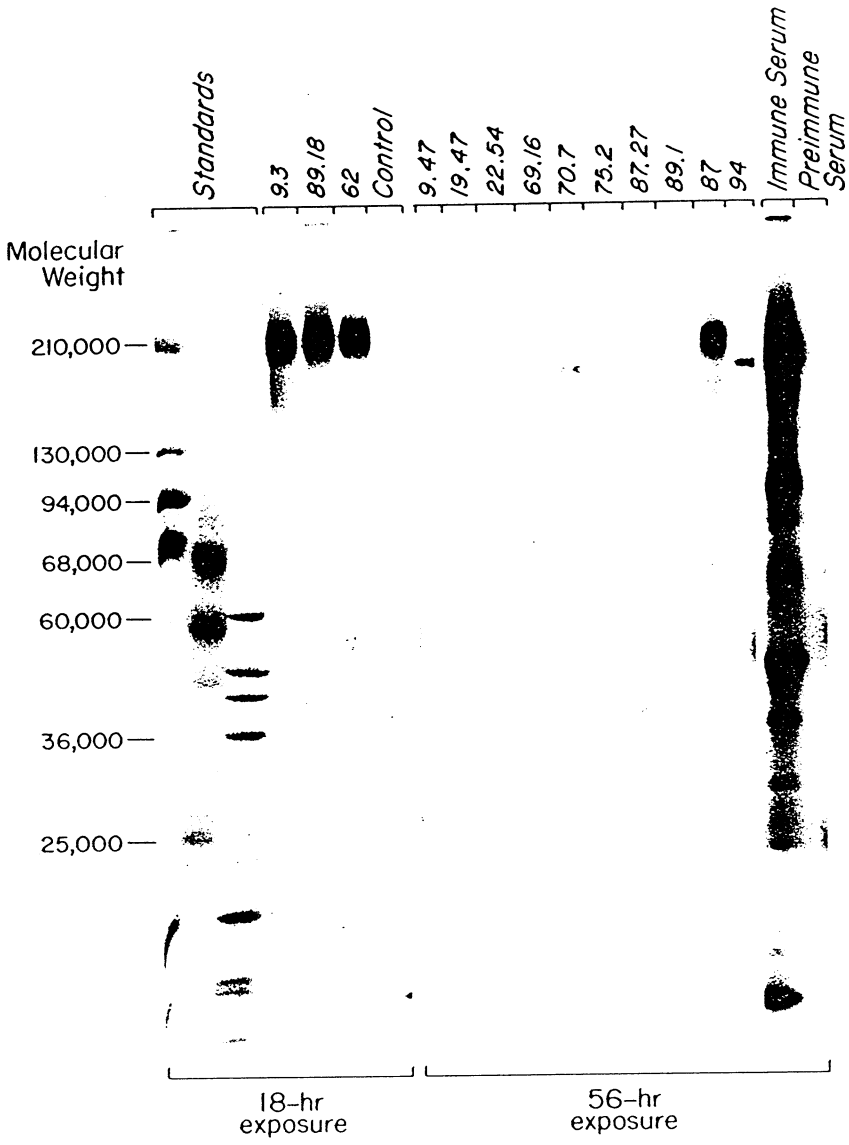

FIGURE 1. SDS 7–15% gradient PAGE analysis of ^{125}I(lactoperoxidase)-labeled Con A-stimulated spleen cell surface, Triton X-100-solubilized molecules precipitated by M1 clone antibodies and rabbit anti-rat IgG.

clonal antibodies precipitated a polypeptide of molecular weight 210,000, while another, M1/70, precipitated two polypeptides of molecular weights 190,000 and 105,000. It was also important to note that a number of monoclonal antibodies did not precipitate any material which could be labeled with ^{125}I. All these react with antigenic determinants that are stable to autoclaving (Table I) and are thus likely to be carbohydrate.

The precipitation experiments illustrate the most important advantage of the monoclonal technique: the ability to use a "dirty" immunogen but to obtain monoclonal antibodies recognizing only a single component of the total mixture. The lack of background precipitation demonstrates the extraordinary specificity of monoclonal antibodies and their utility in protein purification. Because they are pure, monoclonal antibodies are also much more potent than classical antisera. For example, 100 ng of M1/70 antibody, which is obtained in 1 μl of spent culture medium, is sufficient to precipitate maximally its antigen from a lysate of 5×10^5 peritoneal exudate cells. The comparable quantity of alloantiserum normally used to effect precipitation is about 10 μl, containing approximately 100 μg of IgG or 1000-fold more.

E. *Strain Distributions*

The clones listed in Table I have been tested on 6–20 inbred mouse strains of different H-2 types and backgrounds. None show any allospecificity, suggesting that only a small minority of cell-surface antigenic determinants are subject to polymorphic variation. Similarly Parham and Bodmer (1978) immunized mice with human HLA-A2 antigen and found that only 4 of 13 lines that reacted with the highly polymorphic HLA heavy chain recognized polymorphic determinants.

F. *Tumor-Cell Panels*

Tumor-cell lines are useful models for studying differentiation antigens, since each line is usually a homogeneous population of cells arrested in a particular stage of differentiation. Screening of tumor-cell panels proved to be particularly important in the study of two clones, M1/70 and M1/22.25, which gave weak binding to spleen cells. M1/70 gave specific binding of only 2 times background binding, considered barely significant. However, binding plateaued out to a 1000-fold dilution of supernatant, suggesting limitation by the antigen concentration in spleen, rather than antibody concentration. To quantitatively compare the amounts of antigen expressed on a number of tumor lines as well as normal cells the indirect ^{125}I-anti-Ig binding assay was conducted with serial dilutions of cells. For each monoclonal antibody similarly shaped titration curves, plateauing at a characteristic level, were obtained for all positive lines. Antigen concentration was expressed as (cells/ml giving half-maximal bind-

ing)$^{-1} \times 10^9$, or "antigen titer," which is proportional to antigen site number. M1/70 antigen is expressed on the P338D$_1$ macrophagelike tumor line in 100 times greater quantity than on spleen cells but not on T or B lymphomas or the NSI myeloma line (Table II). Further work has confirmed that M1/70 antigen (Mac-1) is a marker of the granulocytic–monocytic line of differentiation.

Similar, though less quantitative, experiments showed that the M1/22.25 Forssman antibody specifically labels embryonal carcinoma cell lines but not thymoma, mastocytoma, myeloma, Abelson or Moloney lymphoma, Friend leukemia, neuroblastoma, fibroblast, or methylcholanthrene-induced sarcoma lines (Stern *et al.*, 1978). Subsequent work demonstrated the presence of M1/22.25 antigen on germinal tissue and temporally and topographically limited expression on early mouse embryos (Willison and Stern, 1978). Thus tumor-cell panel screening is a particularly useful means of characterizing monoclonal antibodies that recognize small subpopulations. Of 12 clones recovered from the M1 hybridization (the anti-HSA, Mac-1, and Forssman clones) 8 recognize antigens present on 25% or less of the nylon-wool, Ficoll–Isopaque purified cells used in priming (see Table IV).

Tumor-cell typing also provided information about the relationship between different antigens. Similarity between M1/9.3 and M1/89.18 antigen was suggested by their identical tumor cell distributions (Table II). This was confirmed by immunoprecipitation of the same antigen of molecular weight 210,000 and competition for the same cell-surface site in antibody cross-inhibition experiments. Differences were suggested between M1/69 and M1/75, both of which recognize an HSA on mouse RBC. M1/69 but not M1/75 antigen is expressed on thymocytes, splenic white cells, and B- and T-lymphoma lines (Table II). The differences were confirmed in cross-inhibition experiments (see Section IV.B).

TABLE II

Antigen Titers on Tumor Lines and Normal Cells[a]

	M1/9.3	M1/89.18	M1/69	M1/75	M1/70
Red blood cells	<0.2	<0.2	150	110	<0.02
Spleen Ficoll–Isopaque pellet	37	40	90	20	(5)
Spleen Ficoll–Isopaque band	170	125	67	<0.4	(3)
Thymocytes	150	130	60	<0.4	<0.8
SIA T lymphoma	950	650	2500	<2	<4
S49 T lymphoma	1100	770	1700	<2	<4
BW 5147 T lymphoma	2900	2000	270	<2	<0.8
R8 CL7 Abelson lymphoma	650	500	570	<10	<4
NSI myeloma	<6	<6	(5)	<2	<0.2
P388D$_1$ macrophagelike line	200	250	(3)	<2	480

[a] Titer = $10^9 \times$(cells/ml giving half-maximal ^{125}I-anti-Ig binding)$^{-1}$. See text for details. Parentheses indicate extrapolated value; weak binding was found at highest cell concentration tested. < Means that binding was negative at highest concentration tested.

