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Chapter 40
Complement fixation by monoclonal antibody–antigen complexes

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The binding of antigen by immunoglobulin is the most readily recognized feature of the immune system [1], but unless such binding leads to further cellular responses [2], little in the way of host defence will be mediated. The so-called 'secondary functions' of immunoglobulins (including opsonization, antibody-dependent cellular cytotoxicity, mediation of mast cell degranulation, and generation of chemotactic factors) are equally important aspects of the immune response, of which antigen binding is only the first step [3]. The integration of antigen binding with these various cellular immune functions is accomplished by the immunoglobulin constant (F_c) region. Thus the F_c domains act as an interface between the process of antigen binding and various effector functions of the immune system.

Probably the most characterized effector function of immunoglobulins is the triggering of the complement cascade [4]. Complement consists of a group of nine proteins which, upon triggering by antigen–antibody complexes, can produce a variety of results including the production of chemotactic factors, cell lysis and viral inactivation. C1q, the first component of complement, interacts with the C_H2 domain of mouse IgG_{2a} and IgG_{2b} [5] (and possibly of IgM; [6]) and, on formation of immune complexes, initiates the sequence of complement reactions. Initial events include enzymatic steps so that considerable amplification of the response takes place, hence the term 'cascade' [7].

The precise mechanism whereby the binding of antigen triggers this system remains unclear and controversial. One of two major theories holds that simple formation of antibody aggregates generates an assemblage of F_c regions requisite for initiation of the cascade [8]. The other theory postulates a 'conformational change' induced in the immunoglobulin that takes place on binding of antigen which acts as a trigger for the cascade [9]. As is often the case with such controversies, there is probably some truth to both theories.

Considerable progress has been made in understanding the molecular processes involved in the interaction of antibody and antigen since the advent of the monoclonal antibody technique pioneered by Köhler & Milstein [10]. The use of well defined antigens together with a single antibody species has led to precise molecular models of the antibody combining site [11]. In a similar manner, application of complement technology previously employed with heterogeneous mixtures of immunoglobulins has demonstrated that, for example, a mouse isotype previously considered non-fixing (IgG1) [12] is really heterogeneous with respect to complement fixation [13–15]. It seems likely that further work with well defined antigens and monoclonal antibodies will finally pinpoint the molecular events involved in the triggering of complement fixation.

Methods for the study of complement fixation

Methods for the study of complement fixation by antibody that have been developed over the past 40 years have been refined considerably in the past few years. Two basic assays for the study of complement fixation are the direct lysis of antibody-coated targets [16] and an indirect complement consumption assay based on the lysis of antibody-coated erythrocytes by residual complement [17]. Both assays are commonly carried out using erythrocytes as targets, which permits quantification by measuring the OD of the supernatant at the Soret absorption maxima of the released haemoglobin. More recently, radioactive chromium release has supplanted haemoglobin release as the method of choice for monitoring the degree of erythrocyte lysis (see below).

In the direct assay, antigen (or hapten) is covalently attached to the target erythrocytes by any one of

several methods. Small haptens can often be purchased as 'reactive' derivatives, as, for example, TNP groups can be introduced by reaction of erythrocytes with trinitrobenzene sulphonate [17]. Larger antigens, such as protein determinants, are conveniently attached by the chromic chloride coupling procedure [19]. Dilutions of antibody (or antisera) are incubated with the target cells for a standard length of time (typically 15–45 min) and then a titred amount of complement is added (again for a set time). Immunoglobulin bound to the cell surface will bind C1q and trigger the complement cascade leading to lysis of the erythrocyte.

In the indirect (or complement consumption) assay, dilutions of antigen are mixed with a standard amount of antibody and incubated in the presence of a set amount of complement for a specified interval (typically 30–60 min). At the end of this incubation, erythrocytes that have been previously coated with IgG anti-erythrocyte antibody are added. During this incubation, the complement not yet consumed by the antigen–antibody complexes will lyse some fraction of the coated erythrocytes.

Both of these assays have benefited from miniaturization and automation made possible by using erythrocytes loaded with radioactive material, typically ^{51}Cr [20]. Reduction in the amounts of reagents required is due to the greater sensitivity of radioactive release compared with haemoglobin optical absorption. The use of radioactivity also permits the use of automatic gamma counters that can count hundreds of samples unattended. These improvements mean that all determinations can be performed in duplicate, triplicate, or more; in addition, more controls and more intermediate points in fixation curves can be determined. This generally results in greater precision in the study of complement fixation.

