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Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens

Hybrid myeloma cell lines secreting monoclonal antibodies to mouse cell surface antigens have been prepared. Spleen cells from a DA rat immunized with B10 mouse spleen cells that had been enriched for T cells were fused to cells from a nonsecreting mouse myeloma line (NSI). The presence in the culture supernatants of antibodies binding to mouse spleen cells was tested by a binding assay with ^{125}I-labeled anti-rat IgG. From a large number of positive cultures, ten independent hybrid clones were purified, each secreting a different antibody. Each antigenic target was analyzed by (a) gel electrophoresis of immunoprecipitated ^{125}I-labeled cell surface molecules, (b) heat stability, (c) strain and species distribution and (d) cross-inhibition of binding of different monoclonal antibodies.

It was concluded that the ten monoclonal antibodies regognized four types of antigen. One was the heterophile, heat-stable, Forssman antigen. The second (mol.wt. 210 000) appears to be a major ^{125}I-labeled lymphoid cell surface protein. The third, a minor component of spleen cells, was precipitated as two polypeptides of mol.wt. 190 000 and 105 000. Five IgG-secreting clones identify the fourth antigen, a heat-stable, possibly glycolipid component expressed on mouse red blood cells and also on thymocytes. Cross-inhibition studies suggest that these last monoclonal antibodies bind to overlapping, but not identical, determinants. The class and chain composition of the monoclonal antibodies were studied by gel electrophoresis, isoelectric focusing and ability to lyse red blood cells and thymocytes.

1 Introduction

Many immunological phenomena are thought to be mediated by cell surface molecules, such as delayed-type hypersensitivity, T and B cell interactions, T cell and macrophage interactions, T lymphocyte cytotoxicity, B lymphocyte activation, antibody-dependent cytotoxicity, phagocytosis, chemotaxis, and lymphocyte homing. Specific cell populations mediating some of these phenomena have been identified [1]. Much has been learned about the role of surface Ig in the stimulation of B lymphocytes by antigen, but the molecules involved in other phenomena have largely remained uncharacterized. Antibodies are widely used as probes to recognize and study such specific surface molecules. However, cell surfaces are complex mosaics containing many types of antigens, and special approaches have been required to obtain specific antibodies.

Many important cell surface antigens have been discovered by alloimmunization between congenic strains of mice [2]. But the construction of congenic strains requires elaborate breeding, many surface molecules may not be polymorphic, and the antisera are weak. These severe limitations are avoided by xenogeneic immunization. The difference between homologous proteins from different species is greater than between the polymorphic variants, so the antisera are stronger and antibodies are elicited to a much wider range of molecules. The problem is to achieve specificity. One approach is to immunize with one tissue and absorb with another. However, the remaining antibodies usually recognize a complex of tissue-specific antigens. Immunization with purified molecules is another approach, but is limited by the difficulty of purifying adequate quantities of surface molecules. Immunization with somatic cell hybrids has also been used [3]. This elegant approach limits the immunizing stimulus to the products of a single chromosome but is limited by the availability of appropriate hybrid cells. The culture of spleen fragments containing single clones of antibody-secreting cells permits the preparation of highly specific antibodies, but only in minute quantities [4, 5].

A more general approach to the problem of obtaining highly specific antibodies to individual cell surface molecules stems from the experiments of Köhler and Milstein [6, 7]. They fused myeloma cells and spleen cells from mice immunized with sheep red blood cells (RBC) to derive continuous hybrid cell lines secreting antibodies to sheep RBC. Such lines can be manipulated in culture so that the multispecific response to a complex immunogen can be resolved into a set of monospecific responses by cloning. Myeloma spleen cell hybrids have been obtained which secrete antibodies to rat major histocompatibility antigens [8], rat cell surface antigens [9], mouse IgD allotypes [10] and mouse H-2K antigens [11].

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Abbreviations: **DMM:** Dulbecco's modified Eagle's medium **FCS:** Fetal calf serum **BSA:** Bovine serum albumin **RBC:** Red blood cells **BBSS:** Buffered balanced salt solution **PAGE:** Polyacrylamide gel electrophoresis **SDS:** Sodium dodecyl sulfate **IEF:** Isoelectric focusing **^{125}I-anti-IgG:** ^{125}I-labeled anti-IgG

In the present report, we explore the application of this approach to the xenogeneic identification of cell surface antigens of the mouse. Numerous antigens have already been identified in the mouse, and a major question is the frequency with which novel antigens could be found by random collection of monoclonal antibodies directed against surface antigens. We describe the isolation of ten clones producing different antibodies which define four cell surface differentiation antigens. Three of these are identified for the first time.

2 Materials and methods

2.1 Cell lines and media

Normal medium for cell culture was Dulbecco's modified Eagle's medium (DMM) and 20 % fetal calf serum (FCS), both from Gibco Biocult Ltd., Paisley, Scotland. For selection against parental myeloma cells, hypoxanthine (10^{-4} M), aminopterin (4×10^{-7} M) and thymidine (1.6×10^{-5} M) were added to the medium (HAT medium, Littlefield [12]). HT medium is the same as HAT medium except that it contains no aminopterin.

P3-NSI/1-Ag4-1 (NSI) is a cell line derived from the mouse myeloma line MOPC 21 [9]. Cells are resistant to 20 μg/ml 8-azaguanine and die in HAT medium. The cells do not secrete Ig chains but contain intracellular κ chains [13].

Cell lines derived from the fusion described in this paper are all prefixed M1. M1/1-M1/96 designate the original 96 two-ml cultures. Clones derived from these cultures by agar cloning contain a second number and, if recloned, a third. Thus, M1/69.16 is a clone of M1/69, and M1/69.16.2 was obtained by recloning M1/69.16.

2.2 Cell counting

Spleen, lymph node and thymus cells were counted after staining for 5 min in 1 mg/ml methyl violet 6 B (Merck, Darmstadt, FRG) in 21 mg/ml citric acid. In this way only white cells were counted.

2.3 Preparation of cells for immunization

Spleen cells from B10 mice were enriched for T lymphocytes by passage through nylon wool [14] and depleted of red cells by centrifugation on Ficoll-Isopaque [15]. The final immunization (see Sect. 2.4) additionally contained B10 spleen cells which had been incubated in the presence of 1 μg/ml concanavalin A in RPMI 1640 with 10 % FCS for two days and then depleted of red cells as above. Cells were washed and suspended in DMM containing 10 mM HEPES, pH 7.4.

2.4 Immunization schedule

DA and AO strain inbred rats, from the breeding colony of the Agricultural Research Council, Babraham, GB, were gifts of Dr. J.C. Howard. DA and AO rats (three of each) were immunized with 10.5×10^6 nylon wool-purified B10 mouse spleen cells (day 1). On day 15, a further injection of 13×10^6 cells was made. The cells were injected in 0.6 ml, half into the peritoneum and the other half into several subcutaneous sites over the hindquarters. Seven days after the second immunization, a serum sample from each rat was analyzed by cytotoxicity against B10 spleen cells. The rat giving the highest titer (4500; reciprocal of dilution giving 50 % of maximal ^{51}Cr release) was given a final immunization on day 31 of 39×10^6 nylon wool-purified spleen cells mixed with 60×10^6 concanavalin A-stimulated spleen cells in 2.0 ml into the tail vein. Three days later, the rat was killed and the spleen removed for fusion.

2.5 Fusion

The fusion was carried out essentially as described by Galfrè et al. [8]. NSI cells (2×10^7) were fused with 2×10^8 rat spleen cells, and after fusion, distributed in 96 two-ml cultures. To cultures M1/1-M1/72, 10^5 untreated rat spleen cells were added while to cultures M1/73-M1/96 were added approximately 3×10^5 nucleated cells, prepared by trypsinization of small pieces of rat spleen fragments remaining from the fusion. After 24 h, 1 ml of medium in each cup was replaced with HAT medium, which was also used for maintenance of cultures for the following six weeks.

2.6 Cloning

Cloning in agar was carried out essentially as described by Cotton et al. [16] except that a 5 % agar stock was used, and this was diluted first with an equal volume of double strength medium (+ 20 % FCS) and then with sufficient single strength medium (+ 20 % FCS) to give 0.5 % agar for the base or 0.25 % agar for the overlay containing cells.

2.7 Binding assay

Supernatants from each culture were assayed after ten days, and thereafter at weekly intervals for their ability to bind B10 lymph node or spleen cells. Supernatants (5 or 50 μl) were incubated with glutaraldehyde-fixed [17] B10 cells (10^5 cells in 5 or 50 μl) for 0.5–1 h. Quantitative titrations showed that glutaraldehyde fixation did not affect any of the specificities detected in this study. The cells, in V-bottom microtiter plates, were washed twice by addition of 200 μl buffered balanced salt solution (BBSS) containing 2 % bovine serum albumin (BSA), centrifugation for 5 min at 200 x g, and aspiration of the supernatant. The cells were then suspended in 5 μl of ^{125}I-labeled anti-Ig (^{125}I-anti-Ig, ~ 30 000 cpm), and after 45 min at 4 °C washed three times, suspended in 150 μl, and transferred to tubes for γ-counting. Control binding was determined by using a rat IgG, anti-rat histocompatibility antigen clone supernatant, usually R4/18.2 [8], as the first antibody. Filler cells were usually added at the beginning of the first wash cycle, to prevent washing losses (5 μl of 5 % suspension). Sheep RBC were used until their reactivity with M1/22.25 and M1/87.27 was discovered. Subsequently, ox RBC or outdated human 0 Rh^- RBC were used.