G. *Fluorescence-Activated Cell Sorter Analysis*

Single-cell suspensions were prepared from lymphoid tissues, labeled with monoclonal antibodies, washed, labeled with affinity purified fluoresceinisothiocyanate (FITC)-rabbit $F(ab')_2$ anti-rat IgG absorbed with mouse IgG, again washed, and subjected to fluorescence-activated cell sorter (FACS) analysis

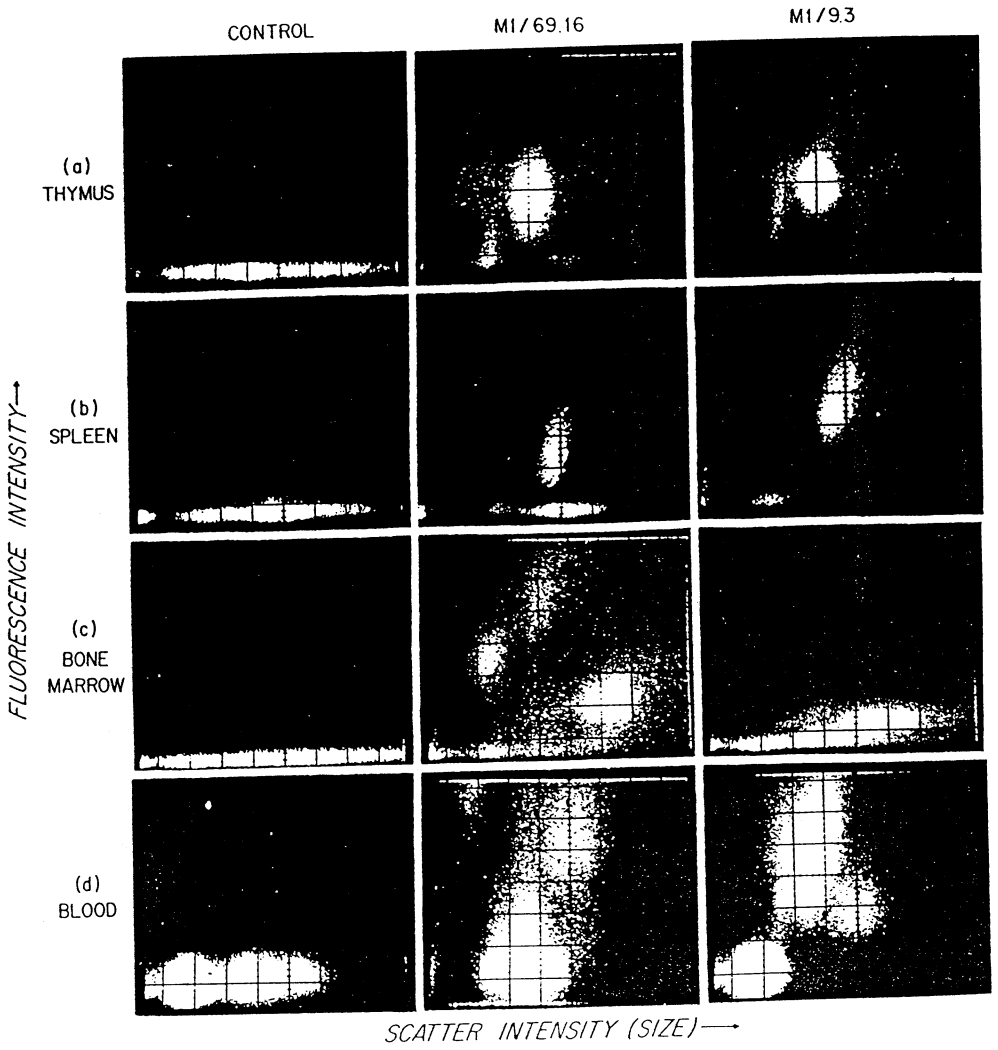

FIGURE 2. FACS two-dimensional resolution of lymphoid tissue cell subpopulations labeled by monoclonal antibodies. Dots mark the intersection of the fluorescence and scatter intensities of individual cells. Cells were purified by Ficoll–Isopaque sedimentation and labeled as described in the text. Some remaining RBC were apparent in bone marrow and blood. An irrelevant rat monoclonal supernatant, R4/18.2, was used as the control. Fluorescence and scatter gains were identical within each tissue but differed between tissues. (a) Thymocytes; (b) spleen cells; (c) bone marrow; (d) blood cells. Populations in increasing order of scatter intensity are RBC, lymphocytes, and monocytes.

(Loken and Herzenberg, 1975). As each cell passes through the FACS laser beam, two parameters are measured: fluorescence emission and light scattering. Fluorescence intensity is directly proportional to the number of FITC molecules bound per cell (and hence in saturating conditions, to the number of antigenic sites); light-scattering intensity is related to cell size but is also influenced by other factors such as cell density and shape. The information from the analysis of 40,000–200,000 cells is displayed in the form of dot plots on a cathode-ray tube of the FACS in Fig. 2. Each cell is displayed as a dot at the intersection of its fluorescence (y axis) and scatter (x axis) intensities. The combination of these two parameters, together with the extraordinary specificity of the monoclonal reagents, resolves in many cases discrete cellular subpopulations. These two dimensional displays or cellular fingerprints are characteristic for each combination of tissue and monoclonal antibody, much in the way that peptide fingerprints are characteristic for each combination of protein and protease. Moreover, just in the way that a subpopulation of pure molecules may be eluted from an area of a chromatogram and chemically characterized, subpopulations of pure cells may be "eluted" from any rectangular subdivision of the dot plot by the selection of appropriate fluorescence and scatter "windows" during sorting. The subpopulations may then be characterized by functional or morphologic criteria. For example, Wright–Giemsa staining of sorted subpopulations from bone marrow shows that M1/69.16 can separate granulocytes from small and large lymphocytes and that M1/9.3 (at higher fluorescence gain) can separate small, mature lymphocytes from immature lymphocytes and granulocytic precursors. Furthermore, it now seems possible to assess the purity of sorted cellular subpopulations by labeling with different monoclonal antibodies and fluorochromes, just as molecular purity is usually checked using analytical separation conditions differing from the preparative conditions.

Even when monoclonal antibodies do not completely resolve cell populations, they can give information about their relative heterogeneity or homogeneity. For example, the quite uniform labeling of blood monocytes, the population with highest scatter in Fig. 2d, supports the idea that during transport from bone marrow to the tissues, monocytes are arrested in a uniform state of differentiation, and that diversification occurs only after crossing the endothelium and encountering particular tissues and levels of inflammation. In contrast, while all lymphocytes are labeled by M1/9.3, it is clear that blood lymphocytes differ in the quantitative amount of M1/9.3 antigen that they express (Fig. 2d), supporting the known heterogeneity of these cells.

III. Properties of Rat Monoclonal Antibodies

A. Quantitation of Immunoglobulin Chains

Large quantities of monoclonal antibodies can be obtained by growth of rat–mouse hybrid lines in tissue culture (Table III). Quantitation is by Mancini single

TABLE III
Properties of Rat Monoclonal Antibodies[a]

Monoclonal antibody	Class or subclass	Complement-mediated lysis[b]	Staph A[d] binding	Active antibody concentration (range, μg/ml)	Chain composition of subclones
M1/22.25	μ	+	–	30–44	HL
M1/87	μ	+	–	62–88	HLK
M3/31	μ	N.D.[c]	–	22–36	HLK
M3/84	γ1	N.D.	+	20–25	HL
M1/9.3	γ2a	–?	–	58–235	HLK, HL
M1/42	γ2a	–?	–	90–106	HLK
M1/84	γ2a	–?	–	51	HL
M3/38	γ2a	N.D.	–	101	HLK
M1/70	γ2b	+	–	50–118	HL
M1/89.18	γ2b	+	N.D.	—[e]	HK?
M1/9.47	γ2b	+	–	98–110	HLK
M1/69	γ2b	+	–	100–252	HLK, HL, HK
M1/89.1	γ2b	+	–	126–174	HLK
M1/22.54	γ2c	+	+	94–119	HLK
M1/75	γ2c	+	+	77–87	HLK

[a]See text for details of determinations.
[b]Springer *et al.* (1978b).
[c]N.D., not determined.
[d]Staph A, protein A-bearing *Staphylococcus aureus*.
[e]The rabbit anti-rat Fab antibody used in single radial immunodiffusion is unreactive with this antibody (see Springer *et al.*, 1978b).

radial immunodiffusion (Ouchterlony and Nilsson, 1978) against rabbit anti-rat Fab. This antibody does not react with the NSI myeloma κ chain or H chain in combination with it (e.g., M1/89.18), and thus the values in Table III reflect the concentration of active half-molecules containing heavy chains associated with specific rat light chains. The presence of either γ or μ specific heavy (H) chains and specific light (L) or myeloma kappa (κ) chains have been investigated by SDS gel electrophoresis, isoelectric focusing (IEF) (Springer *et al.*, 1978b), and a radioimmunoassay detecting κ chain V_L determinants (Springer, submitted for publication). Chain compositions of the subclones and of light-chain loss variants isolated from them are listed in Table III. M1/89.18 appears to be an unusual example of an HK hybrid molecule which retains antigen-binding activity, since specific L chain cannot be detected in either SDS-PAGE or IEF, or by reaction with rabbit anti-rat Fab.

B. *Purification*

Most lines secrete 50–100 μg/ml of antibody, and purification of large quantities is obtained by growth in 5% fetal calf serum (FCS), precipitation with $(NH_4)_2SO_4$ and diethylaminoethyl (DEAE) cellulose and G-200 chromatography. This yields homogeneous material, as shown by SDS-PAGE (Fig. 3) and

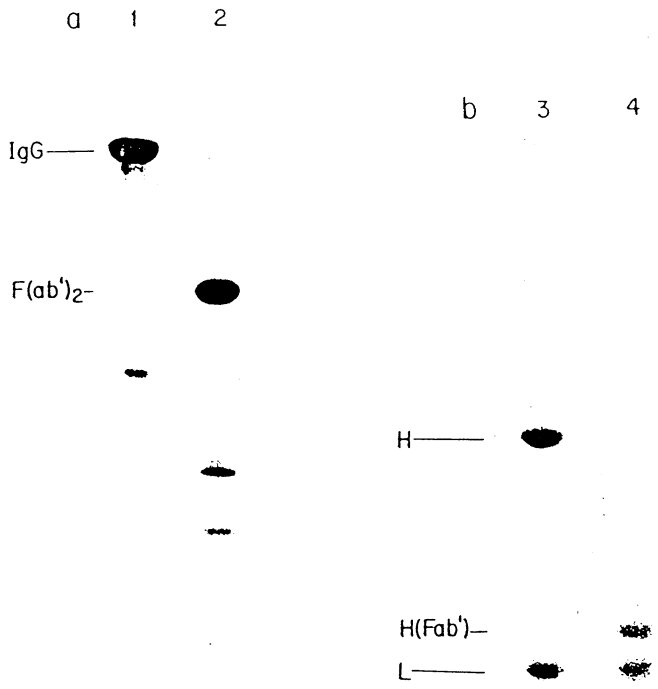

FIGURE 3. SDS–PAGE of purified M1/70 IgG, before and after pepsin digestion. (a) Nonreduced: 1, M1/70 IgG (20 μg); 2, M1/70 F(ab')$_2$ (20 μg). (b) Reduced: 3, M1/70 IgG (10 μg); 4, M1/70 F(ab')$_2$ (8 μg). Samples were prepared in SDS buffer containing 50 mM iodoacetamide (nonreduced) or 5% 2-mercaptoethanol (reduced), subjected to SDS 5–15% PAGE, and stained with coomassie blue. The two gels in (a) and (b) were run on separate occasions. The position of calibration proteins established the identity of bands noted in the figures.

agreement between assays for protein and for rat IgG by Mancini immunodiffusion. F(ab')$_2$ fragments can also readily be obtained (Fig. 3) using standard pH 4 pepsin digestion conditions (Stanworth and Turner, 1978). The inability to grow mouse–rat tumors in normal animals is therefore not a serious drawback of this approach. Athymic nude rats (Festing *et al.*, 1978) present a possible alternative method for large-scale antibody production. Rat myeloma lines suitable for use in cell hybridization have also recently been described (Galfré *et al.*, 1979).

