Monoclonal Antibodies to Alloantigens and to Immunoglobulin Allotypes

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INTRODUCTION

The specificity of the antigen–antibody bond provides an exquisitely precise probe for the identification and analysis of biological molecules. Until recently, though, the usefulness of antibodies has been limited by the variability of the immune response, the extreme heterogeneity of antibodies, and the presence of unwanted antibodies in many antisera. However, the demonstration by Köhler and Milstein (1) of the feasibility of the production of functional hybrids between myeloma cells and normal antibody-secreting cells has essentially solved these problems, and vastly increased the power of the serological approach. It is now possible to generate virtually unlimited quantities of homogeneous, monospecific antibodies to almost any desired antigenic determinant, even if the antigen is not pure. In this paper, we describe the production and properties of hybrid cell lines secreting antibodies to products of the major histocompatibility complex and to immunoglobulin allotypes of the mouse.

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CELL HYBRIDS

The best known example of cell fusion is that of sperm and egg. In general, spontaneous fusion of somatic cells is rare, although the process does occur in myotubes, osteoclasts, and foreign body giant cells (2). However, the incidence of fusion of somatic cells can be greatly increased by certain agents, such as Sendai virus, lyssolecithin and polyethylene glycol (PEG) (2). PEG is now the agent of choice. When the cytoplasmic membranes are fused by chemical treatment, bi- or multi-nucleated cells (heterokaryons) are produced. During the next division, the nuclei fuse, and cell hybrids are formed (2).

The hybrids are then isolated by growth on selective media. For example, normal cells may be fused with tumor cells that have been selected for resistance to thioguanine or azaguanine by virtue of loss of the X-linked "salvage" enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). When the main biosynthetic pathways for purines and pyrimidines are blocked by aminopterin, cells must use HGPRT to convert hypoxanthine and guanine into ribonucleotides. Thus, if the cells are grown in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) (2), only those cells containing HGPRT (i.e., normal parental cells and hybrids) survive. Since normal lymphocytes die after a few days in culture, growth in HAT medium will select for hybrids.

ANTIBODY-SECRETING HYBRIDS

In order to express a differentiated cell function, the two parental cells should be at a similar stage of differentiation. The fusion of unlike cells frequently results in the "extinction" of differentiated function (2,3). Thus, antibody secretion is only maintained if normal antibody-secreting cells are fused with myeloma cells.

The general strategy for production of antibody-secreting hybrids, described more fully elsewhere (1,4) is as follows. Mice are immunized with the desired antigen (or a mixture containing the desired antigen), and, shortly after boosting, the spleen cells are fused with azaguanine-resistant myeloma cells by means of PEG. The cells are then cultured in HAT medium. In the first few days, there is massive cell death, but at 10-14 days after fusion hybrid colonies begin to appear. The culture medium is then tested for antibody to the desired antigen, and positive cultures are cloned by limiting dilution. Positive clones may then be grown in bulk cultures or injected into histocom-
patible mice where they will grow as tumors. The antibodies may be recovered from culture supernates or from serum or ascites fluid of tumor-bearing mice.

During growth of hybrids there is a tendency for chromosome loss (2). Since the loss of antibody secretion probably allows more rapid growth, cultures may be overgrown by nonproducing variants. The best solution to this problem seems to be to clone early and reclone periodically.

PRODUCTION OF HYBRIDS

USE OF NS-1 AS PARENT

The parental cell line used was the NS-1 variant of the P3 (MOPC 21) line (5). Cells of the NS-1 line are resistant to azaguanine, and do not synthesize the MOPC 21 γ, heavy chain. Although NS-1 synthesizes the MOPC 21 κ chain, it is not secreted. NS-1-derived hybrid cell lines secreting the normal parental spleen cell immunoglobulin also secrete hybrid molecules containing the MOPC 21 κ chain. Thus, if the synthesis of light chains from each parental cell occurs at similar rates, and if pairing is random (1), about 25% of antibody molecules will possess only the spleen cell light chain, 25% will possess only the MOPC 21 κ chain, and 50% will possess one of each of the light chains. If the MOPC 21 line were used rather than NS-1, only 6.25% of secreted molecules would be expected to be derived entirely from the spleen cell parent.