Two different labeled antibodies were used. Rabbit $F(ab')_2$ anti-rat Fab was a gift from Dr. A.F. Williams. Rabbit anti-rat IgG antiserum was a gift from Dr. A.J. Munro. The $F(ab')_2$ fragment was prepared by pepsin digestion as described [18]. Iodination was performed either as described by Jensenius and Williams [19] or by iodination of antibody bound to rat

IgG coupled to Sepharose CL 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) a modification of the method of Miles and Hales [20], as described by Herzenberg and Herzenberg [21].

Anti-rat Ig was absorbed to prevent cross-reaction with mouse cell surface Ig, either after iodination by mixing with mouse serum, or before iodination by passing it through mouse serum-Sepharose CL 4B. After it was discovered that the two preparations of ^{125}I-anti-Ig differed in their degree of binding to the M1/clone antibodies, each clone was tested with the best type of ^{125}I-anti-Ig: ^{125}I-rabbit F(ab')$_2$ anti-rat IgG for M1/9.3, M1/89.18, M1/9.47, M1/89.1, M1/69.16, and M1/70.15, or ^{125}I-rabbit F(ab')$_2$ anti-rat Fab for M1/75.21, M1/22.54, M1/22.25, M1/87.27, and M1/19.47.

2.8 Heat stability studies

Glutaraldehyde-fixed cells were washed free of BSA and suspended in phosphate buffered saline (Dulbecco's); sheep and mouse RBC, 5 x 10^8/ml; BW5147 (a T lymphoma line), 10^7/ml; P388D (a macrophage-like line), 2 x 10^7/ml. After vortexing the cells, one aliquot was autoclaved at 120 °C for 15 min and another held on ice. The samples were then serially 5-fold diluted, and 5 µl samples assayed in duplicate in the ^{125}I-anti-Ig binding assay.

2.9 Cytotoxicity assay

RBC and thymocytes were labeled with ^{51}Cr, and the cytotoxicity assay performed essentially as described by Sanderson [22] except in microtiter plates, and 10 µl aliquots of supernatants or sera, cells, and absorbed guinea pig complement were used.

2.10 Iodination and precipitation of cell surface proteins

Concanavalin A-stimulated spleen lymphocytes were purified with Isopaque-Ficoll (Pharmacia) sedimentation and labeled with ^{125}I using a modification of the glucose oxidase-coupled lactoperoxidase method [23]. Cells (10^7) were labeled with 5 mCi ^{125}I, washed and suspended in 1.0 ml buffer containing 5 mg/ml ovalbumin and 5 mg/ml Triton X-100 (Sigma Chemical Co., St. Louis, MO). The 20 000 x g x 2 h supernatant was dialyzed *vs.* 0.14 M NaCl, 0.01 M Tris-HCl, pH 8.0, 0.05 % NaN_3. The yield was 1.2 % of input ^{125}I, or 1.9 x 10^8 cpm in 1 ml. Mouse surface Ig was cleared by adding cross-reacting rabbit anti-rat IgG and then carrier rat serum. Culture supernatants were concentrated 15-fold by precipitation at a 50 % ammonium sulfate saturation. The equivalence point for precipitation with rabbit anti-rat IgG which varied widely for the different supernatants, was determined after addition of trace amounts of ^{125}I-rat IgG. Concentrated supernatants (10 µl or more, to bring the amount of rabbit anti-rat IgG required to at least 50 µl) or 1 µl of immune or pre-immune rat serum were mixed with 20 µl of ^{125}I-labeled surface proteins. After 6 h at 4 °C an excess of rabbit anti-rat IgG containing 0.5 % Triton X-100 was added. After overnight incubation at 4 °C, the volume was adjusted to 0.5 ml with 5 mg/ml ovalbumin, and 5 mg/ml Triton X-100 and precipitates were collected at 3000 x g and washed twice.

Precipitates were boiled in 5 % 2-mercaptoethanol, 1 % sodium dodecyl sulfate (SDS) and analyzed on 7–15 % polyacrylamide slab gels [24], followed by autoradiography of the dried gel. Molecular weight standards were prepared by labeling proteins reduced with dithiothreitol in 1 % SDS with iodo-[^{14}C]acetic acid.

2.11 Analysis of Ig chains by gel electrophoresis and by isoelectric focusing (IEF)

The incorporation of [^{14}C]lysine into secreted Ig chains and their analysis after reduction with 2-mercaptoethanol on SDS-polyacrylamide gels and by IEF were carried out as described previously [25, 26].

2.12 Inhibition of [^{3}H]Ig binding

[^{3}H]lysine was incorporated into secreted Ig [16], and supernatants were dialyzed *vs.* BBSS to remove unincorporated ^{3}H. The minimum concentration of cells which would maximally bind the [^{3}H]Ig in 1 µl or 2 µl of each supernatant was determined and used in inhibition experiments. Fresh (rather than glutaraldehyde-fixed) mouse and control rat target cells were used to allow homogeneous scintillation counting. For the inhibition assay, 5 µl of the optimal concentration of cells were mixed with 50 µl of cold supernatants (concentrated 10 x in an Amicon B15 cell or diluted in DMM-10 % FCS) and shaken at 4 °C for 30 min in a microtiter plate. [^{3}H]Ig (5 µl of a 1:2.5 or 1:5 dilution) was added, and shaking continued for a further 30 min. Cells were washed five times with 200 µl BBSS, 10 % FCS, suspended in 150 µl BBSS, and transferred to 7 ml mini-vials (New England Nuclear, Boston, MA). Two drops of 1 % SDS were added to dissolve the cells, then 4 ml of Bioflour (New England Nuclear), and ^{3}H was determined in a Beckman scintillation counter. M1/22.54 and M1/75.21 agglutinated RBC into a single clump, but this had no relationship to their effectiveness as inhibitors (see Sect. 3.3.6).

3 Results

3.1 Fusion

Both DA and AO rats responded to the challenge of B10 spleen cells, but with sixfold variation in titer. The DA rat with the highest cytotoxic titer (4500) was boosted intravenously, and its spleen cells used three days later for fusion.

All 94 cultures contained hybrid cells as judged by the survival of cells in HAT medium. Ten days after the fusion, at least 92 out of 94 cultures were producing antibody to B10 spleen cells as measured by activity of the culture supernatant in the binding assay (Fig. 1). Cultures M1/73-M1/96 (those that had a feeder of trypsinized cells from spleen fragments) were particularly active at this early stage (Fig. 1). These culture supernatants and a large proportion of the others were also active in cytotoxicity assays.

Culture supernatants were tested weekly by the binding assay, and cells from positive cultures were frozen in liquid nitrogen in 90 % FCS, 10 % dimethyl sulfoxide. On further growth, many cultures lost their activity after two to five weeks. During this period, cultures that were negative in two successive assays were discarded. No attempts to recover them from

earlier frozen stocks have been made so far. After about six weeks, the remaining cultures were adapted to growth in normal medium by transferring them first to HT medium and then, after about five days, to normal growth medium.

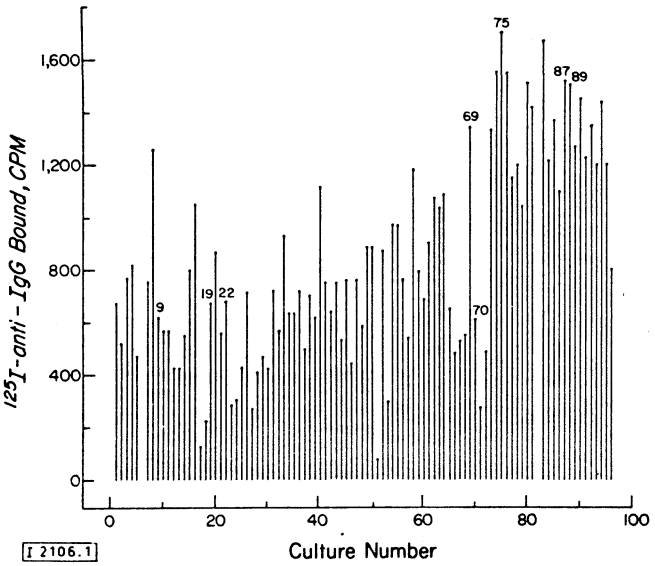

Figure 1. Spleen cell binding activity of M1 culture supernatants. Supernatants were removed from cultures 10 days after hybridization and tested with C57BL/10 spleen cells in the standard binding assay. Cultures numbered in the figure are those from which positive clones were subsequently derived.

