Quantitative Separation of Antigen-Specific Murine Antibodies by Anti-Allotype Chromatography

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The use of Sepharose-conjugated murine anti-Ig^a or anti-Ig^b allo-antisera allowed the quantitative separation of immunoglobulins of the two allotypes. After fractionation of mixtures of anti-(T,G)-A- -L antisera obtained from congenic strains differing in immunoglobulin allotype, it was possible to measure the antigen-binding capacity of specific anti-(T,G)-A- -L antibodies in each allotype fraction. Analysis of artificial mixtures of immune sera obtained from homozygous Ig^a and Ig^b animals showed that this method is quantitative and internally consistent. This method of affinity chromatography was used in the analysis of specific anti-(T,G)-A- -L antisera from tetraparental mice.

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The analytical use of anti-allotype antisera has greatly facilitated the understanding of the genetics of the immunoglobulin system in several mammalian species (8). In theory it should also be possible to use these same reagents in the analysis of the genetic origin of immunoglobulin-producing B cells in cell-mixing experiments for studying the mechanism of the immune response system. There are, however, at least two reasons the application of anti-allotype techniques to this kind of experiment has not been feasible in the mouse system. [1] Attempts to obtain accurate quantitative estimation of levels of specific antibody by the use of anti-allotype precipitation have failed owing to the large experimental error inherent in the method (4). [2] Attempts to prepare murine anti-allotype immunoadsorbents, by, for example, cross-linking the sera with ethyl chloroformate, have usually failed owing to the great lability of the mouse allo-antibodies.

In this paper we report the preparation of anti-allotype affinity chromatography columns capable of quantitative separation of two different allotypes. The columns were prepared by conjugating anti-allotype antibodies to cyanogen bromide-activated Sepharose. Although this general method of conjugation has already gained widespread acceptance in biochemistry and immunology (5, 17), it has been used only infrequently for insolubilizing anti-immunoglobulin antibodies (3, 9, 16).

We have used the affinity chromatography columns preparatively to separate immune sera and artificial serum mixtures into different allotype fractions, which, since they retained full activity, could then be assayed for antigen-binding capacity. The assay system used to quantitate the levels of specific antibody of each allotype consisted of the determination of the levels of anti-(T,G)-A- -L antibody in each fraction. (T,G)-A- -L (poly-(*L*-tyrosyl, *L*-glutamyl)-poly-*D,L*-alanyl- -poly-*L*-lysine) is a branched, multichain, synthetic polypeptide antigen that provokes an immune response controlled by the murine immune response gene *Ir-1A* (13, 14). This assay method was chosen because of its great sensitivity and because it

avoided the problem of partial denaturation inherent in using radioiodinated myeloma proteins as markers (J. H. Freed, unpublished observations). Although the assay method is specialized, the method of preparative affinity chromatography on anti-allotype columns described here is of general applicability.

MATERIALS AND METHODS

Mice. Experimental mice of both sexes were taken from strains maintained in the mouse colony at Stanford: C3H/DiSn, CWB/13Hz (11), C3H.SW/SnHz, and C57BL/10Sn. Tetraparental mice were produced at Stanford by Dr. K. B. Bechtol (1, 2), using in vitro embryo aggregation of C3H/DiSn with its 'double congenic' strain CWB/13Hz.

Antisera. Anti-(T,G)-A--L antisera were prepared by the procedure of McDevitt & Sela (14). Mice were immunized with 10 μg of (T,G)-A--L 509 in complete Freund's adjuvant and were boosted with 10 μg of the same antigen in phosphate-buffered isotonic saline (PBS). Animals were bled 10 days after the second injection, and heparinized plasma was stored frozen at –20°C.

Anti-allotype antisera were produced by the *Bordetella pertussis* method of Herzenberg & Herzenberg (10). Anti-allotype antisera produced in this manner were demonstrated to have binding and precipitating activity against allotypic specificities on IgG(1), IgG(2a), and IgG(2b) (10). These are the major subclasses in which anti-(T,G)-A--L antibodies are found when a Freund's adjuvant immunization protocol is used (12).

Preparation of affinity columns. The 50% saturated ammonium sulfate (SAS) fraction from an anti-allotype serum was washed once with 50% SAS and was then redissolved in 0.1M $NaHCO_3$ so that the optical density at 280 nm for the solution was 20 units. Immediately before the coupling reaction, the pH was raised to pH 8.5–9.0 by cautious addition of 1 N NaOH.

Cyanogen bromide-activated Sepharose-4B was prepared by a procedure modified from that of Cuatrecasas et al. (6), using a Sepharose to cyanogen bromide ratio (wt/wt) of 10:1. The activated Sepharose was collected by filtration and washed rapidly, first with large amounts of water and then with 0.1M $NaHCO_3$.