EFFECT OF THE IMMUNIZATION PROTOCOL

In early experiments, we examined the effect of various forms of immunization on the production of antibody-secreting hybrids. BALB/c mice were immunized with C57BL/10 (IgH) anti-B, pertussis–pertussis complexes, an immunization that generates anti-allootype antibodies (6). There was no clear-cut effect of the different immunization protocols on the number of culture wells with hybrid growth, but there was a marked improvement in the number of wells with anti-Ig-1b antibody when hybridization was performed 3 days after boosting rather than 6 or 8 days. There was also a much greater number of wells secreting anti-Ig-1b antibody when the mice were primed
and boosted once, as opposed to more prolonged immunization. No other anti-allotype positive wells were found.

It is thus our impression that the immunization regimen is best kept short and the hybridization performed 3 days after the last boost. Similarly, good results were obtained in antispleen cell alloantigen immunizations when the mice were primed with $2 \times 10^6$ spleen cells per animal, followed by a similar boost 3 weeks later, and hybridization 3 days after the boost.

**CULTURE CONDITIONS**

Details of the hybridization protocol and cell culture conditions are given elsewhere (4,7). In brief, NS-1 cells from log-phase cultures were fused with immune spleen cells at a 1:4 or 1:2 ratio, using 50% FEG (BDH Chemicals Ltd., Poole, England) and $3 \times 10^6$ cells in 1 ml of serum-free RPMI-1640 medium. Cells were plated out into 96-well microculture plates in RPMI-1640 with 15% FCS, and subjected to progressive HAT selection over a 2 week period. During the second to fourth weeks, depending on the rate of cell growth, supernates were tested for antibody activity (see below). Cells from active wells were transferred to 1 ml cultures, together with $5 \times 10^6$ thymocytes as feeders, and then expanded into larger flasks (50-100 ml). Because of the problem of chromosome loss alluded to earlier, we chose to freeze aliquots of cells at this early stage, so that active clones could be rescued at a later stage if necessary.

Cloning was generally performed by limiting dilution in microculture plates, using $10^6$ thymocyte “feeder” cells per well, and three different dilutions of cells such that each well contained an average of 10, 2, or 0.5 hybrid cells. When the cloning plates showed evidence of vigorous growth, cells from a group in which 20-50% of wells were positive for growth were tested for antibody, and positive cultures expanded as previously. Cells were always diluted gradually; generally not more than a tenfold increase in volume between serial expansions. Loss of production of specific antibody occurred frequently, but in general it was possible to “rescue” failing lines by recloning and re-expansion of the cultures. It was generally observed that those hybrid cell lines that maintained antibody production in 1 ml and flask cultures had high cloning efficiencies and good recoveries of antibody-producing clones.

After cloning, aliquots of cells were frozen, and mice were injected with $2-5 \times 10^6$ cells per mouse. About 90% of clones produced tumors
(hybridomas) within 10–30 days, and of these about 90% produced myeloma-like proteins of the desired antibody activity.

**ANTIBODY ASSAY**

Reactivity against soluble immunoglobulin allotypes was measured by a solid-phase radioimmunoassay using antigen-coated plates (6). Purified myeloma proteins were adsorbed onto wells of flexible plastic microtiter plates (Cooke Lab. Prod., Alexandria, VA), the excess washed off, and any remaining nonspecific binding sites saturated with BSA. Plates were reacted with 5–20 μl of culture supernate for 1 hr at room temperature, washed, and held with 125I-labeled purified anti-Ig-1b or anti-Ig-4b antibodies (6).