Fifteen cultures were then cloned in soft agar, and approximately 48 clones from each culture picked and grown in 2-ml cups. Positive clones were obtained from eight of the fifteen cultures. The clones picked from the agar plates at this stage were likely to be impure, because they had been grown at high cell density. At low density ($\leqslant 10^3$ cells/petri dish) no colonies grew, but at high densities ($\geqslant 10^5$ cells/petri dish) too many colonies grew to be confident that a single agar plug contained only one clone. After one such selection, however, the cloning could in general be repeated with 500–1000 cells/petri dish with cloning efficiencies of 10–20 %.

In some cases (*e.g.* M1/9, see below), the first stage of cloning revealed that there had been two distinct activities within a single culture. No such heterogeneity was ever detected at the second stage of cloning, and so the results obtained from M1/9.3 supernatants, for example, have in general been used interchangeably with those from the true clone M1/9.3.4.

3.2 Identification of positive clones and clones of different antibody specificities from a single culture

3.2.1 General remarks

Positive hybrid cells derived from multiple, independent fusion events were present in most culture wells during the initial stages of growth. Therefore, clone supernatants were assayed in a number of ways to try to determine whether more than one specificity could be recovered from the original culture. The results of some of the different assays used to characterize the antibody activities in the culture supernatants are summarized in Tables 1 and 2. In addition, two sets of experiments were performed at an early stage. In the first, we titrated the supernatants from different clones by serial sixfold dilutions followed by a standard ^{125}I-anti-IgG binding assay (Fig. 2). This gives an indication of the antibody concentration in the supernatants. In some cases, 15-fold concentrated supernatants were also tested to achieve saturation binding. In another set (results not shown), we tested the decrease in ^{125}I-anti-Ig binding capacity at constant supernatant concentration and serial spleen cell dilutions. Here, as cells become limiting, binding is proportional to antigen concentration on the target cells. All these tests not only permitted the separation of different clonal activities derived from a single culture but also early detection of artifacts. For instance, spleen cell dilutions had no effect on the binding of M1/22.25 and of M1/87 antibodies. As will be seen later, these antibodies bind to the sheep RBC used as filler cells. The following sections describe the data pertaining to clones isolated from individual cultures. In the final sections, representative clones from the different cultures are compared in regard to the type of cell surface structures they recognize.

Table 1. Binding of M1 clonal supernatants to spleen cells measured by ^{125}I-anti-IgG and ^{125}I-anti-Fab[a)]

M1 Clones	Counts x 10^{-2}/2 min Anti-Fab	Anti-IgG	Ratio
9.3	92	118	0.8
9.47	38	74	0.5
19.3, 19.6, 19.7, 19.12, 19.22, 19.43, 19.45, 19.47	28–39	17–24	1.6–1.8
22.25	58	29	2.0
22.54	92	47	2.8
69.1, 69.14, 69.16, 69.17, 69.28, 69.35, 69.41, 69.42	94–136	128–170	0.73–0.85
70.15[b)]	0.7	6	0.1
75.2, 75.5, 75.12, 75.21	50–69	20–28	2.5–2.7
87.1, 87.4, 87.11, 87.13, 87.27, 87.29	15–83	6–36	2.3–3.5
89.1	88	119	0.7
89.18 and 17 other clones of M1/89	< 0.3	42–56	< 0.06
Background subtracted	5.8	6.3	

a) Input of ^{125}I was 60 000 counts/2 min. 50 % of the ^{125}I-rabbit F(ab')$_2$ anti-rat IgG and 49 % of the ^{125}I-rabbit F(ab')$_2$ anti-rat Fab was capable of binding to rat serum coupled to Sepharose CL-4B.

b) M1/70.15 binding was measured in a later experiment. The background (subtracted) was 325 for anti-Fab and 255 for anti-IgG.

3.2.2 M1/9

Of 48 clones isolated, only M1/9.3 and M1/9.47 were positive. The binding assay carried out with either ^{125}I-anti-Fab or ^{125}I-anti-IgG as the second antibody, showed the two clones to differ in the ratio of bound anti-Fab to anti-IgG (Table 1). The binding assays carried out with serial dilutions of supernatants (Fig. 2) and with serial dilutions of spleen cells confirmed the difference, since clone M1/9.3 bound twice as many counts as M1/9.47 in the cell dilution ana-

Table 2. Agglutination of mouse RBC[a)]

M1 Clones	−Anti-IgG	+Anti-IgG
9.3	−	−
9.47	−	+++
19.7	−	−
19.47	−	−
22.25	−	−
22.54	+++	+++
69.16	+	+++
70.7	−	−
70.15	−	−
75.2	+++	+++
75.5	+++	+++
89.1	++	+++
89.18	−	−
89.24	−	−

a) Either 50 µl or 5 µl (+ 45µl HEPES-Eagle's + 0.5 % BSA) of supernatant was mixed with 50 µl of 1 % RBC. +++ strong agglutination at both dilutions. ++ and + weaker agglutination with 50 µl and no agglutination with 5 µl. For indirect agglutinations, after 10 min the cells were washed twice, and then 50 µl of a 1:100 dilution of rabbit anti-rat IgG was added.

lysis (not shown). However, the supernatant titer of M1/9.3 was 200 times greater than that of M1/9.47. Binding by M1/9.3 plateaued while that of M1/9.47 did not, hence the M1/9.47 concentration may not have been limiting in the cell dilution analysis, and a firm conclusion on their relative antigen site densities was not possible. (This distinction could be made in other cases, *e.g.* M1/89, see below.) The agglutination assay showed that the two clones have quite different specificities (Table 2). M1/9.47 is active in indirect agglutination of mouse RBC, while M1/9.3 is not. The supernatant titrations rule out antibody concentration as a cause for this difference. Analysis of radioactive supernatants of the purified clones by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3A) and by IEF after reduction (Fig. 3B) show clear differences in their respective H and L chains.

3.2.3 M1/19

Forty-eight clones of M1/19 were isolated and eight were clearly positive. No significant differences between any of these clones could be detected by comparing ^{125}I-anti-Fab and ^{125}I-anti-IgG binding (Table 1). Titration by dilution of supernatant also failed to reveal any difference other than that expected from small differences in antibody concentration in different samples (Fig. 2g). SDS gel analysis of two representative subclones, M1/19.47.1 and M1/19.47.11, showed an H chain with µ-like mobility and an L chain with mobility faster than the parent myeloma chain. M1/19.47 antibody has very low affinity, and it has not been identified as reacting with a specific antigen. Further analysis of this clone has been abandoned.

3.2.4 M1/22

Seventy-two clones of M1/22 were collected. Of 43 tested by binding, two, M1/22.25 and M1/22.54, were strongly positive. In cell dilution analysis, M1/22 binding depended on mouse spleen cell concentrations in contrast to M1/22.25 which appeared not to. Subsequent analysis showed that M1/22.25 bound to the sheep RBC used as filler cells in the

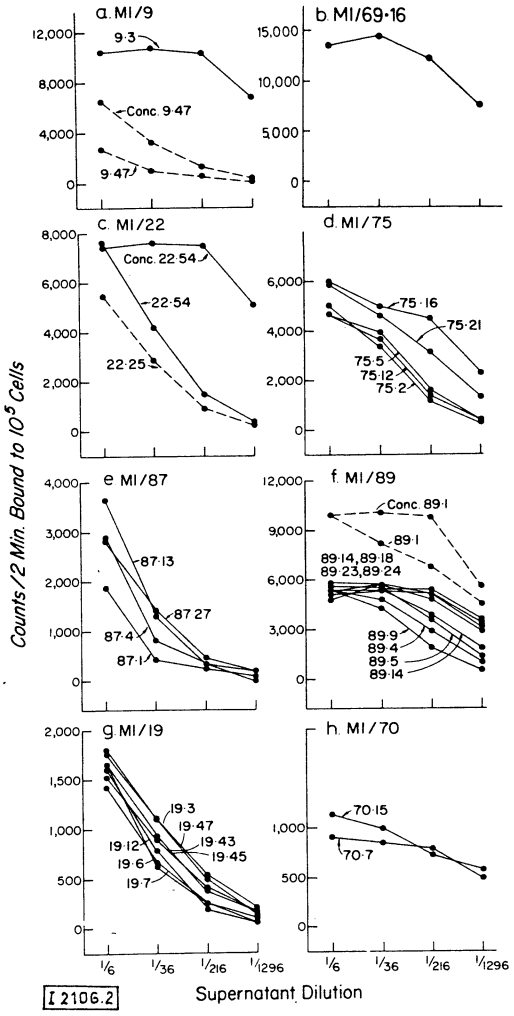

Figure 2. Spleen cell binding activity of M1 clone supernatants studied by supernatant titrations. Supernatants from clones which had been transferred to liquid culture medium were serially sixfold diluted, and 5 µl aliquots were tested with 10^5 spleen cells (in 5 µl) in the standard ^{125}I-anti-Ig binding assay. "Conc." refers to supernatants which were concentrated 15-fold by precipitation at 50 % $(NH_4)_2SO_4$ saturation. Dotted and solid lines distinguish clones from the same well with different antibody specificities.

dilution assay, in addition to binding to mouse spleen cells. M1/22.25 and M1/22.54 are easily distinguished by hemagglutination of mouse RBC (Table 2). In addition, M1/22.25 agglutinates sheep RBC while M1/22.54 does not. M1/22.25 is also lytic for sheep RBC and gives direct Jerne plaques while M1/22.54 does not.

