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Lymphocyte Hybridomas

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Monoclonal Xenogeneic Antibodies to Mouse Leukocyte Antigens: Identification of Macrophage-Specific and Other Differentiation Antigens

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A. Introduction

The identification and study of cell surface molecules which have specific immune functions and are markers of differentiated white blood cell subpopulations is of great interest in immunology. Antibodies are versatile probes with which to study these molecules. However, it is difficult to obtain highly specific antibodies recognizing individual cell surface molecules, because cell surfaces are complex mosaics of many different immunogenic glycoproteins and glycolipids. A general approach to this problem stems from the experiments of Köhler and Milstein (1). They fused myeloma cells and spleen cells from mice immunized with sheep red blood cells to derive continuous hybrid cell lines secreting antibodies to sheep red blood cells. 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. Hybrids have been obtained which secrete antibodies to rat major histocompatibility antigens(2), rat cell surface xenoantigens (3), mouse IgD allotypes (4), and mouse H-2K antigens (5).

In this report, the application of this approach to the xenogeneic identification of cell surface antigens of the mouse is explored. Xeno- rather than allo-immunization was used because the difference between homologous proteins from different species is greater than between the polymorphic variants. Antibodies are thus elicited to a much wider range of molecules. We describe the identification of novel mouse cell surface differentiation antigens, and screening procedures which have been developed to allow detection of antigens present on as little as five percent of the immunizing cells.

I. Immunization and Fusion

DA strain rats received i.p. and s.c. injections of 10×10^6 nylon wool (6) and Ficoll-Isopaque (7) purified B10 spleen cells on days 1 and 15. 40×10^6 of the same cells mixed with 60×10^6 concanavalin-A-stimulated spleen cells were injected in the tail vein on day 31. Three days later the spleen cells were fused with P3-NSI/1-Ag4-1 (NSI) non-secretor myeloma line as previously described (3). The fused cells were distributed into 96 Linbro 2 ml wells and hybrid lines grew up in all. After 10 days and weekly thereafter supernatants from the hybrid cells were tested for binding to glutaraldehyde-fixed spleen cells with ^{125}I-rabbit F(ab')$_2$ anti-rat IgG or anti-rat Fab reagents (3). These reagents were absorbed with mouse IgG and IgM to prevent cross-reaction with surface Ig of the mouse spleen target cells. In the initial

[illegible] Poisson distribution it can be estimated that each culture contained on the average about four independently-fused, successful, specific antibody secreting cells. More clones were positive for binding than for complement-mediated cytotoxicity, and the former assay was therefore adopted for routine use. After further growth over a seven week period, faster growing clones became dominant and some cultures became negative.

II. Screening of Clones

The original 96 2 ml cultures are designated M1/1-M1/96. Clones contain a second number, and if recloned, a third. Positive cultures were cloned in soft agar (1). About 48 clones were generally picked, grown in liquid culture, and tested for activity in the binding assay. Frequently, many positive clones were isolated from a single culture. Because clones from the same culture might differ in their antibody specificities, it became an important problem to distinguish between identical and different clones. One approach was to test ^{125}I-anti-Ig binding capacity at constant supernatant concentration with serial spleen cell dilutions. This method distinguishes between antibodies recognizing antigens which differ in their concentrations, since as cells become limiting, binding is proportional to antigen concentration on the target cells (Fig. 1). Here it can be seen that M1/9.3 and M1/9.47 are different from each other, M1/89.1 and M1/89.23 also differ, but all the M1/75 clones appear identical. This test also allowed the detection at an early stage of an interesting artifact. Binding activity of M1/22.25 (but not M1/22.54) and M1/87 clones appeared unaffected by spleen cell dilution. As will be shown later, the observed binding is to the Forssman antigen on the sheep red blood cells used as filler cells in the binding assay to prevent washing losses. In another set of experiments (not shown), supernatants from different clones were titrated by serial six-fold dilutions followed by the standard ^{125}I anti-Ig binding assay. In some cases, especially when 15x concentrated supernatants were used, saturation binding was achieved. Clones which plateau at different levels of bound ^{125}I-anti-Ig can be distinguished in this manner.