Antibodies to spleen cell allotypes were detected by reacting 4 x 10^2 spleen cells with culture supernates in wells of microtiter plates, washing, and incubation with 125I-anti-allotype antibodies or with 125I-staphylococcal protein A (8). Virtually all of the antibodies detected with the anti-allotype reagent were also detected with protein A. In addition, the protein A detected several clones that were not detected with the anti-allotype reagents (especially IgG2b and IgG3). We did not screen for IgM antibodies.

**ANTIBODIES TO ALLOANTIGENS OF THE MAJOR HISTOCOMPATIBILITY COMPLEX**

The production of strong and specific alloantibodies to products of the major histocompatibility complex (MHC) requires selection of the appropriate mouse strains (both with regard to the H-2 haplotype and background genes controlling overall immune responsiveness), careful selection of mice producing high titer antibody, and a considerable amount of luck (9). In addition, such antisera very frequently contain high titer antibodies to proteins of murine leukemia virus. Viral antigens are frequently present on the surface of neoplastic (10,11) and even normal (10) cells, and may seriously confuse the interpretation of serological data (11). The availability of homogeneous, high titer monospecific antibodies to products of the MHC would greatly facilitate studies of MHC-linked alloantigens on normal and neoplastic cells.

Two hybridizations were performed with spleen cells from mice immunized with allogeneic cells. In one hybridization (H10), donor
<table>
<thead>
<tr>
<th>Strain</th>
<th>H-2</th>
<th>Ig</th>
<th>11-1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>11-2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>11-3&lt;sup&gt;a&lt;/sup&gt;</th>
<th>11-4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>11-5&lt;sup&gt;a&lt;/sup&gt;</th>
<th>11-6&lt;sup&gt;a&lt;/sup&gt;</th>
<th>10-1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>10-2&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CWB</td>
<td>b</td>
<td>b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CKB</td>
<td>k</td>
<td>b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C3W</td>
<td>b</td>
<td>a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+</td>
<td>-</td>
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<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup><sup>125</sup>I-protein A;  
<sup>b</sup><sup>125</sup>I-anti-Ig-1a + anti-Ig-4a;  
<sup>c</sup><sup>125</sup>I-anti-Ig-1b + anti-Ig-4b.
cells were from CWB (H-2^a, Ig^a) mice which were immunized with C3H (H-2^b, Ig^b) spleen cells. CWB and C3H mice differ only in their alleles at the H-2 complex and also at the heavy chain complex; thus this immunization could produce antibody against products of the MHC or allotypes of IgM or IgD receptors (12). The second hybridization (H11) used BALB/c (H-2^a, Ig^a) spleen cells from mice immunized with GKB (H-2^b, Ig^b) spleen cells. This immunization could potentially elicit antibodies against MHC products, IgM, IgD, or numerous C3H "background" gene products.

**LINKAGE ANALYSIS**

Screening of supernates from initial microcultures was performed with a cell binding radioimmunoassay as described previously. The availability of the tetralogy of congenic mice on the C3H background (Table I) greatly facilitated linkage analysis. As shown in Table I, the reactivities of five of the H11 and two of the H10 supernatant antibodies are against antigens linked to the MHC, while one antibody each from H10 and H11 seems to react with surface immunoglobulin. The 10.1 hybrid supernate reacted with cells from all four congenic strains, including CWB, the spleen cell donor. This hybrid cell line apparently is producing an autoantibody against an undefined cell surface antigen, and was not studied further.

It is interesting to note that the great majority of H11 hybrids were directed against products of the MHC or the heavy chain complex, and no antibody against "background" gene products was observed. This