C. Subclass Properties

Rat Ig classes and subclasses were defined by Bazin and co-workers (1974) through the use of a large collection of Ig-secreting ileocecal immunocytomas. Using commercially available typing sera (Miles Laboratories) each of the monoclonal antibodies described here could clearly be placed in one of these subclasses (Table III), confirming the idea that all extant γ subclasses were identified in the Bazin collection. Concentrations of most of the monoclonal antibodies in tissue culture supernatants were sufficiently high to allow typing by direct visualization of precipitin lines in Ouchterlony double immunodiffusion, and the remainder could be typed using 10-fold concentrates.

Monoclonal antibodies offer considerable advantages over myeloma proteins of unknown antibody specificity in assessing the biological properties of Ig classes and subclasses. For example, antibody- and complement-dependent lysis of ^{51}Cr target cells has been used to test for complement fixation (Table III). Antibodies of IgM, IgG2b, and IgG2c classes were clearly lytic in the presence of guinea pig complement. So far no lysis has been observed by three IgG2a subclass antibodies, and the IgG1 antibody has not been tested. Comparison of five antibodies that recognize the same HSA on mouse RBC showed that the three IgG2b's more efficiently utilized complement than the two IgG2c's. The observation that IgG2c's fix complement is in disagreement with the report of Medgyesi *et al.* (1978), who did not have the advantage of using antigen-binding antibodies. It is possible that the 0.5% boric acid purification step used by Medgyesi *et al.* destroyed IgG2c complement-fixing activity, or that heat aggregation does not induce fixation by this subclass.

An interesting property of the IgG2c monoclonals, M1/22.54 and M1/75.21, is their extreme potency in direct hemagglutination. Large clumps of RBCs, several millimeters in diameter, are formed. After unit gravity sedimentation, highly purified splenic white blood cells can be obtained. In contrast, three other monoclonals of the IgG2b subclass that recognize the same HSA on mouse RBC give weak or no agglutination unless anti-rat IgG is added. Strength in agglutination does not correlate with avidity or fine specificity. This suggests that IgG2c's have a more flexible hinge region than IgG2b's.

Another interesting property of the IgG2c subclass is that rats injected with streptococci, a carbohydrate antigen, make almost exclusively antibodies of this subclass (Leslie, 1979). In the case of the HSA, which also appears to be carbohydrate, 2 of 5 clones are IgG2c, compared to 0 of 8 for the anti-protein clones.

Staphylococcus aureus binding properties of all of the rat monoclonals were studied by absorbing neat or 10× supernatants with an equal volume of a 50% suspension of *S. aureus* cowan I strain bacteria in pH 8 buffer (Ey *et al.*, 1978), followed by Mancini immunodiffusion against rabbit anti-rat Fab. Antibodies of the IgG1 and IgG2c subclasses bind, while IgG2a, IgG2b subclasses, and IgM do not. This is in agreement with the results of Medgyesi *et al.* (1978), with the exception that they found 2 of 3 IgM myeloma proteins reactive. ^{125}I-*S. aureus* protein A binding assays have been used to screen for mouse monoclonal antibodies (Oi *et al.*, 1978), but the finding that only 3 of 15 rat monoclonals are *S. aureus*-reactive demonstrates the limitation of the technique in this species.

IV. *Murine Differentiation Antigens Identified by Rat Monoclonal Antibodies*

A. *Forssman Antigen*

The Forssman antigen is widely distributed among animal species and bacteria but not in a phylogenetically ordered manner (Boyd, 1966). Rats are Forssman-negative, while mice and guinea pigs are Forssman-positive. The tissue and species distribution of the antigen recognized by M1/22.25 and M1/87 monoclonal antibodies identify it as Forssman. Absorption by autoclaved guinea pig kidney but not ox RBC distinguishes the heterophile activity from that shown by Paul-Bunnell antibodies, which are found in infectious mononucleosis. Moreover, rabbit antiserum to sheep RBC ("hemolysin"), which classically defines this antigen, competitively inhibits the binding of M1/22.25 and M1/87 to sheep RBC (Springer *et al.*, 1978b). Classically, anti-Forssman antibodies are of the IgM class, as are M1/22.25 and M1/87. The Forssman hapten structure from mammalian species including horse, sheep, goat, dog, and guinea pig is *N*-acetylgalactosaminosyl-(α1-3)-*N*-acetylgalactosaminosyl-(β1-3)-galactosyl-(α1-4)-galactosyl-(β1-4)-glucosyl)(β1-1)-ceramide (Ziolkowski *et al.*, 1975). The findings that the antigen identified by M1/22.25 on murine embryonal carcinoma cells is stable at 100°C, is absent from cells after methanol treatment, and can be labeled by [^{14}C]galactose but not ^{125}I(lactoperoxidase) or [^{35}S]methionine, are consistent with the above glycolipid structure (Stern *et al.*, 1978).

The distribution of the Forssman antigen in spleen was studied by autoradiography of Wright–Giemsa-stained cytocentrifuge preparations. Polychromatophilic erythroblasts are labeled, while mature erythrocytes and cells of the granulocytic and lymphoid lineages are negative (Springer, Secher, Galfré, and Milstein, manuscript in preparation).

The presence of the Forssman antigen on murine germinal tissues was suggested by tumor-cell panel screening (Stern *et al.*, 1978). Only embryonal carcinoma cell lines are positive. Both nullipotent and pluripotent, undifferentiated teratocarcinoma-derived lines are positive. Under appropriate culture conditions, pluripotent lines differentiate into embryoid bodies, which are considered equivalent to the inner cell mass portion of the mouse embryo around the time of implantation. The outer endodermal layer of the embryoid bodies are Forssman-negative, while the inner core of embryonal carcinoma remains positive. Further differentiation of the embryoid bodies occur if they are allowed to attach to a substrate. Multilayered cell cultures with a variety of differentiated cell types are obtained after 2 weeks of growth, less than 1% of which are Forssman-positive. It therefore appears that most of the differentiated derivatives of teratocarcinoma stem cells do not express the antigen. In the adult male mouse, germinal tissues contain the greatest concentration of Forssman antigen. Brain, kidney, spleen, and lymph nodes but not liver or thymus are also positive.

The expression of the Forssman antigen during development of the normal preimplantation mouse embryo has also been studied, using M1/22.25 mono-

clonal antibody (Willison and Stern, 1978). The fertilized egg and embryos up to the 8-cell stage are negative. Antigen is first expressed on trophectodermal cells at the time of blastocoel formation (early blastocyst) but disappears after hatching from the zona pellucida, that is, just before implantation into the uterine wall. The trophectoderm of the blastocyst encloses a fluid-filled cavity called the blastocoel, at one end of which lies the inner cell mass, which gives rise to the embryo proper. The inner cell mass is Forssman–positive, as is its first differentiated product, endoderm, at least soon after its formation. This is in contrast to the endoderm formed by teratocarcinoma cells in culture, which is negative.. This latter endoderm may represent a type (parietal) that is different from that on the blastocoelic surface of the inner cell mass (visceral).

B. *Heat-Stable Antigen*

Another antigen that is expressed in highly specific fashion, yet is found on diverse tissues, was also identified in these studies. This antigen is stable at 120°C and cannot be labeled with ^{125}I using lactoperoxidase or with [^{35}S]methionine. These properties suggest a carbohydrate antigenic determinant present in a moiety devoid of methionines or accessible tyrosines, i.e., a glycolipid. However, since definitive structural characterization of this molecule has not yet been pursued, it will be referred to as heat-stable antigen (HSA).

To investigate whether five different monoclonal antibodies identifying a HSA on mouse red blood cells all recognized the same cell-surface antigenic site, cross-inhibition experiments were carried out (Fig. 4). After preincubating target cells with serial dilutions of unlabeled monoclonal antibodies, binding was measured of added [^{3}H]lysine internally labeled monoclonal Ig. All five monoclonals recognizing a HSA on mouse RBC cross-inhibited binding of the two members of this group that were tested, M1/69 and M1/75, confirming recognition of the same site on these cells. However, differences in inhibitory titers and the slopes of inhibition suggested considerable variation among the clones in avidity. None of the other monoclonals, including the anti-Forssmans, were inhibitory. Despite the fact that all five of these antibodies appeared to recognize the same site on mouse RBC, striking differences between them were noted when inhibition of binding to thymocytes was tested. The most dramatic differences were found between M1/69 and M1/75, with the other three being intermediate. The inhibitory titer of the homologous M1/69 supernatant was the same for RBC and thymocytes, while the other clones gave very shallow inhibitory slopes and much lower inhibitory titers on thymocytes. M1/75 did not appear inhibitory at all. The concentrations of the antibodies employed in these experiments were very similar, between 80 and 120 μg/ml in neat culture supernatants. The results thus reflect true differences in antibody avidity and specificity.