In all cases, a source of complement must be provided and this has traditionally come from guinea-pig serum, a very rich source of complement. However, complement can be derived from rabbit, human or even mouse serum and results may vary somewhat from source to source. To study most accurately the complement fixation that occurs *in vivo* with mouse antibodies, perhaps one should at least compare results obtained using guinea-pig complement with results obtained using mouse complement. A significant point to keep in mind if such studies are undertaken is the variation in complement levels found in different inbred mouse strains.

Direct lysis method: sensitized (labelled) erythrocytes (^{51}Cr)

Labelling sheep red blood cells

Equipment

Centrifuge (CU-5000, IEC).
37 °C water bath (D3, Haake).

Materials

Sheep erythrocytes (Flow Labs).
2,4-Dinitrofluorobenzene (DNFB) (Aldrich Chem. Co.).

Procedure

1 Wash SRBC (0.5 ml packed cells) with gelatin-containing HEPES-buffered saline (gel-HBS) three times.
2 Suspend cells in 10 ml borate buffer.
3 Dissolve 1 mg dinitrofluorobenzene in 0.1 ml acetone stock: 13 μl per 2 ml.
4 Add DNFB solution dropwise with stirring; incubate for 15 min at 37 °C.
5 Wash cells five times with gel-HBS to remove traces of hapten.

Notes and recommendations

Cells are not stable and should be used the same day as preparation. Less DNFB may yield more stable preparations. Use caution when handling DNFB; wear gloves.

Loading cells with chromium

Equipment

Centrifuge (CU-5000, IEC).
37 °C water bath (D3, Haake).
Visible spectrophotometer (PM6, Zeiss).

Materials

Sheep erythrocytes (Flow Labs).
^{51}Cr (New England Nuclear).

Procedure

1 Wash 2 ml suspension of DNP–SRBC with chilled gel-HBS three times.
2 Make 10% suspension (0.2 packed cells to 2 ml).
3 Add 200 μl of ^{51}Cr solution (200 μCi); incubate for 1 h at 37 °C.

4 Wash three times with gel-HBS at 4 °C.
5 Resuspend in 7 ml gel-HBS.
6 Lyse 0.2 ml of suspension into 2.8 ml of 0.1% carbonate (in a cuvette).
7 Measure OD at 541 nm; adjust to 2% suspension by the following formula:

$$\text{Final vol.} = \{OD(541)/0.34\}\ 6.8\ (\text{ml})$$

Notes and recommendations

Use standard radioactive handling and disposal procedures when working with ^{51}Cr.

Assay description

Equipment

Centrifuge (CU-5000, IEC) with plate carriers (Cooke Microtiter System, Dynatech Labs Inc., Alexandria, VA).
Pipettors (Pipettman, Gilson).
Gamma counter with ^{51}Cr window.

Materials

96-Well PVC microtitre plates (Dynatech).

Procedure

1 Twenty-five microlitre aliquots of labelled cell suspension are aliquoted into wells of a 96-well microtitre plate.
2 Twenty-five microlitre dilutions of antibody are added and the plate is incubated for 45 min at 37 °C.
3 At the end of the 45 min incubation, 25 μl aliquots of a 1/20 dilution of guinea-pig serum (exact dilution determined previously by titration) are added.
4 Controls include cells with complement alone, cells with buffer alone, and cells with distilled water to serve as a 100% lysis standard.
5 At the end of a second 45 min incubation at 37 °C the plate is centrifuged (using standard microtitre centrifuge plate holders) for 10 min at 2000 rev./min in a refrigerated centrifuge (4 °C).
6 Fifty microlitre aliquots of supernatant from each well are counted on a gamma counter set to ^{51}Cr.

Notes and recommendations

Use appropriate radioactive procedures for disposing of the used plate and supernatants.

Indirect assay method: complement depletion assay

Load and activate erythrocytes

Equipment

Centrifuge (CU-5000, IEC).
Visible spectrophotometer (PM6, Zeiss).

Materials

Sheep erythrocytes (Flow Labs).
^{51}Cr (New England Nuclear).
Haemolysin solution (Flow Labs).