**TABLE II**

<table>
<thead>
<tr>
<th>MHC Mapping of Hybrid Cell Antibody Reactivity</th>
<th>K</th>
<th>A</th>
<th>B</th>
<th>J</th>
<th>E</th>
<th>C</th>
<th>S</th>
<th>G</th>
<th>D</th>
<th>I-A(2)^a</th>
<th>I-A(17)^d</th>
<th>H-2K^d</th>
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<td>k</td>
<td>k</td>
<td>k</td>
<td>k</td>
<td>k</td>
<td>k</td>
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<td>+</td>
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<tr>
<td>B10.A4(R)</td>
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<td>k</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>b</td>
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<td>+</td>
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<td>+</td>
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<td>s</td>
<td>k</td>
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<td>k</td>
<td>+</td>
<td>+</td>
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<tr>
<td>B10.S</td>
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<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
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<td>s</td>
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<td>s</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B10.AQR</td>
<td>q</td>
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<td>k</td>
<td>k</td>
<td>d</td>
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<td>d</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>B10.T16R</td>
<td>q</td>
<td>q</td>
<td>q</td>
<td>q</td>
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<td>q</td>
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<td>q</td>
<td>q</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B10.A3(R)</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>h</td>
<td>k</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C3H.OH</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
<td>d</td>
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<td>d</td>
<td>k</td>
<td>-</td>
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</tbody>
</table>

H2K monoclonal

H2K antisera

A more detailed analysis of the seven MHC class I clones was carried out using H2 recombinant proteins (Table III). The reactivity of six of these antibodies is consistent with their detecting I-A, I-E, or other subregion products. None of these antibodies detected H-2D, H-2L, or I-E subregion products.
Fig. 2. Autoradiograms of 2-D gels of I-A\(^k\) antigens. Proteins precipitated from (a) C3H extract by normal mouse serum, (b) B10.A(4R) extract by A.TH anti-A.TL, and (c) C3H extract by antibody from clone 10.2.16 were electrophoresed as described in the legend to Fig. 1.

It should be noted that clone 11-1.23 was erroneously typed as an anti-H-2K (7), due to an unexplained false negative on A.TL. This clone is, in fact, positive on A.TL, and also positive on B10.AQR, yet negative on B10.T(6R) (Table II). Thus, the reactivity must be assigned to I-A.

**BIOCHEMICAL ANALYSIS**

To confirm the reactivities of the anti-MHC antibodies, proteins were labeled biosynthetically with \(^{35}\)S-methionine, immunoprecipitated,
and analyzed by two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) (14,15). The first dimension separates the proteins on the basis of their net charge, by means of a technique or nonequilibrium pH-gradient electrophoresis (16). The second dimension was conventional SDS-PAGE, using the Laemmli discontinuous buffer system (14). Earlier studies have shown that this form of analysis produces patterns that are characteristic of both the region or subregion coding for the precipitated antigen and the haplotype (14,15).

Figures 1 and 2 show the excellent correspondence between the gel patterns obtained with monoclonal and conventional antibody. Antibody from the cloned anti-H-2K hybrid cell line 11-4.1 precipitates

Fig. 3. Anti-H-2K and anti-μ chain immunofluorescence histograms. C3H spleen cell stained with rhodamine (R) conjugated rabbit anti-μ and anti-H-2K antibody from clone 11-4.1, followed by fluorescein (F) conjugated rabbit anti-γ, were analyzed by means of the FACS: (a) R-anti-μ profile, (b) F-anti-H-2K profile, (c) F-anti-H-2K profile of μ- cells (cells to the left of channel 15 in (a), indicated by the left arrow), and (d) F-anti-H-2K profile of μ+ cells (cells to the right of channel 30 in (a), indicated by the right arrow).
molecules from 35S-methionine-labeled CKB extracts identical to those precipitated by an alloantiserum (A.TL X C3H.OL)F1, anti-C3H, which is directed against H-2K^b. Similarly, antibody from clone 10-2.16 precipitated the same set of I-A^k molecules from C3H extracts as does A.TH anti-A.TL (anti-1') alloantisera from B10.A(4R).