Analysis of the secreted antibody confirms the difference. M1/22.25 appears to be an IgM, and, uncharacteristically, the unselected purified active clones do not express the parental myeloma κ chain (Fig. 3A). M1/22.54, on the other hand, possesses a γ-size H chain, and its L chain has an apparent mol.wt. clearly larger than M1/22.25. The heterophile pattern of reactivity of M1/22.25, and its IgM structure suggested it to have anti-Forssman antigen specificity (see below).

3.2.5 M1/69

Forty-eight clones of M1/69 were tested in binding assays. Eight gave very high binding (Table 1). All also gave high and

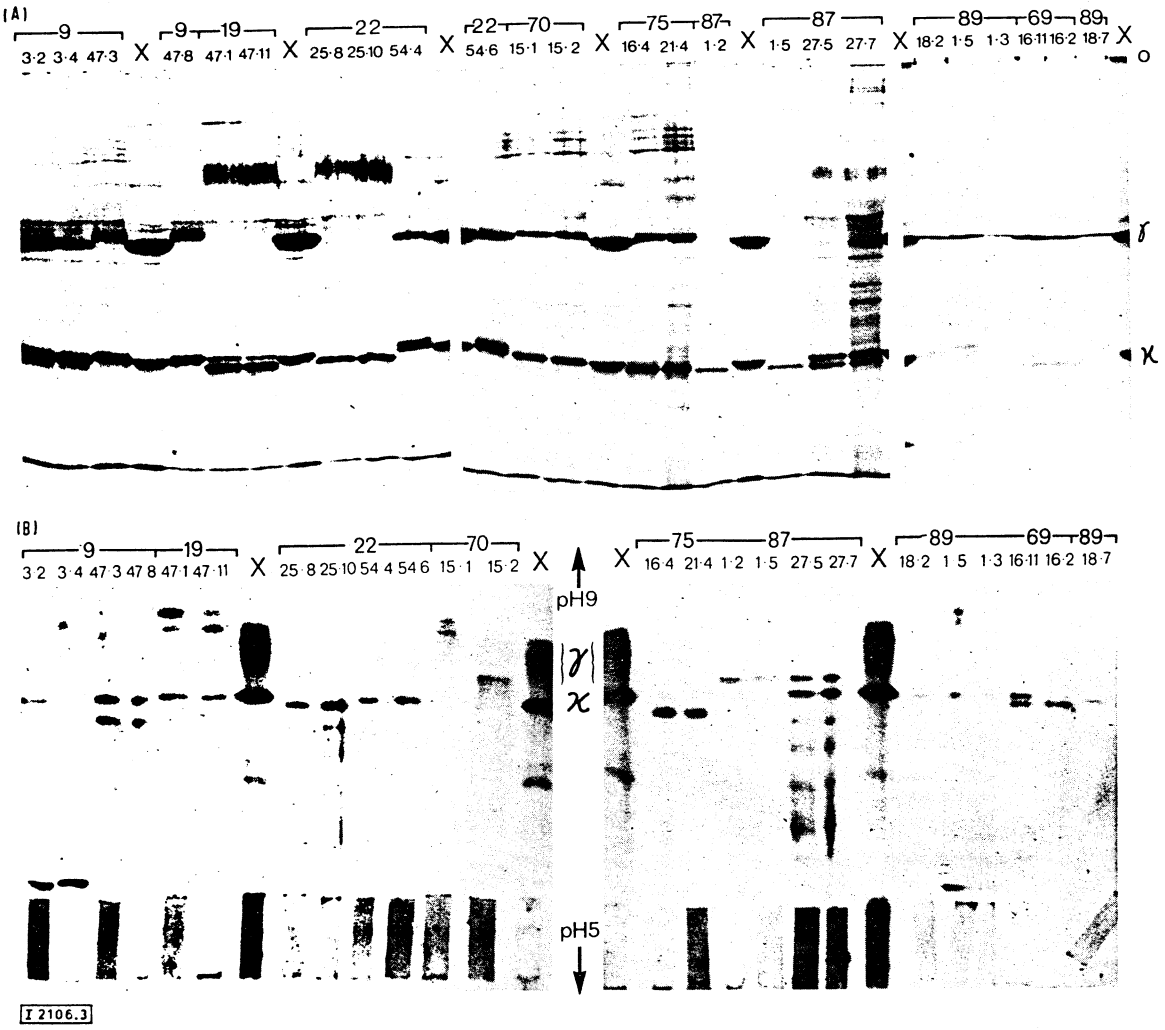

Figure 3. Analysis after reduction of ^{14}C-labeled secreted clone products by SDS-PAGE (A) and IEF (B).

indistinguishable binding to lymph node cells and thymocytes. Only one (M1/69.16) was recloned. A series of subclones was analyzed, most of which expressed two L chains (the myeloma and the specific L chain) which separated on SDS-PAGE, as shown in Fig. 3A. One (M1/69.16.11) was retained as clone M1/69.HLK. In addition, there was an active clone (M1/69.16.2, see Fig. 3A, B) which did not express the myeloma κ chain and was therefore retained as clone M1/69(HL). (The bracket indicates that the recloning step is still lacking). The apparent mol.wt. of the H chain indicated an IgG class antibody.

3.2.6 M1/70

Fourteen clones of M1/70 were tested in the binding assay. Two, M1/70.7 and M1/70.15, gave weak (two times background) binding. Titration of M1/70.7 and M1/70.15 (Fig. 2h) showed that binding plateaued over a wide range of supernatant dilutions, indicating that antigen concentration rather than antibody concentration limited binding. This suggested that M1/70.7 and M1/70.15 recognized an antigen present either in low amounts on spleen cells or on a small subpopulation of spleen cells. Subsequently, it has been discovered that M1/70.15 recognizes an antigen specific to monocytes and macrophages (paper in preparation). Subclones M1/70.15.1 and M1/70.15.2 were derived and their [^{14}C]-labeled antibodies analyzed by SDS-PAGE (Fig. 3A). The antibody secreted contained an H chain of about 50 000 apparent mol.wt., indicating an IgG class. The single L chain band seen in SDS-PAGE has the same mobility as the myeloma κ chain (Fig. 3A). With subclone M1/70.15.1 no myeloma κ chain component was visible on IEF after reduction (Fig. 3B), but the identification of the other components is equivocal. The chain structure of this antibody requires further analysis.

3.2.7 M1/75

Seven out of 34 clones of M1/75 gave strong binding with ^{125}I-anti-IgG. All four tested with ^{125}I-anti-Fab had similar, high, anti-Fab/anti-IgG ratios. The supernatants behaved very similarly in limiting cell titrations. Supernatant titrations (Fig. 2d) showed them to have antibody concentrations within a 15-fold range. Two clones, M1/75.16 and M1/75.21, were recloned and subclones M1/75.16.4 and M1/75.21.4 chosen as representative. Their secreted antibodies appear identical on SDS-PAGE (Fig. 3A) and seem to be of the same IgG class. The myeloma κ chain is present but in very small amounts: perhaps 10–20 % of the specific L chain (see Fig. 3A and B). We shall designate this with the lower case k. Thus, the two clones, which appear identical, are designated M1/75.16.HLk and M1/75.21.HLk.

3.2.8 M1/87

Seven of ten clones of M1/87 tested were positive. All clones bound to and agglutinated sheep RBC as did M1/22.25. However, the binding titer of some of them differed considerably (Table 1, and Fig. 2e). Therefore, cultures with the highest (M1/87.27) and lowest (M1/87.1) antibody titers were selected for subcloning. As in M1/22.25, the antibody secreted by M1/87.27 appeared to be of the IgM class, but the specific L chain was clearly different in SDS-PAGE and in reduced IEF (Fig. 3). Both L and κ chains were expressed, and the clone M1/87.27.5 was adopted as M1/87.HLK.

The subclones derived from the cultures with low activity (M1/87.1.2 and M1/87.1.5) were analyzed by SDS-PAGE (Fig. 3A) which disclosed the presence of the same L chain as in M1/87.27 with no trace of H or κ chain. A large number of previous examples [27] have demonstrated that in the absence of H chains the MOPC 21 κ chain is not secreted. The absence of κ chain in the secreted supernatant was most likely the result of the absence of H chain expression. M1/87.1 therefore appeared to contain a clonal variant which arose during clone purification.