These differences were also noted in two other assay systems. (1) While all five clones are lytic for RBC, M1/69 alone lyses thymocytes (Springer *et al.*, 1978b). (2) M1/69 antigen is expressed on splenic lymphocytes, thymocytes, and T and B lymphomas, while M1/75 antigen is not (Table II).

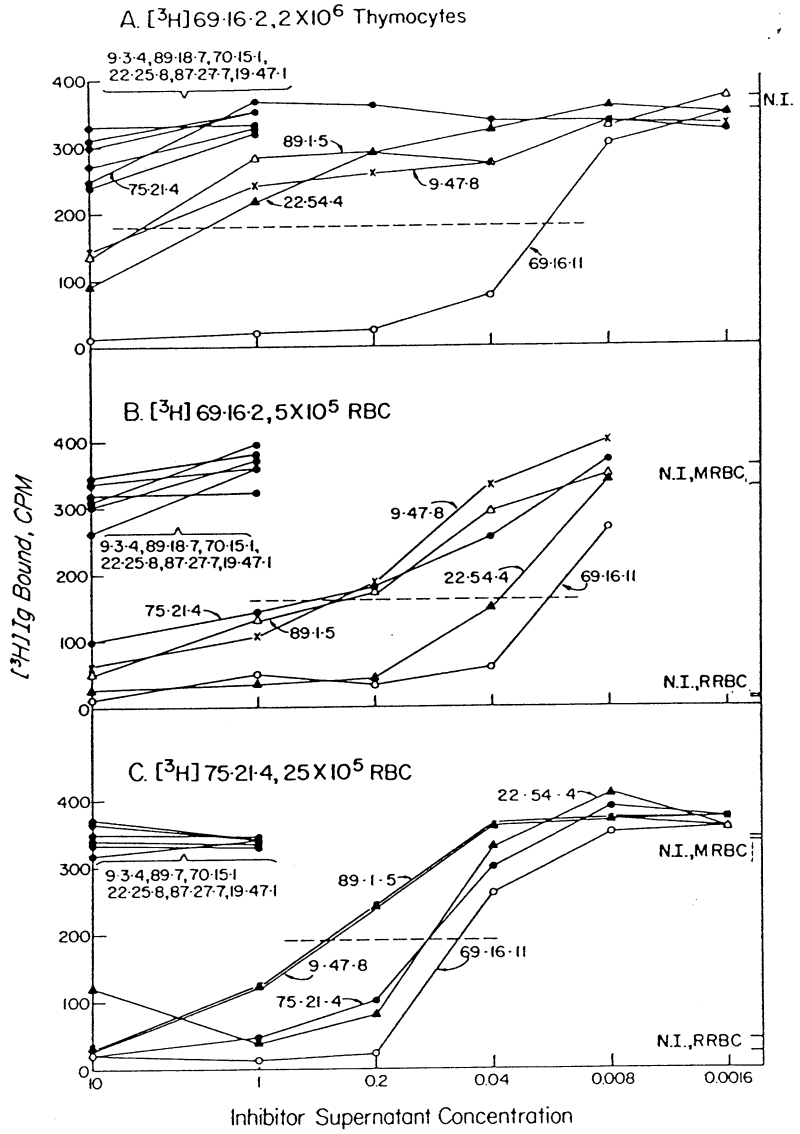

FIGURE 4. Cross-inhibition of binding of [^{3}H]lysine-labeled M1 antibodies by unlabeled clonal supernatants. Target cells were incubated with unlabeled supernatants at various concentrations, then with supernatants from [^{3}H]lysine internally labeled cells, and the amount of cell-bound radioactivity was determined. N.I., no inhibition, DMEM 10% FCS substituted for supernatant, MRBC, RRBC, mouse and rat RBC, respectively.

The working hypothesis is that these clones bind to slightly different (and perhaps overlapping) portions of a carbohydrate that has a common core structure but also some heterogeneity in its glycosylation both on RBC and particularly when dealing with different tissues. Heterogeneity would affect the affinity of each antibody differently, depending on the exact subset of carbohydrate residues recognized, thus giving rise to differences in specificity.

The tissue distribution of M1/69 is RBC > spleen = liver = kidney> lymph nodes = brain (Springer, Secher, Galfré, and Milstein, manuscript in preparation). These results could at least partially be explained by presence of RBC in the tissues. Both mature granulocytes in bone marrow and 16-hr peritoneal exudates and their precursors in bone marrow are strongly positive (Springer *et al.*, 1979). Blood monocytes are also strongly positive (Fig. 2d), but expression is greatly reduced in thioglycollate-induced peritoneal macrophages and is also absent from the macrophagelike line P388D_1 (Table II).

M1/69-positive and -negative lymphoid populations are found in spleen (Fig. 2b). Positive and negative lymphoid populations are also seen in blood (Fig. 2d) (though less well resolved), lymph nodes, and by staining with other clones recognizing HSA: M1/9.47, M1/22.54, and M1/89. The nature of these subpopulations was investigated in nylon wool depletion experiments (Table IV). M1/69+ cells were depleted in parallel with Ig^+ cells. Furthermore, double labeling experiments show that M1/69 + FITC $\bar{a}$-rat IgG or FITC $\bar{a}$-mouse Fab used either separately or together label almost exactly the same percentage of cells. These experiments show that HSA is expressed on peripheral B lymphocytes but not on any appreciable percentage of peripheral T lymphocytes. The situation on thymocytes is quite different, however. Thymocytes are 95% M1/69-positive (Fig. 2a). Thus thymocytes lose HSA expression upon maturation and entry into the peripheral lymphoid circulation. Apparently changes in the expression of a number of different antigens, including the thymus leukemia antigen (Konda *et al.*, 1973) occur at this stage of thymocyte development.

C. *Common Leukocyte Antigen (CLA)*

Two clones, M1/9.3 and M1/89.18, precipitate an antigen of molecular weight 200,000 from spleen cells. The antibodies cross-inhibit one another and thus react with identical or proximal determinants on the same molecule. The antigenic determinant is heat-labile and therefore appears to involve protein.

TABLE IV
Nylon-Wool Depletion of Ig and Heat-Stable Antigen-Bearing Spleen Cells[a]

	Ig	Heat-stable antigen			Mac-1	CLA
	$\bar{a}$ - Fab (%)	M1/69 (%)	M1/9.47 (%)	M1/89.1 (%)	M1/70 (%)	M1/9.3 (%)
Untreated	54	62	59	57	5	96
Nylon-wool passed						
Exp. 1	14	17	15	15	4	99
Exp. 2	19	24	n.d.	n.d.	5	99

[a]Cells in the lymphocyte scatter peak and slightly larger were counted in this analysis. Somewhat higher values for M1/70 positive cells are obtained by including all larger cells. n.d., No data..

The CLA is bound to *Lens culinaris* lectin affinity columns, suggesting it contains carbohydrate; ^{125}I(lactoperoxidase) and [^{35}S]methionine labeling show it contains protein.

When CLA is precipitated from spleen cells, three bands of molecular weights ~230,000, 200,000, and 180,000 are seen (see Fig. 8); only the smaller two of these bands are precipitated from Con-A-stimulated T lymphocytes (Fig. 1). The larger-molecular-weight form may therefore be derived from B lymphocytes. The same phenomenon had previously been described by Trowbridge and Mazauskas (1976), using polyspecific antisera to precipitate what appears to be the same antigen. Trowbridge (1978) has also derived a hybridoma recognizing this antigen, which he calls T200. All hybrids thus far obtained to this protein recognize all three different molecular weight forms. Furthermore, absorption experiments using classical antisera suggest antigenic identity between the molecules on B and T lymphocytes (Trowbridge and Mazauskas, 1976). Thus the difference in molecular weight of the antigen isolated from different sources could reflect a difference in posttranslational modification such as glycosylation, rather than a difference in amino acid sequence.

Tissue absorption studies show that antigen is present on lymph node and spleen cells, but not on brain, kidney, liver, or RBC. T and B lymphomas and a macrophagelike line are positive (Table II). The P815 mastocytoma and Moloney lymphomas bear the antigen, but a Friend erythroleukemia line, a normal fibroblast line, a methylcholanthrene-induced sarcoma, and an embryonal carcinoma line are negative (Stern *et al.*, 1978). The absence of this antigen on fibroblasts and its presence on lymphocytes distinguishes it from the LETS protein, or fibronectin, which has a similar molecular weight (Yamada and Olden, 1978). In fact, CLA and LETS appear to have mutually exclusive tissue distributions. FACS studies show CLA is present on 96–100% of thymocytes, spleen white cells, blood lymphocytes and monocytes (Fig. 2), thioglycollate-induced 18-hr PEC (neutrophilic granulocytes) and 4-d PEC (macrophages). It labels 77% of nucleated bone marrow cells, and sorting studies suggest these include lymphocytes and granulocytes and their precursors but not erythroid precursors. RBC are negative. Since this antigen is common to all lines of leukocyte differentiation, the designation CLA seems preferable to T200 (thymus-dependent 200,000-molecular-weight polypeptide).

D. M1/42 (H-2K) Antigen

Two clones were identified by immunoprecipitation screening of supernatants from M1 cultures that had been stored in liquid N_2 for 1 year (Fig. 5). Culture M1/42 precipitated two polypeptides of molecular weights 46,000 and 12,000, while M1/84 precipitated much smaller amounts of a polypeptide of molecular weight 46,000. Each line was then thawed and has been successfully cloned and recloned.