Previous work (16a) has shown that antisera against the I-A subregion precipitate three distinct families of molecules in the 25,000-33,000 molecular weight range: a basic set, an intermediate set, and an acidic set. The intermediate spot is seen in precipitates of all I-subregions and haplotypes, and shows no evidence of polymorphism. The acidic and basic sets show mobilities characteristic of each I-A haplotype. It is thought that these spots represent three discrete gene products. All three sets are precipitated by antibody from clone

Fig. 4. Contour map in which C3H spleen cells stained with rhodamine-anti-μ and anti-H-2K antibody from clone 11-4.1, followed by fluorescein-conjugated rabbit anti-γ, were analyzed by means of the FACS and data plotted using a computer program written by W. Moore. The interval between adjacent contours is 25 cells. A total of 10,000 cells were analyzed.
10.2.16. Since it is unlikely (though not impossible) that the three molecules have in common the antigenic determinant recognized by clone 10.2.16, the three chains probably exist as a tri-molecular complex on the cell surface. Anti-I-A molecules produced by clones 10-3.6 and 11-5.2 precipitate the same molecules as 10-2.16, adding support to this concept. These data illustrate the power of monoclonal antibodies in the analysis of the fine structure of complex cell surface molecules.

ANALYSIS BY TWO-COLOR IMMUNOFLOURESCENCE USING THE FLUORESCENCE-ACTIVATED CELL SORTER (FACS)

The cell subpopulations recognized by the hybrid cell antibodies were analyzed by two-color fluorescence using the fluorescence-

![Contour map](image)

Fig. 5. Contour map in which C3H spleen cells stained with rhodamine-anti-μ and anti-In antibody from clone 10.2.16, followed by fluorescein-conjugated rabbit anti-γ, were analyzed by means of the FACS, and the data plotted as in Fig. 4. Virtually all μ⁺ cells are I-A⁺.
activated cell sorter (FACS) as previously described (17). Spleen cells from C3H (H-2b) mice were stained with rhodamine-anti-μ to stain most B cells, followed by either anti-H-2K\(^+\) (11-4.1) or anti-I-A\(^b\) (10-2,16), followed by a fluorescein-conjugated rabbit anti-γ. Results are shown in Figs. 3–5. The anti-H-2K hybrid stains most of the spleen cells, although there appears to be a small subpopulation of cells with little or no H-2K or μ. These may represent nonlymphoid cells. Figure 3 also shows that B cells possess slightly more H-2K than do T cells. On the other hand, the anti-I-A\(^b\) antibodies stained virtually all of the μ\(^+\) cells, but did not detectably stain μ\(^-\) cells (Fig. 3).

PURIFICATION AND CHAIN COMPOSITION OF HYBRID CELL ANTIBODIES

The great majority of hybrid cell lines bound staphylococcal Protein A, as detected in the cell binding assay. Thus, affinity chromatography on protein A-Sepharose (8,18) provided a simple and efficient one-step procedure for isolation of antibody from culture supernates or serum. In accordance with recently published work (18) all mouse IgG subclasses were found to bind at pH 8.0 (although we prefer pH 8.6 which provides more firm binding of IgG\(_\gamma\)). Selective elution of IgG subclasses was obtained by stepwise elution at pH 6.0 (IgG\(_\gamma\)), 4.0 (IgG\(_\alpha\)), and 2.2 (IgG\(_\delta\)). Yields from culture supernates were typically 10–20 μg/ml, but were sometimes as high as 50 μg/ml. There was no detectable binding of proteins from the FCS in the culture medium. Figure 6 shows the degree of purity obtainable.

In order to analyze the antibody heavy and light chain composition, hybrid cell antibodies were also analyzed by 2-D PAGE. A typical pattern is shown in Fig. 7. Normal mouse IgG purified from whole serum by affinity chromatography on protein A-Sepharose is heterogeneous in charge. In contrast, IgG\(_\gamma\) from mice bearing the MOPC 21 tumor is much simpler. The presence of two to three spots for each chain probably reflects post-translational modifications such as deamidation, and causes movement towards the more acidic end of the gel. A similar analysis of IgG isolated from the clone 10-3.6 is shown in Fig. 7. As expected from the contribution of the NS-1 parent, one of the two light chain spots corresponds to the position of the MOPC 21 κ chain. On the other hand, the single pair of heavy chain spots is distinct from the expected position of the MOPC 21 heavy chain, which is absent.