A 1.0-g portion of liquid-free activated Sepharose was added for each 1.0 ml of the antiserum solution. The coupling mixture was stirred very gently overnight at 4°C. Any remaining active sites were sealed, using 1M glycine in 0.1M $NaHCO_3$ (adjusted to pH 9). After being washed, the conjugated resin was treated for 10 min with 200 μl of normal mouse serum (NMS) of the same allotype as the serum just conjugated to the column, to block any site that might irreversibly bind mouse immunoglobulin.

Buffers for affinity chromatography. All buffers contained 5×10^{-5}% pentachlorophenol; buffers 1–3 contained normal rabbit serum (NRS) as a source of carrier protein.

1. Binding and washing buffer (pH 7.6): 0.05M Tris chloride, pH 7.6, containing 3.0 g bovine serum albumin and 5.0 NRS per 100 ml solution; adjusted to pH 7.6 and centrifuged at 13,000 *g*.

2. Acid eluting buffer (pH 3.1): 0.58 ml glacial acetic acid, 0.877 g NaCl, and 1.0 ml NRS per 100 ml solution.

3. Column regenerating buffer (PBS + 1% NRS): 0.190 g $Na_2HPO_4 \cdot 7H_2O$, 0.040 g $NaH_2PO_4 \cdot H_2O$, 0.80 g NaCl, and 1.0 ml NRS per 100 ml solution.

4. Acid neutralizing buffer: 67 ml saturated Na_2HPO_4 and 33 ml PBS.

Affinity chromatography. The use of an anti-Ig^a column (bed volume, 1.0 ml) is given as an example: The column was prewashed with buffer 2 and then with buffer 1. A 10-μl aliquot of serum-containing Ig^a and Ig^b immunoglobulins was added to the column, which initially was run slowly to allow binding and then at the rate of 3–6 ml/hr. The first four 2.0-ml fractions, collected using buffer 1, contained the *b*-allotype immunoglobulins. The next four fractions, eluted with buffer 2 and containing *a*-allotype immunoglobulins, were collected into tubes containing 2.0 ml of buffer 4. The column was regenerated immediately, using 8 ml of buffer 3.

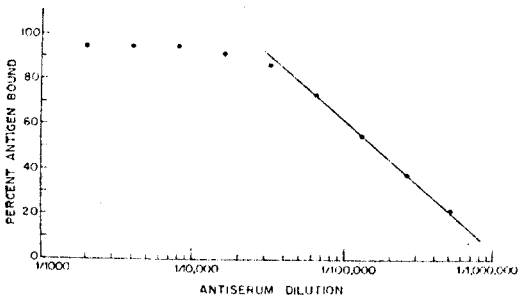

Fig. 1. Standard titration curve for the (T,G)-A--L-binding capacity of a known mixture of immune sera from C3H.SW (Ig^a) and CWB (Ig^b) mice. The sera were titered separately and mixed so that, of the titer shown, 23% is due to the C3H.SW serum and 77% is due to the CWB. An identical aliquot of this mixture was fractionated on an anti-Ig^a affinity column. The Ig^a and Ig^b fractions were assayed for (T,G)-A--L-binding capacity over six- to eight-fold serial dilutions. By referring the binding capacities obtained to the linear portion of the standard titration curve, it was possible to calculate that 25% of the recovered (T,G)-A--L-binding activity was in the fractions bound by the column (Ig^a) and 75% was in the fractions washed through the column (Ig^b).

Analysis of fractions for (T,G)-A--L-binding capacity. The modified Farr assay described by McDevitt & Tyan (15) was used to analyze the anti-(T,G)-A--L-binding capacity of the column fractions. The assay was carried out as follows: ^{125}I-labeled (T,G)-A--L 509 was used at a 0.01-μg/ml concentration, and the rabbit anti-mouse immunoglobulin (RAMIg) was used at a concentration that had been shown to give maximal precipitation of anti-(T,G)-A--L antibody at a 1:1,000 serum dilution. All dilutions of antibody (column fractions and the standard) were made in 1:1,000 NMS in order to maintain equivalence with RAMIg.

The (T,G)-A--L-binding capacities of the first three fractions eluted with buffer 1 and buffer 2 were determined, using serial twofold dilutions over a six- to eight-fold range. These binding capacities were used to determine an equivalent anti-(T,G)-A--L antiserum concentration for each fraction by comparison to the standard titration curve (see Fig. 1). The equivalent antiserum concentrations were used to calculate the absolute amount of anti-(T,G)-A--L antiserum (in μl) in each fraction.

These values were added to determine the total amount of anti-(T,G)-A--L activity recovered from the columns and to calculate the percentages of *b*-allotype (not bound) and *a*-allotype (bound and eluted) anti-(T,G)-A--L activity recovered.