The antigen precipitated by M1/42 from C57BL/6J spleen cells has been preliminarily identified as H-2K^b in coprecipitation experiments. Preprecipita-

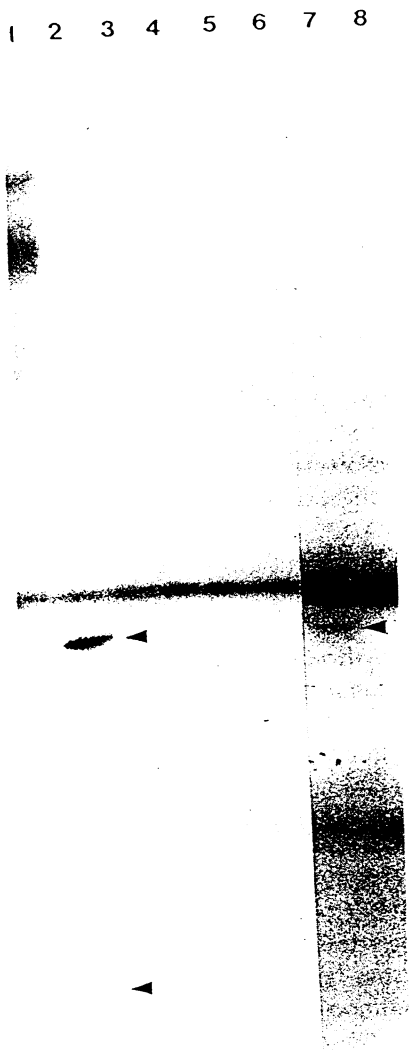

FIGURE 5. SDS 7–15% gradient PAGE of ^{125}I-labeled surface proteins precipitated by frozen M1 culture supernatants. Equal quantities of ^{125}I(lactoperoxidase)-labeled spleen cells and 4-day thiglycollate-induced PEC that had been solubilized with Triton X-100 were mixed together and immunoprecipitated with 100 μl of clonal supernatants and rabbit anti-rat IgG, reduced, prepared for PAGE, and autoradiographed, as described previously (Springer *et al.*, 1978b). 1, M1/9.3.4; 2, M1/42; 3, M1/81; 4, M1/94; 5, M1/7; 6, M1/79; 7, M1/84; 8, M1/55. Lanes 7 and 8 were exposed 4× longer to emphasize the band precipitated by M1/84. Bands appearing in all lanes are FC-receptor-bound mouse IgG H and L chains that cross-react with the rabbit anti-rat IgG sandwich reagent.

tion with M1/42 removed material reactive with anti-H-2K^b but not anti-H-2D^b allosera. Thus M1/42 appears specific for the K end of H-2^b. M1/42 does not recognize an allodeterminant because ^{125}I-indirect binding assays and immunoprecipitation show it is equally reactive with mice of *b*, *k*, *d*, and *s* haplotypes. It is thus possible that the M1/42 antigen is a general marker of H-2K-ness; confirmation of this idea must await mapping of the reactivity in a number of different strains. While alloreagents were essential for the identification of histocompatibility antigens, it seems that xenoreagents may actually offer several practical advantages. For example, a single reagent could be used to isolate H-2K for biochemical analysis from a number of different haplotypes. Preliminary results

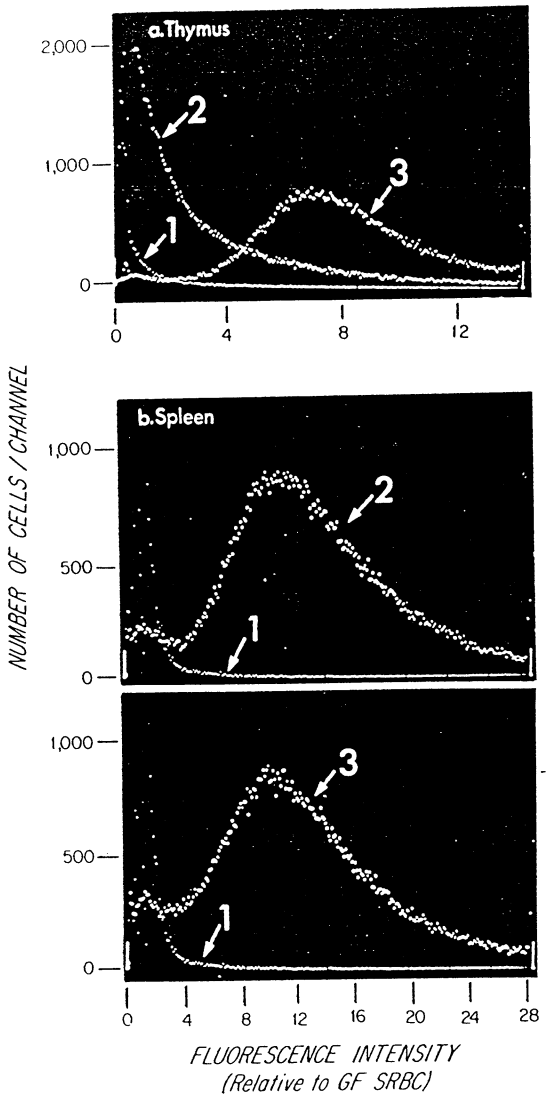

FIGURE 6. FACS analysis of M1/42 and M1/84 antigen expression on thymus and spleen cells. Cells were labeled with monoclonal antibodies and then with FITC anti-rat IgG as described in the text. Fluorescence intensity was standardized with gluteraldehyde-fixed sheep RBC (GF SRBC). 1, control (NSI supernatant with 50 μg/ml of added rat IgG); 2, M1/42; 3, M1/84.

suggest that M1/42–Sepharose columns are useful for the preparation of large-scale quantities of immunologically active H-2K molecules (Mescher and Springer, unpublished).

The use of monoclonal xenoantibodies also simplifies procedures for the labeling of cells with fluorochrome-tagged antibodies. The binding of a large number of different rat anti-mouse monoclonal antibodies can be studied, using a single indirect mouse Ig-absorbed FITC anti-rat Ig antibody preparation. The use of indirect anti-mouse Ig reagents is not feasible with mouse alloantisera because of the problem of cross-reaction with B-lymphocyte-surface Ig or Fc-receptor-bound Ig. Alloantibodies must either be individually labeled with fluo-

rochromes or haptenated and used with indirect fluorochrome–antihapten antibodies (Wofsy *et al.*, 1978).

One example of the use of such xenoreagents to alloantigens is in FACS studies of H-2 expression on T lymphocytes in the thymus and spleen (Fig. 6). H-2 is expressed by 88% of thymocytes. By far the majority of thymocytes express only small quantities while a small proportion of cells express varying amounts up to about 20 times more. In contrast, spleen cells have a quantity of H-2 antigen that is similar to the highest amount seen on thymocytes, and the distribution is much more uniform. H-2 expression increases during thymocyte maturation (Konda *et al.*, 1973, Beller and Unanue, 1978), and thus the distribution of thymocyte H-2 fluorescence intensity probably represents a maturation gradient.

E. *M1/84 Antigen*

In contrast to M1/42 antigen, the quantity of M1/84 antigen on thymocytes and spleen cells is very similar (Fig. 6), and the distribution is quite uniform in both tissues. The M1/84 polypeptide of molecular weight 46,000 is not identical to that of M1/42 antigen, H-2K^b, or H-2D^b, as shown by coprecipitation experiments. M1/84 labels a smaller proportion (80%) of nucleated bone marrow cells (Fig. 7) than M1/42 (97%) and thus shows some tissue specificity. It is present on all lymphocytes, granulocytes, and monocytes.

F. *Mac-1 Antigen*

Screening of a tumor-cell panel revealed that M1/70 monoclonal antibody reacts with a macrophagelike line, P388D$_1$, but not with B or T lymphomas, thymocytes, or RBC (Table II). Spleen cells contain 100-fold less of the antigen than the P388D$_1$ line. FACS experiments show that M1/70 antigen is expressed on 44% of bone marrow cells, on peritoneal exudate macrophages (Fig. 7), on blood monocytes, granulocytes, and 8% of spleen cells (Springer *et al.*, 1979). No expression is found on lymphocytes, thymocytes, or nonlymphoid tissues. The specificity of M1/70 for macrophages in peritoneal exudates is demonstrated by comparison to M1/84 (Fig. 7), which stains the smaller lymphocytes and polymorphononuclear cells as well as the larger macrophages. Differentiation from blood monocyte to thioglycollate-induced macrophage is accompanied by a large increase in M1/70 expression in parallel with a large decrease in M1/69 expression. Peritoneal exudate cells (PEC) express 8-fold more M1/70 than positive cells isolated in single-cell suspension from spleens, and about 5- to 10-fold more M1/70 than the 44% of positive cells in marrow (Fig. 7). The latter cells are granulocytic precursors. Monocytic precursors constitute only 0.3% of nucleated bone marrow cells (Van Furth, 1975). Because of its specificity for granulocytes and mononuclear phagocytes and because macrophages are its richest source M1/70 antigen has been designated Mac-1. Mac-1 is the first discrete molecule to be described that is specific to phagocytes.