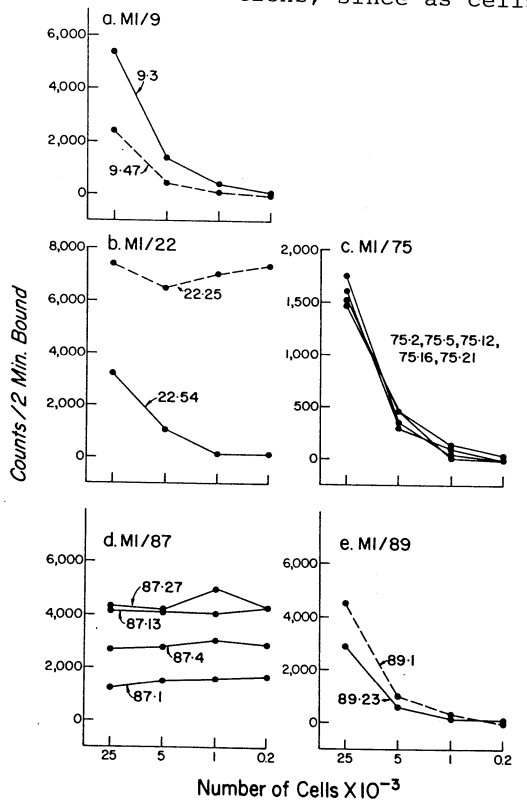

Figure 1. Binding Activity of M1 Clone Supernatants Studied by Spleen Cell Titrations. Supernatants from clones which had been transferred to liquid culture medium (5µℓ) were mixed with the indicated number of glutaraldehyde-fixed (3) spleen cells (in 5µℓ) in V well microtiter plates. After shaking 45 min. at 4°, 5µℓ of 5% filler RBC (sheep here, ox or outdated human O in later experiments) were added and samples were washed twice by adding 200µℓ buffered saline containing 2% bovine serum albumin, (BSA-saline), centrifuging for 5 min. at 200g, and aspirating the supernatant. The cells were suspended in 5µℓ (∿30,000 cpm) of mouse Ig absorbed ^{125}I rabbit F(ab')$_2$ anti-rat IgG or Fab (prepared by iodinating the appropriate antibodies while bound to rat IgG-Sepharose (8)) in BSA-saline. After shaking 45 min. at 4°, cells were washed 3 times as above and transferred to tubes for γ-counting. The background (subtracted) was determined for each spleen cell concentration using a rat anti-rat histocompatibility antigen clone supernatant (4) as a control.

Supernatant titrations were used to guide cloning and recloning. Once multiple identical clones were identified, one or two clones with highest antibody titer were usually subcloned. Again, the subclone supernatants were titrated and the strongest subclones saved. The importance of this procedure in isolating stable subclones is emphasized by the examples of M1/69 and M1/87 clones. While M1/69.16 and M1/69.17 clones were initially both highly positive, one month later the titer of M1/69.16 was 1,000 fold greater than M1/69.17. Ten M1/69.16 subclones were derived and the two most active were saved. One, M1/69.16.2, has lost the myeloma κ chain. Seven M1/87 clones were positive. Clone M1/87.27 had five-fold more supernatant activity than M1/87.1. Later, two subclones were randomly isolated from each and analyzed. Subclones M1/87.27.5 and M1/87.27.7 were both active, while subclones M1/87.1.2 and M1/87.1.5 are both inactive and have lost their specific heavy chains, but retain the specific light chain. The observation that heavy chain loss variants can overcome antibody secreting cells suggests that they may have a selective growth advantage and emphasizes the importance of recloning and screening antibody titer.

Of about 500 picked clones, 47 were positive in the binding assay. By elimination of duplicate clones, the number was reduced to 10. The characteristics of the monoclonal antibodies and the antigens they recognize are summarized in Table 1. Two different types of

Table 1. Summary of M1 Clone Characteristics

Clone	Tissue Distribution	^{125}I-Antigen Precipitated	Stability at 120°C	Agglutination MRBC	Agglutination SRBC	Heavy Chain Class
M1/9.3	White blood cells	210,000 MW	-	-	-	γ
M1/89.18	White blood cells	210,000 MW	-	-	-	γ
M1/70.15	Macrophages	190,000 MW 105,000 MW	-	-	-	γ
M1/75.21	Complex	None	+	+	-	γ
M1/22.54	Complex	None	+	+	-	γ
M1/89.1	Complex	None	+	+	-	γ
M1/9.47	Complex	None	+	+	-	γ
M1/69.16	Complex	None	+	+	-	γ
M1/22.25	Forssman	None	+	-	+	µ
M1/87.27	Forssman	None	+	-	+	µ

clones were isolated from each of three cultures. The differences between M1/9.3 and M1/9.47, between M1/89.18 and M1/89.1, and between M1/22.54 and M1/22.25 are confirmed by many criteria.