Procedure

1 Wash 2 ml of SRBC suspension with chilled gel-HBS three times.
2 Make a 10% suspension (0.2 ml packed cells to 2 ml).
3 Add 200 μl of ^{51}Cr solution (200 μCi).
4 Incubate for 1 h at 37 °C; then wash three times with gel-HBS at 4 °C.
5 Make 5% cell suspension; add an equal volume of 1:200 diluted haemolysin solution.
6 Incubate for 0.5 h at 37 °C; then incubate for 0.5 h at 0 °C.
7 Wash three times with gel-HBS and resuspend in gel-HBS to yield a 3% suspension (7 ml).
8 Lyse 0.2 ml of suspension into 2.8 ml of 0.1% carbonate solution (in a cuvette).
9 Measure OD at 541 nm; adjust to 2% suspension by the following formula:

$$\text{Final vol.} = \{OD(541)/0.34\}\ 6.8\ (\text{ml})$$

Titring guinea-pig serum

Equipment

Centrifuge with plate carriers (CU-5000, IEC).
Pipettors (Pipettman, Gilson).
Gamma counter with ^{51}Cr window (Gamma 4000, Beckman).

Materials

Guinea-pig.
Activated ^{51}Cr sheep erythrocytes.

Procedure

1 Obtain fresh guinea-pig serum by heart puncture bleeding; allow clotting to occur for 1 h on ice; remove

clot by centrifugation and store 100 μl aliquots of serum at -70 °C.

2 Make dilutions (in gel-HBS) of an aliquot of freshly thawed guinea-pig serum (on ice) in wells of a microtitre plate; total volume of 75 μl; typically 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 35, 40, 45, 50 μl of 1 : 50 diluted serum is a sufficient series; carry out in triplicate.

3 Incubate for 45 min at 37 °C.

4 Add 50 μl of the 2% ^{51}Cr loaded, activated erythrocyte solution to each well and incubate for a further 45 min at 37 °C.

5 Pellet the non-lysed erythrocytes by centrifuging the plate; count 50 μl aliquots of supernatant on a gamma counter set for ^{51}Cr.

6 Tabulate the chromium counts for all samples; calculate the fractional lysis (= y) for each dilution; compute the quantity $\log\{y/(1-y)\}$ for each dilution; plot these values versus the log(dilution); the intercept is the 50% lysis (= one C_H50 unit) point; typical data are plotted in Fig. 40.1.

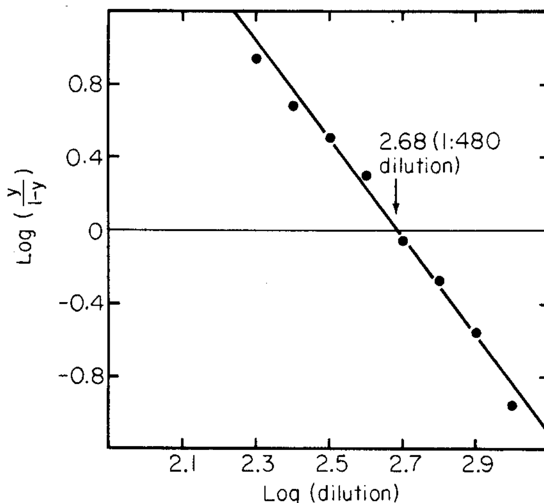

Fig. 40.1. Titration plot of guinea-pig complement to determine C_H50 units.

7 From the plot, determine the amount of serum necessary to cause 50% lysis of the erythrocytes; this is one C_H50 unit; twice this amount is used in the standard complement consumption assay.

Notes and recommendations

Complement is very labile; accordingly, clotting should take place only on ice, and serum should be aliquoted and frozen at -70 °C immediately.

Assay description

Equipment

Centrifuge (CU-5000, IEC) with plate carriers (Cooke Microtiter Systems, Dynatech Labs Inc., Alexandria, VA).
Pipettors (Pipettman, Gilson).
Gamma counter with ^{51}Cr window (Gamma 4000, Beckman).

Materials

96-well microtitre plate, activated ^{51}Cr sheep erythrocytes, titred guinea-pig serum.

Procedure

1 Twenty-five microlitre antibody aliquots (10 μg per 25 μl) are added to wells of a 96-well microtitre plate.

2 Dilutions of antigen in 25 μl are added next.

3 Finally, guinea-pig complement (two C_H50 units) in 25 μl aliquots is added and the plate incubated at 37 °C for 45 min.

4 Controls on the plate include: antigen dilutions with complement; antibody with complement (no antigen); complement alone; buffer alone; water alone to act as a 100% lysis standard. A typical assay is diagrammed in Fig. 40.2.

5 After the 45 min incubation period, 50 μl aliquots of a solution of 2% activated ^{51}Cr loaded sheep erythrocytes are added to each well and the plate is incubated for a further 45 min at 37 °C.