3.2.9 M1/89

All 19 clones of M1/89 tested in the binding assay were positive. The existence of two distinct types of clones was demonstrated in several ways: (a) M1/89.1 bound ^{125}I-anti-Fab and ^{125}I-anti-IgG about equally well, whereas all the other supernatants bound only ^{125}I-anti-IgG (Table 1); (b) binding plateaus at distinctly different levels in supernatant titrations (Fig. 2f) and cell dilution analysis (not shown) suggested that M1/89.23 had a higher antigen site number than M1/89.1; (c) M1/89.1 agglutinated mouse RBC while M1/89.18 and M1/89.24 did not (Table 2). Clones M1/89.1 and M1/89.18 were chosen as representative of the two different kinds of specificities for recloning. Analysis of the secreted antibodies by SDS-PAGE and IEF showed that M1/89.1 has both the specific rat H and L chains and the mouse myeloma L chain. M1/89.18 seemed to secrete only one L chain component, co-migrating with the myeloma κ chain (Fig. 3). However, we cannot exclude the presence of a specific L chain in M1/89.18 in much lower amounts than the κ chain or co-migrating with it in SDS-PAGE and IEF. The H chains of M1/89.18 and M1/89.1.5 both appear to be of γ class.

The best evidence of a difference between the two clonal products comes from studies of the antigenic targets (Sect. 3.3). As will be shown below, a clear distinction could be seen in the analysis of iodinated membrane components recognized by the different antibodies, in the heat stability of the antigens, and in cross-inhibition studies.

The lack of reactivity of M1/89.18 with the absorbed ^{125}I-anti-Fab reagent is surprising. One possibility would be that M1/89.18 contains the rare rat λ chain, and that the absorbed anti-Fab is anti-κ. It is also possible that M1/89.18 has completely lost its rat L chain, and that the anti-Fab is mainly rat L chain-specific. This requires that the artificial mouse myeloma L chain–rat H chain combination retains antigen-binding activity but accounts for the absence of a specific L chain in SDS-PAGE or IEF. Residual activity of a similar nature has precedents in anti-sheep RBC monoclonal antibodies [27].

3.3 Characterization of target cell surface molecules

3.3.1 SDS-PAGE

Concanavalin A-stimulated spleen cells were surface-labeled with with ^{125}I using lactoperoxidase, and detergent-solubilized material was mixed with clone supernatants, precipitated with anti-rat-IgG serum, and analyzed by SDS-PAGE (Fig. 4).

M1/9.3 and M1/89.18 precipitate a protein running as a doublet at 210 000 mol. wt. A trace of the 210 000 mol. wt. protein was also precipitated by M1/9.47, but this is probably because before final purification, M1/9.47 was contaminated with a few M1/9.3 cells. Supernatants of M1/9.47 subclones do not inhibit the binding of M1/9.3 antibody (see below).

Antibody M1/70.7 precipitated iodinated material, present in very small amounts, running as two bands of 190 000 and 105 000 mol. wt. No ^{125}I-labeled material was precipitated by clones which agglutinate mouse RBC or sheep RBC, *i.e.* M1/22.54, M1/69.16, M1/75.2, M1/87.27 and, presumably (see above), M1/9.47.11. Since the number of antigenic sites detected by M1/69.16 on lymph node cells is on average 25 000 per cell [28], a protein target should have been visible on SDS-PAGE unless it was devoid of accessible tyrosine residues. Alternatively, the antigens were glycolipids or other nonproteinaceous structures.

The antibodies secreted by several uncloned cultures were also studied by precipitation. The 210 000 mol. wt. protein appears to be a frequently recognized antigen. Supernatants from cultures M1/62 and M1/87 also precipitated it. While several clones from culture M1/87 were active in sheep RBC agglutination, no clones recovered from this culture recognized the 210 000 mol. wt. protein. Such clones would have been positive in mouse spleen cell binding but negative in sheep RBC binding. Another culture supernatant, M1/94, precipitated two polypeptides similar to those precipitated by M1/70.7.

Precipitation with the antiserum from the rat contributing the spleen for the fusion revealed many additional bands. Thus, the hybrid clones isolated in this experiment represent only a few out of many clones responding to the immunization.

3.3.2 Heat stability studies

Since carbohydrate structures are generally much more stable to heat than proteins, the antigenic targets were studied for heat stability. Glutaraldehyde-fixed RBC or cultured cell lines were heated in an autoclave at 120 °C for 15 min. The cell concentration at which half-maximal binding occurs for heated and unheated cells was determined with serial cell dilutions, and expressed as percentage of remaining antigen (Table 3).

The antigen(s) on mouse RBC recognized by clones M1/9.47, M1/89.1, M1/69.16, M1/75.21 and M1/22.54 are stable at 120 °C. The cross-reactive antigen on sheep RBC recognized by M1/22.25 and M1/87.27 is also heat-stable. In contrast, the antigens recognized by clones M1/9.3, M1/89.18, and M1/70.15 on cultured cells are totally destroyed by autoclaving. Some clumping of the cultured cells, but not the red cells, occurred after heating. Since M1/89.1, M1/69.16 and

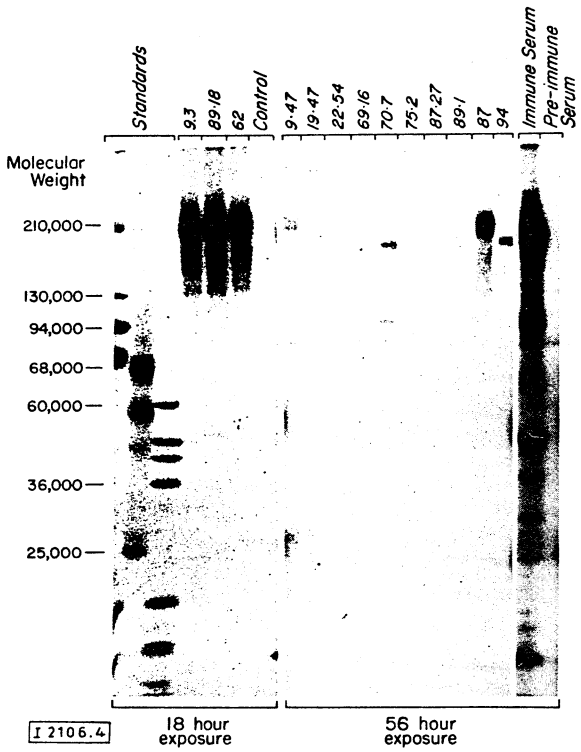

Figure 4. SDS-PAGE analysis of ^{125}I-labeled spleen cell surface molecules precipitated by M1 clone antibodies. Concanavalin A-stimulated spleen cells were labeled with ^{125}I using the glucose oxidase-coupled lactoperoxidase technique, solubilized with Triton X-100 and precleared by precipitation with cross-reacting rabbit anti-rat IgG. Precipitates were boiled in SDS and 2-mercaptoethanol, and analyzed on a 7–15 % polyacrylamide gradient gel containing SDS. The gel was dried and then autoradiographed for 18 and 56 h. "Control" precipitate was with 10 µl of R4/18.2 rat anti-rat histocompatibility antigen supernatant [8]. "Immune serum" and "pre-immune serum" are from the rat contributing the spleen for the fusion. Labeled bands appearing on the edges of the "control" and "9.47" lanes are from heavily labeled material run in intervening lanes which were cut out. Mol. wt. markers labeled with iodo[^{14}C]acetic acid are myosin, 210 000; β-galactosidase, 130 000; phosphorylase, 94 000; BSA, 68 000; catalase, 60 000; glyceraldehyde-3-phosphate dehydrogenase, 36 000; IgG L chain, 25 000.

Table 3. Heat stability of antigens[a] (120 °C for 15 min)

M1 Clones	% Antigen remaining			
	Mouse RBC	Sheep RBC	BW 5147 [23]	P388 D_1[b]
9.3	–	–	0	0
89.18	–	–	0	0
70.15	–	–	–	0
9.47	100	–	–	–
89.1	95	–	20	–
69.16	72	–	43	33
75.21	73	–	–	–
22.54	74	–	23	–
22.25	–	114	–	–
87.27	–	97	–	–

a) Glutaraldehyde-fixed target cells were either autoclaved at 120 °C for 15 min or kept on ice. Cells were serially diluted in the ^{125}I-anti-Ig binding assay. Percent antigen remaining = (unheated/heated cell concentration giving half-maximal ^{125}I-binding) x 100. (–) not determined.

b) See Dawe, C. J. and Potter, M., *Am. J. Pathol.* 1957. *33*: 603.

M1/22.54 measured on the cultured cells remained about 30 % intact, the loss of M1/89.18, M1/9.3 and M1/70.15 on the cultured cells appears to be due to heat denaturation rather than covering up of the cell surface.