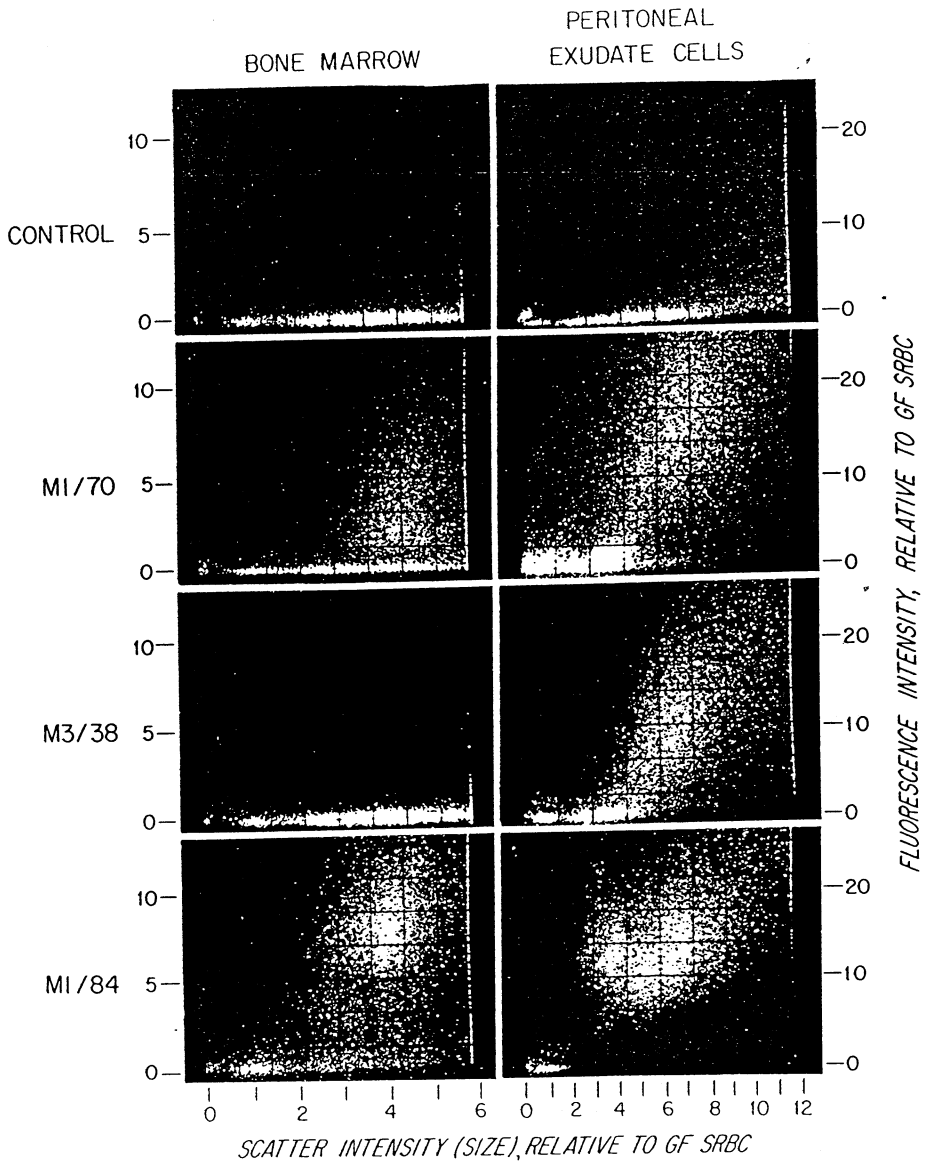

FIGURE 7. FACS dot plot analysis of Mac-1 (M1/70), Mac-2 (M3/38), and M1/84 antigen expression in bone marrow and thioglycollate-induced 4-day PEC populations. Scatter and fluorescence are scaled relative to gluteraldehyde-fixed sheep RBC (GF SRBC). Mouse RBC and macrophages appear at scatter intensities of 1 and 7, respectively. See text for labeling procedures.

The reactivity of M1/70 is not an artifactual association with the avid Fc receptor of macrophages, since (1) indirect ^{125}I-anti-Ig binding is highly specific, 34× the background level of normal rat IgG as control, (2) it cannot be inhibited by high concentrations of normal or heat-aggregated IgG, (3) [^{3}H]lysine-labeled M1/70 antibody binds to mouse but not rat PEC, and (4) M1/70 F(ab')$_2$ frag-

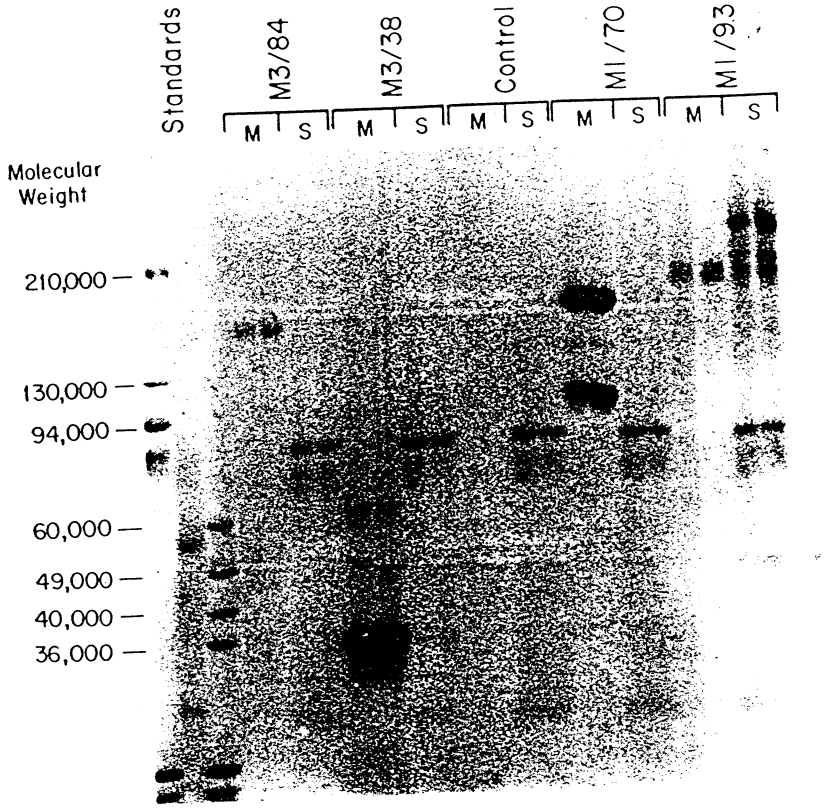

FIGURE 8. SDS 5–12% PAGE autoradiograph of immunoprecipitates from ^{125}I(lactoperoxidase) spleen cells and 4-day thioglycollate-induced macrophages. Equal quantities of spleen (S) and macrophage (M) Triton X-100-solubilized antigens were indirectly precipitated using monoclonal supernatants or NSI supernatant +50 μg/ml rat IgG as control. Two different concentrations of antibodies were employed in each experimental group: 30 or 100 μl of clonal supernatant and 10 or 33 μl of rabbit anti-rat IgG, electrophoresed in left or right gel lanes, respectively. Samples were reduced with 5% 2-mercaptoethanol. In addition to bands specifically precipitated from spleen cells, μ, δ, and light chains were precipitated by cross-reaction with the rabbit anti-rat IgG. Preclearing with the mouse IgG cross-reactive fraction of rabbit anti-rat IgG coupled to Sepharose had removed IgG bound to macrophages but not sIgD or sIgM.

ments (Fig. 3) titrate to the same endpoint as M1/70 IgG in labeling and antigen-precipitation experiments.

Precipitation experiments confirm that Mac-1 is present in much larger quantities on PEC than on spleen cells (Fig. 8). The same polypeptides are precipitated from spleen cells, but in amounts only visible on prolonged exposure of the autoradiogram. Mac-1 contains two polypeptides of molecular weights 190,000 and 105,000. The same results are obtained using either ^{125}I(lactoperoxidase) or [^{35}S]methionione labeling of adherent cells, showing that both chains are exposed to the exterior and are synthesized by the macrophage. The amounts of radiolabel incorporated are consistent with equimolar amounts of each chain. The two chains are not linked by disulfide bonds. The

working hypothesis that the two chains are noncovalently associated is currently being tested. At least one chain contains carbohydrate because Mac-1 antigen is retained by *L. culinaris* lectin columns. The antigenic determinant recognized by M1/70 appears to involve protein because it is labile to heating.

G. *Never Repeating the Past: A Cascade Procedure for Successive Generations of Novel Clones*

The discovery of a macrophage-specific clone in the M1 hybridization was serendipitous and probably largely due to chance, since spleen cells purified similarly to those used in the immunization, by nylon wool filtration, contain only 4–8% of M1/70$^+$ cells (Table IV). In recent experiments (Springer, submitted for publication) we attempted to deliberately elicit and screen for further macrophage-specific hybridomas using a novel cascade procedure (Fig. 9). Rats were immunized with thioglycollate-induced murine macrophage detergent-solubilized membrane proteins that had been purified by the following procedures. Since most if not all surface proteins are glycosylated, the immunogen was partially purified by *L. culinaris* lectin affinity chromatography. Then, to direct the response toward macrophage-specific antigens, antigens with wide tissue distributions that had previously been identified in the M1 hybridization experiment were removed from the immunogen by filtration through Sepharose containing covalently attached M1/9.3 and M1/69 monoclonal antibodies. After removal of detergent, priming in complete Freund's adjuvant, and a final intra-

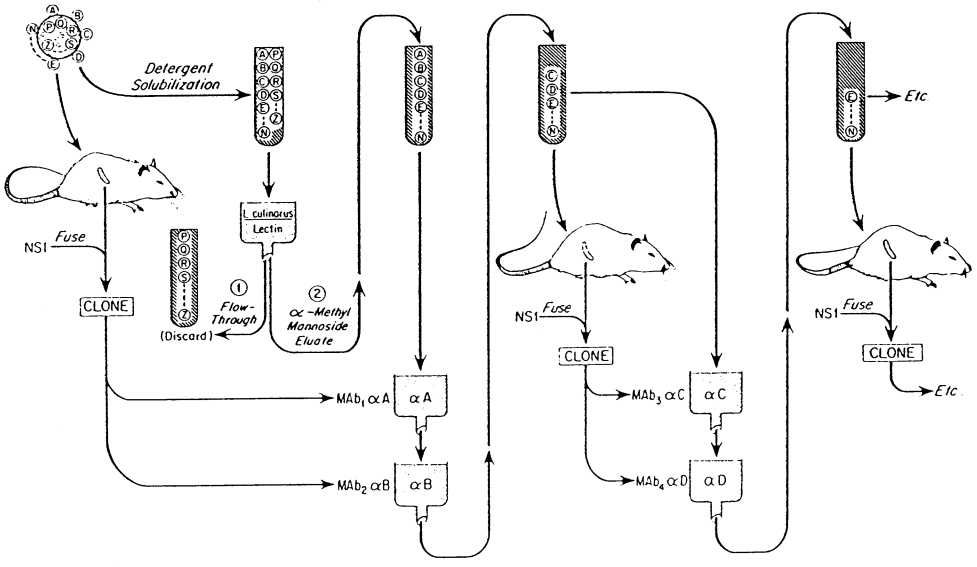

FIGURE 9. Cascade generation of monoclonal antibodies to cell-surface antigens. By combination of cell hybridization and immunoadsorbent techniques, successive generations of novel antibodies may be obtained until finally an entire collection of cell-surface antigens is identified.