6 At the end of the second incubation, the plate is centrifuged (using standard microtitre centrifuge plate holders) for 10 min at 2200 rev./min in a refrigerated centrifuge (4 °C).

7 Fifty microlitre aliquots of supernatant are withdrawn and counted on a gamma counter set to ^{51}Cr.

Notes and recommendations

For maximal reproducibility, complement should be thawed immediately before use and then diluted into ice-cold buffer. It should be added to all wells as quickly as possible; then the plate should be rapidly brought to 37 °C.

Complement fixation materials: antibodies and antigens

The following section describes the materials essential to complement analysis: antibodies and antigens. Monoclonal antibodies specific for small defined haptens permit close control of the types of molecular

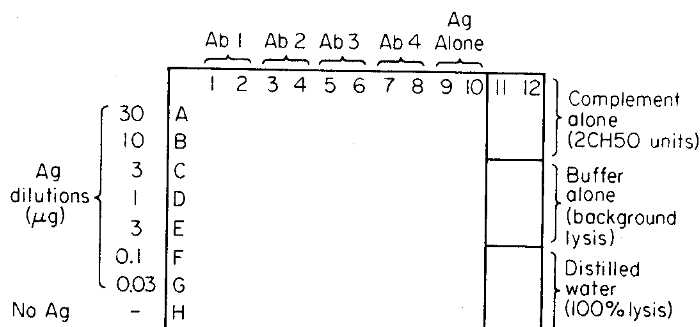

Fig. 40.2. Typical plate layout for standard complement fixation assay.

complexes generated on combining antibody with antigen. In particular, it is possible to generate monovalent antigens by employing certain proteins, and such antigens permit further dissection of the relationship of binding and/or aggregation to complement fixation.

Monoclonal antibodies

One of the greatest recent advances in the study of the antibody combining site has been the development of methods for preparing antigen-specific monoclonal antibodies by somatic cell hybridization. Large amounts of homogeneous antibody of predetermined specificity can be isolated with relative ease [21]. In like manner, such antibodies also provide very useful material for the study of antibody effector functions, such as complement fixation. For example, it is possible to compare groups of monoclonal antibodies of different isotypes that bind the same hapten to carefully assess variation in complement fixing ability with isotype.

Furthermore, using such monoclonal secreting cell lines it is possible to isolate rare variants that express the same V_H region, but different C_H regions [22]. Several such IgC_H switch 'families' have been isolated [23] and it appears that this method can be used to generate variants of any hybridoma cell line (see Chapter 109 in this handbook). Antibodies isolated from these cell lines provide very useful tools for the investigation of structure–function relationships and antibody effector functions [24].

In order to prepare antigen–antibody complexes of well-defined sizes and compositions, it is often convenient to work with antibodies that bind small haptens [15]. In this way, the hapten can be attached to proteins (or cells) at characterized levels of substitution and this variable can be controlled (or investigated). In particular, large proteins with just a single antigenic determinant can be prepared and used to study questions of antibody cross-linking and its relationship to complement fixation.

Defined antigens

Antigens consisting of small haptens covalently linked to large protein carrier molecules are very useful in the study of complement fixation. Certain haptens, such as DNP, have the added advantage of a large visible absorption [25] which permits rapid determination of the degree of substitution of the carrier protein by hapten. In addition, there exist certain proteins with only one reactive determinant [26] which makes production of monosubstituted antigens relatively straightforward.

Method: labelling proteins with haptens

Equipment

Rocker ('Labquake', Labindustries, Berkeley, CA).
UV-visible spectrophotometer (PM6, Zeiss).
Pipettors (Pipettman, Gilson).
Milligram balance (Mettler, Zurich).

Materials

2,4,6-Trinitrobenzene sulphonic acid (Aldrich Chem. Co.).
Dimethylformamide (Kodak).
Desalting column (PD-10, Pharmacia).

Procedure

Protein at 20 mg/ml in coupling buffer.
1a Add 1 mg 2,4,6-trinitrobenzene sulphonic acid per 25 mg protein (use 10 mg/ml stock in dimethylformamide), or
1b Add 25 mg 2,4-dinitrobenzene sulphonic acid per

25 mg protein (use 100 mg/ml stock in dimethylformamide), or

1c Add 25 mg dansyl chloride per 25 mg protein (use 100 mg/ml stock in dimethylformamide).

2 Rock for about 2 h at room temperature: length of time determines degree of substitution; protect from light.

3 Remove hapten with a desalting column (i.e. Pharmacia PD-10 column; apply maximum of 2 ml per column).

4 Dialyse against Tris/saline overnight at 4 °C; centrifuge at 10 000 rev./min to remove any precipitate; sterile filter; store at 4 °C in the dark.