3.3.3 M1/22.25 and M1/87.27 recognize Forssman antigen

None of the M1 antibodies are absorbed by typed human RBC or inhibited by AB substance from porcine and equine stomachs (Table 4). Therefore, none of these antibodies are anti-ABO blood group or anti-Rh blood group. M1/9.47, M1/75.21, M1/22.54, M1/89.1 and M1/69.16 were not absorbed by horse, ox, sheep or goat RBC or by autoclaved guinea pig kidney, but of course are absorbed by mouse RBC. M1/22.25 and M1/87.27 are not absorbed by horse, ox or mouse RBC, but are completely absorbed by sheep or goat RBC or by autoclaved guinea pig kidney. Sheep and goat RBC and guinea pig kidney contain the Forssman antigen. Oxen are Forssman-negative. Horses (and guinea pigs) are Forssman-positive but lack this antigen on their RBC [29]. The absorption by guinea pig kidney but not ox RBC distinguishes the heterophile activity as due to Forssman rather than Paul Bunnell antibodies [28]. Mice are Forssman-positive, but the lack of absorption by mouse RBC shows this antigen is very weak or absent from mouse RBC. In another experiment, it was determined whether M1/87.27 and M1/22.25 have the same specificity as Forssman antibodies elicited by immunizing rabbits with autoclaved sheep RBC stroma (Table 5). Pre-incubating mouse spleen cells with the rabbit anti-sheep RBC serum blocked the binding of M1/87.27 and M1/22.25 but not of other clones. – These results show that the M1/22.25 and M1/87.27 antibodies react with Forssman antigen.

3.3.4 Cytotoxicity of M1 antibodies

M1 supernatants were tested for their ability to lyse ^{51}Cr-labeled target cells in the presence of guinea pig complement (Table 6). All those which agglutinated mouse RBC also lysed RBC in the presence of complement, but only M1/69.16 was capable of lysing thymocytes. Of the two antibodies recognizing the 210 000 mol. wt. protein, M1/9.3 and M1/89.18, only M1/89.18 lysed thymocytes and neither lysed mouse RBC. M1/69.16 was lytic for thymocytes at lower complement concentrations than M1/89.18. Lysis by M1/69.16 was still complete at 1:96 final dilution of complement (but nil without) while lysis by M1/89.18 fell off between dilutions of 1:24 to 1:96. None of the other clones M1/22.25, M1/87.27, M1/19.47, or M1/70.15 were lytic for mouse thymocytes or mouse RBC. However, M1/22.25 and M1/87.27 hybrid cells gave direct Jerne plaques on sheep RBC.

3.3.5 Are any of the M1 antibodies allospecific?

Xenoimmunization between rat and mouse has been shown to elicit a significant proportion of allospecific antibodies which are revealed after absorption with mice congenic to the immunizing strain [30]. Moreover, a large number of mouse RBC blood groups have been detected [2], and it seemed possible that the RBC-agglutinating clones might react with their allodeterminants. Therefore, a number of mouse strains informative for the major histocompatibility complex, Thy-1 and blood group markers were tested (Table 7). Clones M1/9.3, M1/89.18, M1/22.25, and M1/19.47 were tested by ^{125}I-

Table 4. Species specificity of agglutinating hybrid supernatants[a]

Absorbing material	Mouse RBC agglutination					Sheep RBC agglutination	
	9.47	75.21	22.54	89.1	69.16	22.25	87.27
Typed human RBC[b]	+++[c]	+++	+++	+++	+++	+++	+++
AB substance[d]	+++	+++	+++	+++	+++	+++	+++
Horse RBC	+++	+++	+++	+++	+++	+++	+++
Ox RBC	+++	+++	+++	+++	+++	+++	+++
Sheep RBC	+++	+++	+++	+++	+++	—	—
Goat RBC	+++	+++	+++	+++	+++	—	—
Guinea pig autoclaved kidney	+++	+++	+++	+++	+++	—	—
BALB/c RBC	—	—	—	—	—	+++	+++

a) To 50 µl of diluted supernatants (1/5 M1/9.47, 1/20 M1/75.21, 1/10 M1/22.54, 1/20 M1/89.1, 1/10 M1/69.16, neat M1/22.25, neat M1/87.27) was added 25µl of absorbing material: 6 x 10^9/ml typed human RBC, 1 mg/ml AB substance (Sharpe and Dohme), 20 x 10^9/ml horse RBC, 11 x 10^9 ox RBC, 2 x 10^9 sheep RBC, 28 x 10^9 goat RBC, 25 % autoclaved homogenized guinea pig kidney, or 5 x 10^9 mouse RBC. After 30 min the samples (in microtiter plates) were centrifuged at 1 000 x g for 5 min and 10 µl aliquots were removed. The aliquots were mixed with 50 µl of 1 % C57BL/10 RBC or sheep RBC, 5 µl of 1/20 rabbit anti-rat IgG was added (except for M1/22.25 and M1/87.27), plates were shaken 1 min, and agglutinations were read several hours later.

b) Seven different samples of outdated, typed human RBC were tested: 0 Rh^-, 0 Rh^+, B Rh^-, B Rh^+, A Rh^+, B Rh^-, B Rh^+, A Rh^-, A Rh^+, and AB Rh^+. Identical results were obtained with all.

c) + + +: strong agglutination; ——: no agglutination.

d) From porcine and equine stomach.

Table 5. Rabbit anti-sheep RBC serum inhibits binding of M1/22.25 and M1/87.27 to mouse spleen cells[a]

M1 Clones	Anti-sheep RBC (cpm)	Control (cpm)	Inhibition (%)
22.25	33	1 561	98
87.27	−15	1 018	100
9.47	1 546	1 559	1
9.3	4 575	4 818	5
Background subtracted	(261)	(192)	

a) Glutaraldehyde-fixed mouse spleen cells (10^5 in 5 µl) were pre-incubated with 5 µl of undiluted rabbit antiserum to autoclaved sheep RBC stroma (anti-sheep RBC) or 5 µl of normal mouse serum (control) for 45 min at 20 °C, and then binding of the clone supernatants tested by the normal ^{125}I-anti-Ig binding assay.

Table 6. Complement-dependent lysis by M1 antibodies

M1 Clones	C57/BL10	
	Thymocytes	RBC
9.3	—	—
89.18	+	—
9.47	—	+
22.54	—	+
69.16	+	+
75.21	—	+
89.1	—	+
22.25	—	—
87.27	—	—
19.47	—	—
70.15	—	—

anti-Ig binding to spleen cells, M1/70.15 was tested by binding to peritoneal exudate cells, and M1/9.47, M1/22.54, M1/69.16, M1/75.21, and M1/89.1 were tested by indirect RBC agglutination. All recognized the 30 mouse strains tested. Thus, the antigenic determinants are not polymorphic, at least within the wide range of strains tested.

Table 7. Mouse strains tested for allospecificity

A/J	AKR/J	AU/SsJ	BALB/c
BDP/J	BUB/BnJ	CBA/CaJ	C57BL/10J
CE/J[a]	C3H/HeJ	C57L/J	C58/J
DBA/1J	DBA/2J	I/LnJ	LG/J
LP/J	MA/MyJ	NZB/BINJ	P/J
PL/J	RF/J	RIII/2J	SEA/GnJ
SEC/IReJ	SJL/J	SM/J[a]	St/bJ
SWR/J	129/J		

a) Tested only for M1/9.47, M1/22.54, M1/69.16, M1/75.21 and M1/89.1. Target cells described in text.

3.3.6 Cross-inhibition of M1 antibodies

The ten antibodies recognize at least four different classes of antigen. Cross-inhibition experiments were carried out to identify groups of M1 antibodies recognizing the same antigenic determinant. Target cells were incubated with unlabeled and then with ^{3}H-labeled clonal supernatants. If the two antibodies bound to identical or proximate sites on the same surface structure, the ^{3}H-antibody binding should be inhibited, while binding to independent structures on the same cell should not cause inhibition.

^{3}H-M1/9.3.4 binding to thymocytes was inhibited by M1/89.18.7 and M1/9.3.4, but not by any of the other supernatants (Fig. 5B). This was not surprising, since both clones precipitated the 210 000 mol. wt. protein from cell surfaces. Similar results and inhibitory titers were obtained when the reciprocal experiment (using ^{3}H-M1/89.2) was done (results not shown). Inhibition of ^{3}H-M1/69.16.2 binding was studied with both RBC and thymocytes as targets because, as stated above (Table 6), M1/69.16 is lytic for thymocytes. Preliminary titrations showed that thymocytes have 25 % as much M1/69.16 antigen on their surface as RBC. Tests were therefore done with equal concentrations of antigens, *i.e.* 5 x 10^5 RBC or 2 x 10^6 thymocytes. ^{3}H-M1/69.16.2 binding to RBC was inhibited by all the antibodies recognizing the heat-stable antigen, *i.e.* M1/9.47, M1/22.54, M1/89.1.5, M1/69.16.11 and M1/75.21.4, but not by any of the others (Fig. 5D). However, there are considerable differences in competitive avidity between the antibodies as judged by the slope of their inhibition curves and their inhibitory titers. The M1/69.16 and M1/22.54 supernatants were more effective competitors of ^{3}H-M1/69.16 binding to RBC, and the supernatants tested had an inhibitory titer one order of magnitude higher than M1/9.47.8, M1/89.1.5, and M1/75.21.4.