RESULTS

Specificity of binding

To test for specificity and completeness of binding, 10-μl aliquots of high-titered anti-(T,G)-A--L antisera obtained from primed and boosted C3H.SW (Ig^a) and CWB (Ig^b) mice were passed through 1.0-ml bed volume anti-Ig^a and anti-Ig^b columns. Each fraction obtained from the columns was assayed for (T,G)-A--L-binding capacity as described in Materials and Methods (Fig. 1). As the results

Table I. Analysis of anti (T,G)-A--L antisera on anti- Ig^a and anti-Ig^b affinity chromatography columns

Antiserum	Allotype	Anti-Ig^a column			Anti-Ig^b column		
		% Ig^a*	% Ig^b*	% Recovery**	% Ig^a*	% Ig^b*	% Recovery**
C3H.SW	*a*	99	1	83	85	15	98
		98	2	81			
CWB/13	*b*	12	88	104	25	75	72
		10	90	110			
C57BL/10	*b*	10	90	93			

* % Ig^a and % Ig^b values are percentages of the recovered (T,G)-A--L binding activity.
** % Recovery is the anti-(T,G)-A--L serum equivalent (in μl) recovered from the column divided by the amount of anti-(T,G)-A--L serum applied to the column (10 μl) and expressed as a percentage.

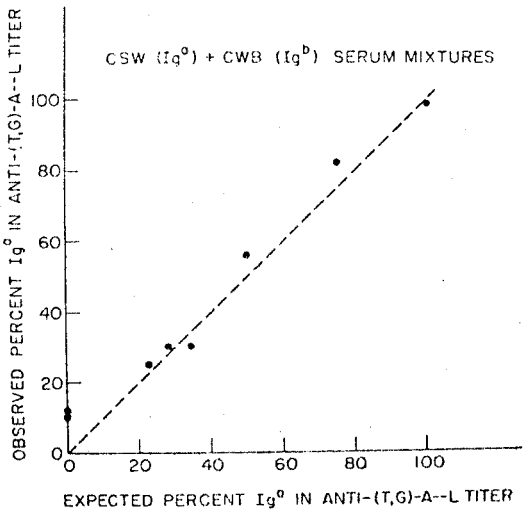

Fig. 2. Separation of artificial mixtures of C3H.SW and CWB anti-(T,G)-A--L antisera on the anti-Ig^a affinity chromatography column. Each point represents a fractionation and quantitative assay of a known allotype mixture of anti-(T,G)-A--L antisera. The dotted line indicates the theoretical curve for perfect separation at all allotype compositions.

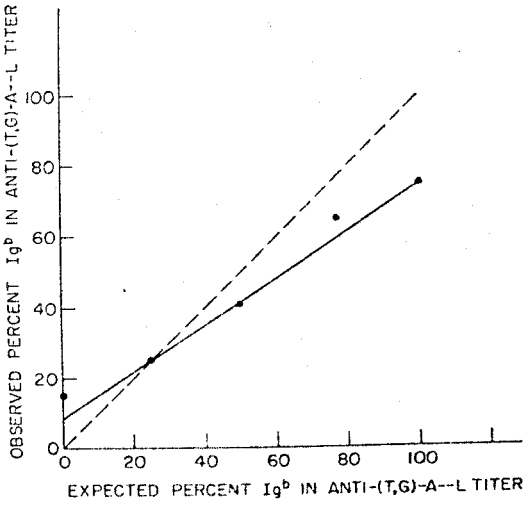

Fig. 3. Separation of artificial mixtures of C3H.SW and CWB anti-(T,G)-A--L antisera on the anti-Ig^b affinity chromatography column. The dotted line indicates the theoretical curve for perfect separation at all allotype compositions. The solid line is the correlation line used to correct the data obtained from analysis of the tetraparental mouse sera on the anti-Ig^b column.

in Table I show, the anti-Ig^a column quantitatively bound the specific anti-(T,G)-A--L antibodies from 10 μl of C3H.SW (Ig^a) serum while allowing 90% of the globulins from CWB (Ig^b) serum to pass through. Prewashing the column with Ig^b NMS did not decrease this binding nor did running the CWB anti-(T,G)-A--L antiserum admixed with 20 μl of Ig^b NMS. The anti-Ig^b column was less specific: only 75% of CWB (Ig^b) serum was bound, whereas 15% of the C3H.SW (Ig^a) was bound nonspecifically.

Quantitative separation of artificial mixtures of sera

Each of the columns was used to separate artifical mixtures of C3H.SW and CWB anti-(T,G)-A--L antisera. (The two sera were mixed in ratios based on their (T,G)-A--L-binding capacity and not by volume. This was necessary because antibody concentrations in various fractions were determined by (T,G)-A--L-binding capacity and not by the number of micrograms of antibody present.) The results for the anti-Ig^a column are presented in Fig. 2. As the data show, the anti-Ig^a column gives nearly quantitative and specific separation for all allotype compositions except those under 20% Ig^a.