venous boost, spleen cells from one rat were fused with NSI myeloma cells and aliquoted into 600 microculture wells. After several weeks the cultures with highest binding activity to PEC in indirect ^{125}I-anti-rat IgG binding assays were chosen for further growth. The specificity of their antibodies was investigated by precipitation of ^{125}I-PEC cell-surface proteins followed by SDS-PAGE analysis. Supernatants from five of the M3 cultures precipitated polypeptides of molecular weights 190,000 and 105,000 and thus demonstrated the same specificity as M1/70. Other supernatants showed specificity for quite different polypeptides. A polypeptide of molecular weight 32,000 was precipitated by 11 cultures, two of which, M3/31 and M3/38, have been isolated as stable subclones. An antigen of molecular weight 110,000 was recognized by one culture, M3/84, which has also been cloned. However, none of the clones recognized the CLA polypeptide of molecular weight 210,000 despite the fact that this antigen appears to be highly immunodominant and is present on macrophage surfaces. This suggested that the cascade purification procedure successfully subtracted this antigen from the mixture of molecules used in immunization. Radioimmunoassays also demonstrated removal of the antigen by the immunoadsorbent column.

The cascade procedure described here eliminates the problem of immunodominant antigens. It should allow continual successive generations of novel monoclonal antibodies to be obtained until finally, the last (and probably most weakly immunogenic) cell-surface antigen is identified. This procedure is also proposed for the identification of all intracellular antigens (Milstein and Lennox, 1979).

H. Mac-2 and Mac-3 Antigens

The antigen of molecular weight 32,000 precipitated by M3/31 and M3/38, designated Mac-2, and the antigen of molecular weight 110,000 precipitated by M3/84, designated Mac-3 are precipitated in much greater quantities from PEC than from spleen cells (Fig. 8). The same polypeptides are seen after labeling with either ^{125}I(lactoperoxidase) or [^{35}S]methionine. The *L. culinaris* experiments show that these antigens contain carbohydrate, and thus they are glycoproteins. Mac-2 (and Mac-3), in contrast to Mac-1, are not expressed on bone marrow cells (0–1% positive) (Fig. 7). These results rule out expression on granulocyte precursors, but not on promonocytes, which constitute only 0.3% of nucleated bone marrow cells. Mac-2 and Mac-3 appear to be expressed on the monocytic line of differentiation at some stage after divergence from the granulocytic line, while Mac-1 is found on both branches. Mac-2 and Mac-3 antibodies label 6–9% of spleen cells and 0–2% of thymocytes. In peritoneal exudates, macrophages but not smaller cells such as polymorphs or lymphocytes are labeled (Fig. 7). Peritoneal exudate macrophages are stained much more brightly than positive cells in spleen or blood. Thus, as in the case of Mac-1, expression is greatly increased during differentiation of monocytes to stimulated peritoneal macrophages.

V. *Patterns of Differentiation Antigen Expression*

A. *"Jumping" Differentiation Antigens*

In considering the antigens that have been identified in these studies, two strikingly different types of tissue distribution patterns emerge. "Lineage" antigens, exemplified by CLA, Mac-1, Mac-2, and Mac-3, are expressed in an ontogenetically or functionally orderly fashion. "Jumping" antigens (Milstein and Lennox, 1980), exemplified by the Forssman and HSA antigens are not. Distantly related tissues often will share expression of a jumping antigen while more closely related tissues do not. These two classes should perhaps be thought of as extremes in a continuum, with some antigens such as H-2 lying somewhere in between. However, as will be discussed below, it appears that jumping antigens share other properties besides their occurrence on disparate tissues. They thus may constitute a truly distinct class of surface antigens with different functional properties than lineage antigens.

A number of jumping antigens have previously been described. Perhaps the best known is Thy-1 antigen, which has been thoroughly characterized with allo- and xenoantisera and to which both rat anti-mouse and mouse anti-mouse monoclonal antibodies have been obtained (Hämmerling *et al.*, 1978; Marshak-Rothstein *et al.*, 1979). Thy-1 expression in the mouse is shared by disparate tissues such as fibroblasts, brain cells, and lymphocytes. Subclasses of lymphocytes differ in expression, with T but not B cells being positive. Dramatic changes in Thy-1 expression occur during brain cell development. Thy-1 is not found on 19-day fetal brain cells but is well expressed on a subpopulation of brain cells 10 days after birth or after cultivation of fetal cells *in vitro* (Mirsky and Thompson, 1975). Another example of a jumping antigen is asialo-GM_1 ganglioside. This glycosphinogolipid is found on peripheral T lymphocytes but not on thymocytes or peripheral B lymphocytes (Stein *et al.*, 1978). This is exactly the reverse of the situation for the HSA. A number of jumping antigens have also been identified by monoclonal antibodies in the rat. W3/13, a glycoprotein of molecular weight 95,000, is found on T lymphocytes and plaque-forming cells but is absent from B lymphocytes (Williams *et al.*, 1977, Milstein and Lennox, 1980). MRC 0×2, a glycoprotein of molecular weight 60,000, is found on brain, thymocytes, and B but not T lymphocytes (Milstein and Lennox, 1980). Solter and Knowles (1978) identified a jumping antigen by syngeneic immunization of mice with teratocarcinoma cell lines. An IgM monoclonal antibody was obtained which recognizes a stage-specific embryonic glycolipid, distinct from the Forssman specificity. Its topographic distribution on pre-implantation embryos resembles that of the Forssman antigen, although the time of first appearance, at the 8-cell stage, is earlier. It is found on mouse brain and kidney and mouse and human sperm and teratocarcinomas, but not on sheep RBC. Similarly, the peanut agglutinin, which is specific for terminal D-galactose residues, reacts with preimplantation em-

TABLE V
Jumping Antigens Can Have Wide Tissue Distributions Yet within a Given Line of Differentiation Mark a Specific Developmental Stage

Line of differentiation	Antigen	Negative stage	Positive stage	Negative stage
Erythroid	Forssman	?	Polychromatophilic erythroblast	Erythrocyte
Embryonic trophectoderm	Forssman	Morula	Early blastocyst	Late blastocyst
T lymphocyte	Heat-stable	?	Thymocyte	Peripheral T lymphocyte

bryos, cells of the spermatogenic series, and embryonal carcinoma cells but not on their differentiated derivatives (Reisner *et al.*, 1977). This lectin is also reactive with 80–90% of thymic but not peripheral lymphocytes or RBC (London *et al.*, 1978). While perhaps broader, the specificity of this lectin might include that of the Forssman and/or the Solter and Knowles (1978) antibodies.

A striking property of jumping antigens is that within a given line of differentiation, change in antigen expression is often correlated with cellular migration to a different tissue (Table V). Loss of Forssman antigen expression in the mouse erythroblast precedes exit from hematopoietic tissue into the circulation and in the trophectoderm precedes implantation in the uterus. A decrease in HSA occurs after blood monocytes cross the endothelium and differentiate into stimulated macrophages. Loss of HSA from thymocytes occurs slightly before or concomitantly with entry into the peripheral lymphoid circulation. Appearance of asialo-GM_1 ganglioside occurs at the same time (Stein *et al.*, 1978).

Adhesive properties also change during thymocyte maturation. Stamper and Woodruff (1976) have demonstrated selective adhesion of recirculating lymphocytes during emigration from blood to lymph nodes. Migration to lymph nodes occurs via specialized high endothelial venules and not through other vascular endothelia in lymph nodes. In experiments measuring binding of lymphocytes to fixed sections of lymph nodes, avid binding to the specialized venule endothelia was observed for lymphocytes from thoracic duct, spleen, and lymph nodes, but not for thymocytes. Thoracic duct lymphocytes were found to adhere about 100 times more frequently to specialized venule endothelia than to normal vascular endothelia. Adhesive interactions appear to play a particularly important role in guiding the migration of individual cells in embryogenesis and in hematopoietic and lymphoid systems (see Marchase *et al.*, 1976, for a review). While there is no direct evidence that jumping antigens are involved in the control of adhesive specificity, the numerous correlations between changes in jumping antigen expression and cellular migration make this an intriguing possibility.

All the glycolipid antigens identified here belong to the jumping antigen category. Glycoproteins may be expressed as either jumping or lineage antigens. Whether carbohydrate plays in general a more important role in the function of

jumping antigens than of lineage antigens is unknown. It is interesting, however, that the most thoroughly studied example of a glycoprotein jumping antigen, Thy-1, is a small glycoprotein with a high percentage of carbohydrate, 30% (Williams *et al.*, 1976), and that certain pleiotrophic mutations in carbohydrate biosynthesis prevent the surface expression of Thy-1 but not a number of other cell-surface glycoproteins (Trowbridge *et al.*, 1978).

The different ways in which lineage antigens and jumping antigens appear to be deployed during development is schematically summarized in Fig. 10. Combinatorial use of lineage and jumping antigens allows a very economical means for achieving cell-surface diversity. Jumping antigens, perhaps in different combinations, might fulfill similar functions in quite different tissues. This economy would have important consequences for host defense. The probability of cross-reaction between viral or bacterial and host cell surface antigens would be reduced, as would the probability that an appropriate receptor for virus would be expressed.