The isolated proteins were tested for isotype using subclass-specific sera. In every case, light chains were κ in type. The heavy chain types are listed in Table III.
Fig. 6. SDS-PAGE of reduced immunoglobulins. (a) MOPC 21; (b) C.B17-12; (c) 11-4.1; (d) 11-5.2; (e) 10-4.22; (f) 11-3.24; (g) 11-2.13; (h) 11-2.12; (i) 11-6.3. Samples (a) and (b) were isolated from serum of tumor-bearing mice by ion exchange chromatography and gel filtration. Samples (c)-(h) were isolated from culture supernates by affinity chromatography on protein A-Sepharose. Sample (i) consisted of culture fluid from clone 11-6.3.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Isotype</th>
<th>Specificity</th>
<th>Titer (x 10^5)</th>
<th>Maximum lysis (%)</th>
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</thead>
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<td>γ2a</td>
<td>I-A(2)</td>
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<td>69</td>
</tr>
<tr>
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<td>γδ</td>
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<td>68</td>
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<td>I-A(17)</td>
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<td>γ2a</td>
<td>H-2K(new)</td>
<td>200</td>
<td>98</td>
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Fig. 7. 2-D PAGE analysis of light and heavy chains from (a) normal BALB/c serum Ig purified on protein A-Sepharose; (b) MOPC 21 myeloma protein; (c) antibody from clone 10-3.6 (anti-I-A') purified from culture supernates on protein A-Sepharose. The gels were run as described in the legend to Fig. 1, and the proteins visualized by Coomassie Blue staining. Ovalbumin (OA), 45,000 daltons, was added as a molecular weight marker.

ANTIBODIES TO IMMUNOGLOBULIN ALLOTYPES

IgD ALLOTYPES

In addition to the antibodies reacting with MHC determinants obtained from the H10 and H11 hybridizations, two cultures were found to react with lymphocyte surface antigens linked to the heavy chain
complex (see Table 1). Since these antibodies potentially could recognize allotypic determinants on either IgM or IgD receptors (12,13), further experiments were carried out to identify the antigen.

BIOCHEMICAL ANALYSIS

Spleen cells from BALB/c (Ig\(^a\)) or BAB/14 (Ig\(^b\)) mice were surface radio-iodinated with \(^{125}\)I by the lactoperoxidase technique, and the membrane proteins solubilized in the non-ionic detergent NP-40. Antigens were precipitated and analyzed by SDS–PAGE. Results are shown in Fig. 8.

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**Fig. 8.** Autoradiogram of SDS–PAGE analysis of immunoprecipitates of NP-40 solubilized BALB/c (Ig\(^a\)) and BAB/14 (Ig\(^b\)) spleen cells, labeled with \(^{125}\)I by the lactoperoxidase technique. Reactivities of antibodies used for immunoprecipitation are indicated at the top of the gel. Anti-Ig-5a is from clone 10-4.22 and anti-Ig-5b from clone 11-6.3. Fixed *S. aureus* bacteria were used to bring down the antigen–antibody complexes. Since the 11-6.3 antibody (IgG) does not bind strongly to *S. aureus* protein A, a small amount of rabbit anti-mouse \(\gamma\) chain was added to facilitate binding of the 11-6.3 complexes to the bacteria.
When antibody from clone 10-4.22 was tested against BALB/c spleen cells, bands corresponding in mobility to $\delta$ and light chains were seen. No bands were seen using BAB/14 cells. Conversely, clone 11-6.3 precipitated $\delta$ and light chains from BAB/14 cells but not from BALB/c cells. Thus, it was concluded that both the clones producing antibodies against allotype-linked surface structures were recognizing allotypic determinants of $\delta$ chain. Clone 10-4.22 recognizes the Ig-$5^e$ allele and 11-6.3 the Ig-$5^e$ allele.