Four classes of antigen are recognized. M1/9.3 and M1/89.18 precipitate a heat labile antigen of 210,000 M.W. from detergent solubilized, ^{125}I-labeled spleen cells. M1/70.15 precipitates two polypeptides of 190,000 and 105,000 M.W. and the antigenic determinant is heat labile. M1/75.21, M1/22.54, M1/9.47, and M1/69.16 recognize a molecule on mouse RBC and other tissues which is stable at 120°C and is not labeled by ^{125}I. These characteristics suggest it is a glycolipid. M1/22.25 and M1/87.27 recognize a heat-stable antigen which is not labeled by ^{125}I and is found on sheep red blood cells as well as mouse tissues. Mice and sheep are Forssman positive while rats are Forssman negative. Absorption of M1/22.25 and M1/87.27 activity by guinea pig kidney but not ox red blood cells suggests these clones react with the Forssman antigen.

III. Screening for Differentiation Antigens using Tumor Cell Panels

Tumor lines are useful models for studying differentiation antigens, since each line is usually a clone of cells arrested in a particular stage of differentiation. Therefore, the monoclonal antibodies were used to quantitatively compare the amounts of antigen expressed on several tumor cell lines (Table 2, see legend for details). M1/89.18 binds to a number of white blood cell tumor lines but not to the NS-1 myeloma or red blood cells. M1/69.16 shows a different pattern, reacting with red blood cells, thymocytes, T lymphomas and an Abelson lymphoma but not a myeloma or a macrophage-like line. Fluorescent labeling experiments (12) show M1/69.16 is expressed on mature B but not T lymphocytes and thus the T lymphomas resemble thymocytes but not mature peripheral T lymphocytes in this respect. M1/69.16 typing may be of interest to investigators doing T-T fusions who are looking for cultured lines with mature T lymphocyte characteristics. A macrophage-like line, P388D$_1$, is the only line positive

Table 2. Antigen Titers on Tumor Lines and Normal Cells

	M1/89.18	M1/69.16	M1/70.15
Red blood cells	<0.2	150	<0.02
Spleen (Ficoll-Isopaque purified)	125	67	(3)
Thymocytes	130	60	<0.8
SIA T lymphoma (9)	650	2,500	<4
S49 T lymphoma (9)	770	1,700	<4
BW5147 T lymphoma (9)	2,000	270	<0.8
R8CL7 Abelson lymphoma (10)	500	570	<4
NS-I myeloma (3)	<6	(5)	<0.2
P388D$_1$ macrophage-like line (11)	250	(3)	480

The binding assay (see legend to Fig. 1) was conducted with serial 5-fold dilutions of glutaraldehyde-fixed tumor cells, beginning at 1 to 10 x 10^7 cells/ml. Antigen titer = 10^9 x (cells/ml giving half-maximal ^{125}I-anti-Ig binding)$^{-1}$. ( ): extrapolated to half-maximal binding.

antigen than spleen white cells. Testing with the tumor panel thus shows that M1/89.18, M1/69.16, and M1/70.15 recognize differentiatic antigens and that lymphoma, myeloma, and macrophage-like lines diffe in the mosaic of antigens they express.

Further testing has shown that M1/70.15 recognizes an antigen found on macrophages and their precursors but not on lymphocytes (12). This antigen is expressed in low amounts on only 5-8% of the cells in spleen. Binding by M1/70.15 antibody to spleen cells in the standard assay was only 2 times background, which is just marginally detectable. It is interesting that the Forssman clones, which also gave only barely detectable binding to mouse spleen cells give strong binding to teratocarcinomas but not to many other types of tumor cell lines tested (13). The ability of the myeloma hybrid procedure to produce monoclonal antibodies recognizing antigens present in low amounts or on minor subpopulations of the immunizing cells, has been observed repeatedly. Because M1/70.15, M1/22.25, and M1/87.27 binding to spleen cells was barely above the threshold for detection, we suspect that other interesting clones may have been missed because the screening procedure was not sensitive enoug We thus propose initial screening with tumor cell lines. Each line expresses a different mosaic of surface antigens and the problem of minor subpopulations could be avoided.

B. Concluding Remarks

The cell surface antigens of the mouse are better characterized tha 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 screenin hybrid myeloma cultures with a similar cell population to the one used in the immunization. No serious attempt to select for specif 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 xenoantise 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. Thus 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.

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