A common characteristic of Thy-1 antigen and the Forssman antigen, and perhaps of jumping antigens in general, is species variation in the tissue distribution patterns. In the mouse Thy-1 is expressed on thymocytes and on T but not B lymphocytes, while in the rat it is found on 40% of bone marrow cells and is expressed on thymocytes but not on most peripheral T lymphocytes (Williams *et al.*, 1976). Expression of the dog Thy-1 homologue is similar to that of the mouse except that kidney is strongly positive. The human homologue is present on kidney, absent from thymus and lymphoid compartments, and as in the mouse, rat, and dog, is present on brain (Dalchau and Fabre, 1979). It has long been known that Forssman antigen distributions vary considerably among species. In the guinea pig Forssman antigen is found on nearly all tissues but not on erythrocytes, while exactly the opposite type of distribution is found in the sheep and goat (Boyd, 1966). This suggests considerable flexibility in the manner in which jumping antigens are employed. Perhaps this is allowed by redundancy in the antigens that regulate any particular function, such as adhesion. If so, evolutionary changes in their expression could have subtle and potentially important effects on organismal development.

Jumping antigens have been identified with monoclonal antibodies, both in

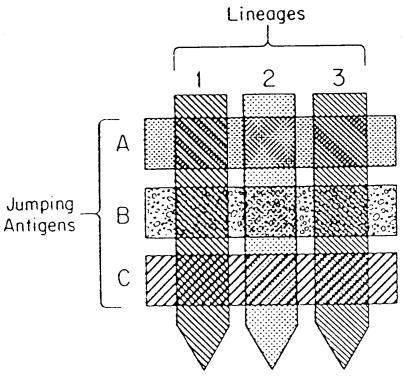

FIGURE 10. Combinatorial use of lineage and jumping antigens helps generate cell-surface diversity. The course of development is portrayed as occurring from the top to the bottom of the figure.

the mouse and in the rat, with a surprisingly high frequency. This is probably explained by the very wide window through which the cell surface is seen using the monoclonal approach in conjunction with xenogeneic immunization and the indirect ^{125}I-anti-Ig binding assay. By comparison, alloimmunization selects for polymorphic antigens and xenoimmunization with one tissue and absorption with others selects for lineage antigens. Identification of antibodies by their ability to precipitate ^{125}I- or [^{35}S]methionine-labeled antigens selects against glycolipids.

The possibility of chemical dissimilarity between jumping (or lineage) antigens isolated from different tissues must always be considered, especially since monoclonal antibodies recognize only one out of many possible determinants on a molecule. Gross dissimilarity between the antigens recognized in different tissues has not been found, such as would be predicted by the multiple recognition theory of Richards *et al.* (1975). However, some smaller differences have been noted. For example, the HSA on RBC bears both the M1/69 and M1/75 determinants, while the HSA on white blood cells bears only the former. Furthermore, the CLAs on T cells and B cells all bear both M1/9.3 and M1/89.18 determinants, but the differing molecular weight of this antigen when isolated from B or T lymphoid sources suggests other differences, perhaps in glycosylation. Moreover, Williams *et al.* (1976) have found striking differences in the carbohydrate composition of brain as opposed to thymus Thy-1 antigen. This heterogeneity in the antigens themselves introduces a further complexity in the analysis of cell surfaces.

The existence of jumping antigens also demonstrates the pitfalls of assigning ontogenetic relatedness between cells on the basis of a single surface marker. A number of shared antigens should be demonstrated before conclusions are drawn.

B. *"Lineage" Differentiation Antigens*

The more circumscribed distribution of lineage antigens distinguishes them from jumping antigens and suggests them to be important in the unique functions of the cells on which they are expressed. Unlike jumping antigens, species variation in pattern of expression would not be expected. In this regard it is of interest that Mac-1 cross-reacts with human cells and exhibits the same specificity for monocytes in humans as found in the mouse (Ault and Springer, manuscript in preparation). Breadth of expression of lineage antigens appears to approximately reflect different levels of specialization during differentiation (Fig. 11). Thus the expression of CLA is restricted to leukocytes, Mac-1 to "professional" phagocytes (granulocytes and mononuclear phagocytes) and Mac-2 and Mac-3 to mononuclear phagocytes. Sometimes closely related sublines do not all express an antigen. For example, erythrocytes, granulocytes, monocytes, and megakaryocytes share a pluripotent stem cell, which differs from the lymphocyte stem cell, and both of these stem cells are in turn derived from a more primitive progenitor cell (Cline, 1975). Yet all leukocytes share the CLA, whereas erythrocytes and

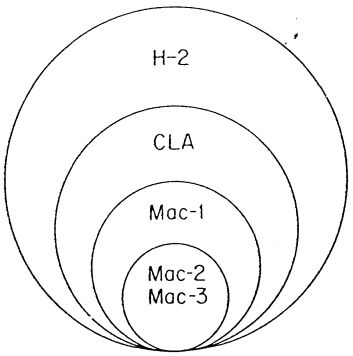

FIGURE 11. Expression of lineage antigens correlates with specialization during differentiation.

their immediate precursors do not. The pattern of expression does not exactly recapitulate ontogeny, but is closer to what would be expected on the basis of functional relatedness. Similarly, monocytes and B but not T lymphocytes share expression of Ia antigens and trypsin-resistant Fc receptors for aggregated IgG (FcRII). Unkeless (1979) has prepared a rat monoclonal antibody that blocks the macrophage FcRII but not FcRI site, and also the FcRII site on B cells. Quantitative changes in expression or even complete loss may also occur during development of the lineage. For example, the absence of CLA from certain myeloma lines but its expression on B cells and B lymphomas suggests that in differentiation of B cells to plasma cells, expression of this antigen may be lost.

In the rat a number of lineage antigens have also been identified using monoclonal antibodies. MRC OX1 recognizes a CLA, which in molecular weight and tissue distribution appears similar to the CLA in the mouse (Sunderland *et al.*, 1979). W3/25 is specific for a T-lymphocyte functional subset (White *et al.*, 1978). Cells mediating help for B cells and graft-vs.-host reactivity are contained within the W3/25-positive subpopulation of 80% of thoracic duct lymphocytes. The 10% subpopulation of unlabeled T lymphocytes contains the allogeneic suppressor cells. This and similar reagents should be of great value in the study of T-lymphocyte subsets.

The value of monoclonal reagents in the study of differentiation is also well illustrated by the derivation of antibodies to Mac-1, -2, and -3. This is the first time that individual molecules marking the phagocyte (Mac-1) and mononuclear phagocyte (Mac-2 and -3) lines of differentiation have been identified. Mononuclear phagocytes are a very interesting system for studying differentiation. Their precursors are formed in bone marrow and are transported to tissues in the blood, where they have a half-life of about 22 hr. Upon crossing the endothelium, they differentiate into cells with diverse functional and morphological characteristics, such as alveolar macrophages, peritoneal macrophages, thymic macrophages, liver Kupffer cells, bone osteoclasts, and epidermal Langerhans cells (Hobart and McConnell, 1975). Differentiation to "activated" tumoricidal and microbicidal macrophages may occur in response to inflammatory stimuli such as mediators released by T lymphocytes (Bianco and Edelson, 1977). The possibility is being tested whether mononuclear phagocytes localized in certain tissues

lack Mac-1, -2, -3 expression. If so, this would provide further evidence for the diversity of these cells. In peritoneal exudates, all macrophages express Mac-1, -2, and -3 antigens. Ia antisera, however, define peritoneal exudate macrophage subpopulations (Cowing *et al.*, 1978), and it remains possible that further Mac antigens identifying these subpopulations will be found.

VI. Concluding Remarks

Studies utilizing monoclonal antibodies are beginning to reveal interesting patterns of antigen expression on developing cell surfaces. Two extremely different types of antigen distributions, with perhaps also some intermediate types, were noted in this study. "Jumping" antigens have tissue distributions which are patchy and widely scattered, often undergo change during cellular migration, and are often different for the homologues in other species. Carbohydrate often appears to be a prominent structural component. Their distribution suggests a role in modulation of generalized membrane phenomena such as intercellular adhesion. "Lineage" antigens have tissue distributions which are much more restricted, may undergo changes in expression during maturation and are similar for the homologues in different species. Their distribution suggests participation in the specialized functions of the cells on which they are expressed. It will be interesting to learn whether these generalizations can be extended as more antigens on leukocytes and other types of cells are studied.

The ease with which novel surface antigens have been identified up to now strongly suggests that only a small fraction of the total are presently identified. The ultimate goal of identifying the entire array of cell-surface antigens seems possible in the next few decades, particularly with the availability of cascade schemes for the production of antibodies against antigens of successively lower immunodominance. Using a complete set of monoclonal antibodies, the total ebb and flow of antigen expression during differentiation and development could be quantitatively described.

The study of differentiation is just one of the many uses of monoclonal antibodies. Analysis of tissue distribution is an important step in assessing the likely functional importance of a particular antigen before more detailed chemical and functional characterization is carried out. Monoclonal antibodies appear particularly well suited for studying the structure and function of membrane molecules. This is because unlike enzymes or Fc receptors, most cell-surface molecules may not retain biological activity after isolation. For example, in the macrophage, many functions such as phagocytosis, chemotaxis, tumor killing, or induction of immune responsiveness no doubt require the interactions of a number of different molecules in the context of the whole cell. Monoclonal antibodies should therefore be highly valuable not only for the isolation of individual cell-surface molecules but also to probe or modify the activity of these molecules in their native environment on the cell surface. In this regard, Mac-1, -2, and -3 antibodies are currently being tested for inhibition of a panel of

macrophage-specific, surface-associated activities. Parallel studies on the structure and membrane integration of Mac-1, -2, and -3 antigens are also being carried out.

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