FACS ANALYSIS

Clones 10-4.22 and 11-6.3 were analyzed by two-color fluorescence using the FACS as described earlier. Results are shown in Fig. 9. Several conclusions may be drawn. The great majority of $\mu^e$ cells were

![Contour map with antibody staining](image)

Fig. 9. Contour map in which C3H spleen cells stained with rhodamine-anti-$\mu$ and anti-Ig-$\delta$ (anti-$\delta^e$) antibody from clone 10-4.22, followed by fluorescein-conjugated rabbit anti-$\gamma$, were analyzed by means of the FACS, and data plotted as in Fig. 4. Three predominant populations are seen: T cells ($\mu^e\delta^e$) lie near the origin; the majority of B cells are $\mu^e\delta^e$, while a minority of B cells are $\mu^e\delta^e$. 
also δ⁺. No cells were seen which possessed IgD but not IgM. There was, however, a distinct set of cells which possess IgM but not IgD. These cells may represent a mixture of immature and activated B cells (12). Among the μ⁺δ⁺ cells, there was positive correlation between the amount of μ and the amount of δ. Finally, the mean intensity of μ staining on the μ⁺δ⁺ cells was greater than that of the μ⁺δ⁺ cells.

SPECIFICITY ANALYSIS

The monoclonal anti-IgD allotype antibodies simplify the analysis of Ig-5 antigenic specificities, since unlike alloantiseria (12) they can be used on any strain possessing an appropriate Ig-5 allele, regardless of background. Two Ig-5 specificities have been described using conventional antisera. Specificity 1 is present on cells of Ig⁺δ⁺ and Ig⁺δ⁺ haplotype, but not on Ig⁺ (12). Specificity 2 is present on Ig⁺ but not on Ig⁺ (12). Specificity 3 is defined by the H6/31 monoclonal antibody (19), which

<table>
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<tr>
<td>Genetic Analysis of Anti-IgD Antibodies</td>
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<tr>
<td>Ig Haplotype</td>
</tr>
<tr>
<td>BALB/c</td>
</tr>
<tr>
<td>NMS</td>
</tr>
<tr>
<td>11-5.3⁺</td>
</tr>
<tr>
<td>NMS</td>
</tr>
<tr>
<td>10-4.22⁺</td>
</tr>
</tbody>
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*Second-step was 1111-anti-Ig-4a.
*Second-step was 1111-protein A.

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<th>TABLE V</th>
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<tr>
<td>IgD Specificities</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>a</td>
</tr>
<tr>
<td>b</td>
</tr>
<tr>
<td>c</td>
</tr>
<tr>
<td>d</td>
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reacts with Ig^a and Ig^e haplotypes, but not Ig^e (T. Pearson and L. A. Herzenberg, unpublished). Specificity 4 is defined by clone 10-4.22, which reacts with Ig^e but not Ig^e or Ig^e (Tables IV and V). Thus, hybrid cell antibodies provide a powerful means of analysis of the complexity of antigenic determinants on single molecules, and of dissecting the nature of genetic polymorphisms.

**Blocking of ^125^I-Ig (lb) 2.9 Binding to Ig (lb) (C.BP101) Myeloma Protein**

Fig. 10. Anti-Ig (lb) blocking curve. Various concentrations of unlabeled Ig (lb) 2.4, 3.1, and 2.9 were used (abscissa) to block the subsequent binding of ^125^I-Ig (lb) 2.9. One hundred percent of counts bound represents the number of ^125^I-Ig (lb) 2.9 bound when medium (1% BSA-PBS, pH 7.5) was used in the blocking step of the assay.
Ig-1 (γm) ALLOTYPES