The data for the anti-Ig^b column are given in Fig. 3. Since the experimental points deviate somewhat from the line indicating perfect separation, a correlation line was drawn through the experimental points to allow extrapolation. In the following experiments, the data from the anti-Ig^b column have been corrected using this correlation line.

Analysis of antisera from tetraparental mice

Both the anti-Ig^a and anti-Ig^b columns were used to determine the allotype composition of antiserum samples obtained from C3H $\longleftrightarrow$ CWB tetraparental mice (1, 2) immunized with 10 μg (T,G)-A--L in complete Freund's adju-

Table II. Analysis of selected tetraparental mouse anti-(T,G)-A--L antisera on anti-Ig^a and anti-Ig^b affinity chromatography columns

Tetraparental mouse no.	Anti-Ig^a column*			Anti-Ig^b column		
	% Ig^a	% Ig^b	% Recovery	% Ig^a	% Ig^b	% Recovery
424	27	73	100	35	65	88
423	36	64	96	22	88	92
405	33	67	96	33	67	76
1977	100	0	76	100	0	78

* The data reported for the anti-Ig^a column have appeared in a preliminary report (7). For explanation of % Ig and % Recovery, see Table I.

vant. Table II lists data from four representative examples. As these data show, both anti-Ig^a and anti-Ig^b columns give approximately the same allotype composition for the anti-(T,G)-A--L antibodies in each of the sera tested. This suggests that the method of allotype separation is internally consistent and that there are not large amounts of anti-(T,G)-A--L antibodies, such as IgM, that are not bound by either column.

DISCUSSION

The data presented in this paper demonstrate the use of affinity chromatography with mouse anti-allotype sera in the quantitative separation of specific antibodies according to allotype. Although only a single application of this method is explored in this paper, the method is obviously of general application in the separation of serum mixtures containing immunoglobulins with different allotypes.

Recently, three groups have reported the use of Sepharose-insolubilized antibodies to study immunoglobulins in other species. Bennich & Johansson (3) have used a *class*-specific anti-human IgE affinity column to concentrate IgE from serum. Wang et al. (16) have used an insolubilized anti-light chain column to remove undigested IgM contaminants from $(Fc)_{5\mu}$ fragments produced by trypsin digestion. Gottlieb et al. (9) have used rabbit anti-allotype and anti-idiotype antisera insolubilized on Sepharose to measure the serum levels of allotypic or idiotypic specificities by radioimmunoassay. These papers suggest further application of the murine anti-allotype conjugated Sepharose, namely concentration or removal of minor allotype components in sera from chimeras or allotype heterozygotes, and analytical determination of allotype levels in serum or serum mixtures.

One of the major advantages of using Sepharose-conjugated anti-allotype sera is that the columns may be reused several times before there is a significant loss of binding activity. Although mouse allo-antibodies are relatively fragile molecules and normally do not survive processes such as cross-linking with ethyl chloroformate (L. A. Herzenberg, unpublished observation), they can usually be coupled to cyanogen bromide-activated Sepharose with no loss of activity. Once they are coupled, the antibodies appear to be stabilized by the Sepharose matrix, and the columns may be kept for several weeks even at room temperature. The slight loss of activity as the column is used repeatedly may be corrected for by occasional calibration with standard serum mixtures.

Two minor disadvantages are associated with anti-allotype affinity chromatography. Obtaining a high recovery of antibody from the col-

umns requires the presence of a protein carrier in the binding and eluting buffers; it is, however, usually possible to choose this carrier protein such that its presence will not affect later experiments. The second problem is the apparent loss of specificity of the anti-Ig^b antiserum when coupled to Sepharose. It is not known whether this loss of specificity is characteristic of this particular antiserum or whether it is a more general phenomenon associated with anti-Ig^b antisera. However, once an anti-Ig^b column is calibrated, this nonspecificity can be compensated for by using a correction curve similar to that shown in Fig. 3.

The anti-allotype affinity chromatography system allows preparative separation of antiserum mixtures into allotype fractions. Since the antibodies are eluted from the column in fully active form, they can be assayed for antigen-binding capacity. The data in Table II show the application of the technique to the analysis of specific anti-(T,G)-A--L antibodies obtained from tetraparental mice. In earlier experiments by Cheseboro et al. (4), anti-(T,G)-A--L sera from radiation chimeras and tetraparental mice were first treated with anti-allotype sera, and the remaining anti-(T,G)-A--L titer was determined and was assumed to be due to antibodies of the other allotype. Owing to technical problems, this indirect method failed to detect accurately less than 25% of either allotype in the serum. Clearly, the affinity chromatography method, which has the capability of accurately detecting Ig^a relative concentrations between 10% and 100% (*cf.* tetraparental mouse 1197 in Table II), offers a considerable increase in sensitivity over the earlier method.

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