Differences are even more pronounced in the inhibition of ^{3}H-M1/69.16.2 binding to thymocytes (Fig. 5C). The inhibitory titer of the homologous M1/69.16 supernatant was the same for RBC and thymocytes, as expected, since cell numbers had been adjusted to give the same concentration of antigen. In contrast, the other clones gave very shallow inhibitory slopes and much lower inhibitory titers. M1/22.54.4 was 50-fold less inhibitory on thymocytes than on RBC, M1/9.47.8 and M1/89.1.5 were 10-fold less inhibitory, and M1/75.21.4 did not reach 50 % inhibition.

On the other hand, when inhibition of ^{3}H-M1/75.21.4 binding to RBC was studied, a different pattern of inhibition emerged (Fig. 5E). M1/69.16.11 was still the best inhibitor, but M1/75.21.4 and M1/22.54.4 were only slightly less potent, while M1/89.1.5 and M1/9.47.8 were considerably less potent. These results show that while M1/69.16, M1/22.54, M1/89.1, M1/75.21, and M1/9.47 all seem to bind to the same antigen on RBC (and with the possible exception of M1/75.21 on thymocytes), there is considerable complexity and/or heterogeneity in the actual determinant recognized and in the corresponding antibody avidity.

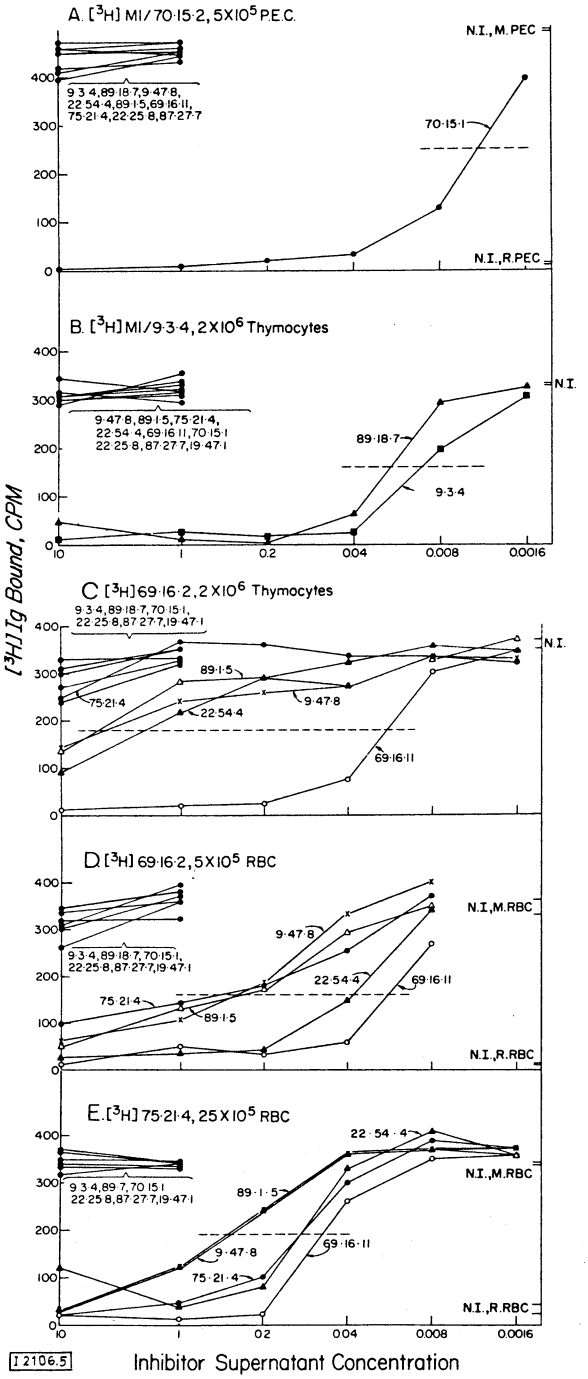

Figure 5. Inhibition of binding of ^{3}H-M1 antibodies by unlabeled clone supernatants. Target cells were incubated with unlabeled supernatants at various concentrations, then with ^{3}H-labeled supernatant, and the amount of cell-bound radioactivity was determined: (A) [^{3}H] M1/70.15.2 and 5 x 10^5 peritoneal exudate cells; (B) [^{3}H]M1/9.3.4 and 2 x 10^6 thymocytes; (C) [^{3}H]M1/69.16.2 and 2 x 10^6 thymocytes; (D) [^{3}H]M1/69.16.2 and 5 x 10^5 RBC; (E) [^{3}H]M1/75.21.4 and 25 x 10^5 RBC. Half-maximal binding is indicated by the broken lines. "N.I.", no inhibition, DMM 10 % FCS substituted for supernatant. "M.PEC", "R.PEC", mouse and rat peritoneal exudate cells, induced by injection of 1.5 ml or 20 ml Brewer's thioglycollate, respectively [29]. "M.RBC", "R.RBC", mouse and rat RBC, respectively.

4 Discussion

A much larger number of positive cultures, and of isolated clones, were obtained in this fusion experiment than in most previous ones [6–11]. Since, when first tested, there were at most two negative cultures out of 94, it can be estimated from the Poisson distribution that there were an average of four independently fused, positive, successful hybrid cells per well. In the 24 cultures (M1/73-M1/96), which contained a different feeder cell population, the efficiency must have been considerably larger, since all of them were not only strongly positive for binding but also for cytotoxicity. Thus, at least 400 specific antibody-secreting, viable hybrid cells were produced by the fusion of 2 x 10^8 rat spleen cells and 2 x 10^7 mouse myeloma cells. Allogeneic immunizations with rats also gave rise to a high frequency of positive hybrids [8], and a mouse anti-rat fusion experiment [9] elicited a large but lower number of specific hybrid cells. However, allogeneic immunization in the mouse results in a much lower frequency of positive hybrids [10, 11]. The critical factor in the production of

specific hybrids appears to be the strength of the antibody response which, in the case of the mouse surface antigens, is elicited by xenogeneic immunization.

This experiment differs from previous hybridizations in another respect. The anti-mouse (C57BL/10) antibodies secreted by the mouse (BALB/c)-rat hybrids have the potential to be self-reactive. This may have affected the type of clones isolated [28].

The high frequency of positive hybrids in this experiment was a mixed blessing, since it resulted in the presence of multiple antibody specificities within single cultures and complicated the analysis of clones. Different types of screening procedures were utilized, which allowed us to detect and isolate two types of clones with different specificities from each of three different cultures. Analysis was facilitated by the rapid and sensitive ^{125}I-anti-Ig binding assay [31]. This assay is particularly useful for xenogeneic antibodies, since the ^{125}I-anti-Ig can be absorbed to remove cross-reaction with surface Ig on the target cells. The sensitivity of the assay is demonstrated by its identification of M1/70.15 binding to spleen cells. Subsequent analysis of this clone has shown that it is present in relatively small amounts on only 8 % of spleen cells, and is present on peritoneal exudate macrophages in much larger amounts [28].

The characteristics of the ten representative clones are summarized in Table 8. Clones were classified into four groups based on their reaction with either: (a) a 210 000 mol. wt. polypeptide; (b) 190 000 and 105 000 mol. wt. polypeptides; (c) a species-specific heat-stable antigen, or; (d) the Forssman antigen. This classification was confirmed by cross-inhibition experiments. The antibodies inhibit binding by clone antibodies of the same group but not of other groups. It is interesting that the ten clones not only certainly arose by independent fusion events, but also appear to originate from independent B cell clones. All the antibodies within a group can be distinguished either by their characteristics in binding assay (Fab/IgG binding ratio), their ability to fix complement, their binding competition properties, their mobilities in SDS-PAGE, or their isoelectric points. However, only the last two provide definite evidence of structural differences. For instance, M1/9.47 and M1/75.21 have very similar H and L chains in terms of electrophoretic properties. But the former has a high amount of myeloma κ chains and the latter only a trace. This could affect all other properties listed except that M1/75.21 (almost no κ chain) is less (and not more) inhibitory of ^{3}H-M1/69.16 binding to thymocytes than M1/9.47 (Fig. 6C).