The value of monoclonal antibodies in the analysis of antigenic specificities is also illustrated by five clones producing antibody against the Ig-1b allotype. These five clones [Ig-1b, 1.2, 1.7, 2.4, 2.9, and 3.1] were obtained from BALB/c mice immunized with immunoglobulins from C57BL/10 mice, as described earlier. Specificity analysis was carried out by a binding assay in which the antigen (C.BPC101 IgGm myeloma protein) was bound to flexible plastic plates. The plates were then incubated with unlabeled antibody (inhibitor), followed by 125I-labeled antibody. Each antibody was capable of blocking itself (i.e., the labeled and unlabeled antibodies competed for the same sites, as expected). However, Ig(1b)2.9 was not blocked by Ig(1b)3.1, and the binding of Ig(1b)2.9 was slightly but reproducibly enhanced by Ig(1b)2.4 (Fig. 10). These results suggest the presence of at least two antigenic specificities on Ig-1b which are not on Ig-1a. Conventional antisera distinguish only one specificity between these alleles.

The nature of the antibody-induced enhancement of binding is not understood, and is currently under investigation. We favor the idea that antibody induces a conformational change in the antigen which facilitates binding. The chain composition and reactivities of the anti-allotype clones are given in Table VI.

CONCLUSIONS

There are many advantages of monoclonal antisera over conventional antisera (1,4). Especially important are: (a) the need to purify the antigen is eliminated; (b) the specificity, titer, and yield of antibody are extremely high; (c) problems of reproducibility are eliminated.
Increasing clinical applications will be found for monoclonal antibodies in addition to their use in the research laboratory. The use of monoclonal antibody against HLA antigens will greatly add to the precision of tissue typing, and monoclonal antibodies against drugs or hormones will improve and simplify radioimmunoassay procedures.

Macromolecular antigens often possess multiple antigenic determinants. Thus, a single antigen molecule may be capable of binding simultaneously several different antibodies from a conventional antisera. In contrast, a given monoclonal antibody may be expected to interact with a single antigenic determinant. If all of a given species of surface molecule were equally accessible to antisera, this property would allow quantitation of surface molecules (20).

Moreover, the number of monoclonal antibody molecules bound per cell may be much less than that bound by conventional antisera. Directly conjugated fluorescent monoclonal antibodies have given relatively weak staining. Attention will have to be paid to ways of improving fluorescence intensity. The hapten-sandwich technique of Wofsy and colleagues (21,22) should provide the necessary amplification, although it may be anticipated that some monoclonal antibodies may have their combining site damaged by haptenation.

Precipitation would seem impossible where the antigen has only one antigenic site per molecule. Even when there are two sites (e.g., IgG, chain allotypes), only linear arrays rather than lattices would be formed. However, the anti-Ig-1b monoclonal antibodies form strong precipitation lines in Ouchterlony analysis. This phenomenon is currently being investigated. Possible explanations include (a) precipitation occurring with long chains of antigen–antibody complexes or (b) presence of multiple identical antigenic determinants in each heavy chain, reflecting origin of the domains by gene duplication.

The inability of monoclonal antibodies to bind more than one determinant has implications for functions involving cross-linking, such as capping, complement fixation, and precipitation. If the density of a cell surface antigen is low, or if it is not favorably oriented, or if it exists only as a monomer, complement fixation by monoclonal antibody may be inefficient. However, the anti-H-2 and anti-la hybrid antibodies have extremely high cytotoxic titers. Monoclonal antibodies may also be useful in resolving the question of which immunoglobulin subclasses fix complement.

The extreme specificity of monoclonal antibodies should allow much more detailed analysis of cell surface proteins than is possible with conventional antibodies. The precise correspondence between antigen and antibody should allow the dissection of molecular com-
plexes, such as histocompatibility antigens and Ia antigens. The use of allo-immunizations rather than xeno-immunizations, while restricting analysis to those molecules which are polymorphic, facilitates gene mapping, and will probably lead to the definition of many new loci.

Antibodies have been used widely to study the conformation of protein antigens (23). Monoclonal antibodies may extend the value of this approach, as illustrated in the case of the anti-IgD, allotype antibodies. Finally, the production of homogeneous antibodies to haptns and fluorophores should allow a better understanding of the antibody combining site.

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