The M1/9.3 and M1/89.18 antibodies recognize the same surface antigen of 210 000 mol. wt. The two determinants recognized are not necessarily identical, but must be close enough together so that binding to one prevents binding to the other. The antigenic determinants are susceptible to heat denaturation. M1/89.18 lyses thymocytes but M1/9.3 does not. Since both antibodies seem to have similar specificities and avidities, this suggests that M1/89.18 and M1/9.3 belong to different γ chain subclasses which differ in their abilities to fix complement. Although antisera specific for the 210 000 mol. wt. protein have not previously been obtained by allo- or xenoimmunization, this protein appears to be one of the major ^{125}I-labeled surface proteins recognized by the whole rat anti-mouse lymphocyte serum. Not surprisingly then, this or a very similar protein has been observed previously as a component of lymphocyte cell surfaces after labeling with ^{125}I [32] or galactose oxidase treatment and sodium boro[^{3}H]-hydride reduction [33]. It has been suggested that this is the main protein affected by brief trypsin treatment, under conditions which modify lymphocyte migration patterns in lymphoid tissue [32]. High mol. wt. immunodominant proteins have also been observed in mouse and rabbit anti-rat lymphocyte immunizations [34].

M1/22.25 and M1/87.27 antibodies react with the Forssman antigen. This is a heterophile antigen, *i.e.* it is distributed widely among many animal species (and bacteria), but not in a phylogenetically orderly manner [29, 35]. Mice, but not rats, bear the Forssman antigen. The xenogeneic response to this antigen may have been expanded by prior bacterial sensitization. The stability of this antigen to autoclaving is explained by its glycosphingolipid structure [36]. The Forssman antigen is present on sheep RBC which are agglutinated and lysed by M1/22.25 and M1/87.27. However, these antibodies do not agglutinate, nor are they absorbed by mouse RBC. Subsequent studies confirm that the Forssman antigen is absent from mouse RBC [28] but, of course, is present on other cells in the spleen. In addition, M1/22.25 antigen is present in teratocarcinoma cell lines and in a subpopulation of embryonic

Table 8. Summary of M1 clone characteristics

M1 Clones	Antigen recognized (mol. wt.)	Cross inhibition group	Lysis of thymocytes	Agglutination and lysis Mouse RBC	Sheep RBC[a]	Heavy chain class	Chain composition of subclones[b]
9.3	210 000 polypeptide	A	—	—	—	γ	HLk
89.18	210 000 polypeptide	A	+	—	—	γ	HK? [c]
70.15	190 000 105 000 polypeptides	B	—	—	—	γ	HL?
75.21	Heat-stable	C	—	+	—	γ	HLk
22.54	Heat-stable	C	—	+	—	γ	HLK
89.1	Heat-stable	C	—	+	—	γ	HLK
9.47	Heat-stable	C	—	+	—	γ	HLK
69.16	Heat-stable	C	+	+	—	γ	HL, HLK
22.25	Forssman	None of above	—	—	+	μ	HL
87.27	Forssman	None of above	—	—	+	μ	HLK

a) Lysis tested by Jerne plaques only on M1/22.25 and M1/87.27.
b) H and L: specific heavy and light chains. K and k parental myeloma κ chain in normal or trace amounts, respectively.
c) See text.

cells, making the M1/22.25 antibody a very useful reagent for embryological studies [37]. It is interesting that the Forssman antigen elicited clones secreting IgM antibodies, while the other three classes of antigens analyzed in this study elicited IgG antibodies. Forssman antibodies are normally found in the macroglubulin (19 S) fraction [35].

Five clones, M1/75.21, M1/22.54, M1/89.1, M1/9.47 and M1/69.16, recognize an antigen which is stable to autoclaving. No radioactive band was detected on SDS-PAGE of antibody precipitates of solubilized spleen cells labeled with ^{125}I. These findings suggest that the antigenic determinant is carbohydrate, and that it may be a glycolipid. All these clones agglutinate RBC, and all bind to the same region of the membrane as shown by cross-inhibition studies. However, the five antibodies differ markedly in competition studies. M1/69.16 was the best competitor of ^{3}H-M1/75.21 and ^{3}H-M1/69.16 binding. The clone M1/69.16.11 was used as a competitor because its chain composition was HLK, allowing a more meaningful comparison with other HLK clones. While M1/75.21 was practically as good as M1/22.54 and M1/69.16 for the inhibition of ^{3}H-M1/75.21, it was the poorest inhibitor of ^{3}H-M1/69.16 binding.

Clearly, we are dealing with a number of variables, namely the avidity of each antibody, the precise antigenic determinants and the concentration of antibody in the culture supernatants. The latter probably plays a minor role, since most clones secrete similar amounts of protein, and supernatants were collected at similar cell densities. Furthermore, the concentration would affect the inhibition titer rather than the slopes and, more important, the differences between antibodies would be more consistent in pattern. While M1/69.16 was consistently a better inhibitor, and therefore is likely to have a higher avidity for antigen, the differences between M1/75.21 and M1/22.54 cannot be explained by differences in avidity only. Our working hypothesis is that these clones bind to slightly different (and perhaps overlapping) portions of a carbohydrate which has a common core structure but also some heterogeneity in its glycosylation, particularly when dealing with different tissues. Heterogeneity would affect the affinity of each antibody differently, depending on exactly what carbohydrate residue is recognized by each antibody. Taking M1/69.16 as a standard for the concentration of antigen, all the other clones were much poorer inhibitors of ^{3}H-M1/69.16 binding to thymocyte than to RBC. Inhibition by M1/75.21 was undetectable on thymocytes. This could be a reflection of the heterogeneity in glycosylation but could also result from a different environment or the lower density of the heat-stable antigen on thymocytes. It is important to stress that from the pattern of lysis of RBC and thymocytes it might have been erroneously concluded that M1/69.16 recognizes a different antigen from the other antibodies. The actual explanation seems to lie in the avidity of the antibodies and the specificity for particular forms of the heat-stable antigen.

The antibodies to the heat-stable antigen were not absorbed by RBC from several species or different human ABO blood groups. However, many non-ABO blood group antigens have been identified in the mouse [2]. It seemed likely that our xenoantibodies could show such alloantigenic activity. A variety of mouse strains, informative for blood groups, were tested, but no differences were detected. This antigen is also found on a subpopulation of lymphocytes [28]. To our knowledge, no antigen with these properties has been previously described.

Five out of the ten clones isolated are specific for the heat-stable antigen. Thus, surprisingly, a previously unidentified antigen appears to dominate the response. Perhaps exposure to a cross-reacting bacterial polysaccharide also expanded the response to this antigen. Early in our analysis we looked with interest at supernatants reacting with both white and red blood cells as candidates for anti-H-2 antibodies. H-2 antigens are on both red and white blood cells [2] and were expected to dominate the xenogeneic response [29]. Further analysis shows that the heat-stable antigen is more densely expressed on red than white cells, while the reverse is true for H-2 antigens [2]. Testing with the binding assay on red and white blood cells can be used in future screening to recognize the clones producing antibodies to the heat-stable antigen.

The clone M1/70.15 recognizes a heat-labile antigen and precipitates two polypeptide chains of 190 000 and 105 000 mol. wt. Further analysis will be required to determine whether these are two different polypeptides or one is produced by proteolytic degradation of the other. In addition, the amount of ^{125}I incorporated into this antigen in concanavalin A-stimulated spleen cells is much less than that in the 210 000 mol. wt. protein. In spleen cells, the antigen recognized by M1/70.15 is much less abundant than the antigen recognized by M1/89.18 and M1/9.3 [28]. More recent analysis of the distribution of the antigen reactive with M1/70.15 reveals it is specific for macrophages and their precursors [28]. This antigen has not been previously identified by allo- or xenoantisera.

The cell surface antigens of the mouse are better characterized than any other species, and at least 20 surface markers have been identified, mainly by alloantisera. Of the four types of antigens recognized by the antibodies from the ten clones isolated in this study, specific antisera to only the Forssman antigen had been previously prepared. So far, our experiments to produce monoclonal antibodies to differentiation antigens have been based on screening hybrid myeloma cultures with a similar cell population to the one used for immunization. No serious attempt to select for specific target antigens was made. In spite of this simple approach, monoclonal reagents to three novel types of mouse white blood cell antigens, not previously available as specific allo- or xenoantisera, have been obtained in the present study. This not only represents a significant extension of our catalogue of cell surface reagents, but implies that a large number remain yet to be identified.

The ability of the hybrid myeloma procedure to produce monoclonal antibodies recognizing antigens present in low amounts or on minor subpopulations of the immunizing cells, has been observed repeatedly. We strongly suspect that interesting clones may have been missed because the screening procedure was not sensitive enough. The suspicion arises because the testing of some clones was at the limit of detection. Therefore, we believe that an improvement in the screening procedure would be a welcome addition to the method. In another publication, we argue that such improvement could be the use of a panel of tumor cell lines during random screening [37]. Each line expresses a different mosaic of surface antigens, and the problem of minor subpopulations could be avoided.

The present study further proves that obtaining clones of hybrid cells secreting xenoantibodies is a powerful technique for identifying novel surface antigens, even in a well-studied animal such as the mouse. In subsequent reports we show that

these monoclonal antibodies are excellent reagents for studying the distribution of these antigens on white blood cell subpopulations [28] and for embryological studies [37].

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