5 Measure OD at 280 nm and 340 nm (TNP or DNS) or 360 nm (DNP) of a suitable dilution.

Corrections: TNP absorbs at 280 nm (34% of 340 OD)
DNP absorbs at 280 nm (32% of 360 OD)
DNS absorbs at 280 nm (45% of 340 OD)

Absorption constants:
Ig OD (0.1%) at 280 nm = 1.4
BSA OD (0.1%) at 280 nm = 0.7

Extinction coefficients: TNP ε (340) = 12 500
DNP ε (360) = 17 400
DNS ε (340) = 3400

Molecular weights: Ig 150 000
BSA 68 000

Notes and recommendations

Derivatization may reduce the solubility of proteins (especially at lower pH). For example, DNP-bovine IgG is soluble beyond 0.1 mg/ml only in low ionic strength buffers (0.01 M-phosphate, pH 7.4).

Production of monosubstituted antigen

Ribonuclease

Ribonuclease is a useful protein for preparing monosubstituted antigens because it contains a single cysteine group [26]. Therefore any reagent capable of specifically reacting with the sulphydryl group will label this protein in a single site.

Lima bean trypsin inhibitor

Lima bean trypsin inhibitor (LBTI) is a small protein (9 kDa) which contains a single tyrosine residue [27]. Thus labelling reagents capable of reacting specifically with tyrosine will yield monosubstituted antigens with this protein. Probably the simplest general tyrosine labelling technique is nitration with tetranitromethane to yield mononitrotyrosine [28], followed by reduction with sodium dithionite to yield monoaminotyrosine [29]. The pK of this aromatic amino group is much lower than any other amino group in proteins so that at low pH it can become the unique target for nucleophilic labelling reagents (such as fluorodinitrobenzene, FDNB) [30].

Method: production of DNP_1-LBTI antigen

Equipment

Fraction collector (FC-80K, Gilson).
UV monitor (UV-1, Pharmacia).
Gradient former (GM-1, Pharmacia).
UV-visible spectrophotometer (PM6, Zeiss).

Materials

Lima bean extract (Sigma).
Tetranitromethane (Aldrich Chem. Co.).
Sodium dithionite (Baker Chem. Co.).
1-Fluoro-2,4-dinitrobenzene (Aldrich Chem. Co.).
Disposable desalting columns (PD-10, Pharmacia).

Procedure

1 Prepare lima bean trypsin inhibitor from crude lima bean extract by gel filtration followed by ion-exchange chromatography [27].

2 Label purified LBTI with a fivefold molar excess of tetranitromethane for 5 h at room temperature; isolate the labelled protein on a desalting column.

3 Reduce the nitrated protein with a tenfold molar excess of sodium dithionite in 50 mM-Tris buffer (pH 8.0) for 1 h at room temperature; reduction can be followed by disappearance of yellow nitro group (428 nm); isolate the reduced protein on a desalting column.

4 Label the aminotyrosyl LBTI with a fivefold excess of fluorodinitrobenzene in 0.05 M-acetate buffer (pH 4.5) for 12 h at room temperature; isolate DNP-labelled protein on a desalting column.

5 Quantify the degree of labelling by measuring the OD at 360 nm (molar extinction coefficient for DNP is approximately 17 400).

6 BSA can be labelled similarly (with somewhat less assurance of only one DNP per protein) by carrying out the initial nitration with a twofold excess of tetranitromethane. One tyrosine on BSA is considerably more reactive than any of the rest.

Analysis of antibody–antigen complexes by HPLC

One of the most powerful tools to emerge recently for the analysis of antigen–antibody complexes is size permeation chromatography on the high-performance liquid chromatograph (HPLC) [31]. Columns are now

commercially available with separation ranges in the 100–2 000 kDa range which make them quite suitable for the analysis of soluble immune complexes. Most HPLC set-ups include a very sensitive absorbance detector for monitoring the column effluent so this should be adjusted to monitor at 280 nm. Much greater sensitivity (about tenfold) can be obtained by monitoring at 218 nm, but great care must be taken with the purity of buffer constituents at this wavelength. In addition, certain buffer constituents (i.e. Tris) absorb at this wavelength.

Probably the most difficult problem encountered with HPLC analysis of antibodies and soluble immune complexes is clogging of the column, which is usually irreversible and expensive. This can be avoided by very careful pre-treatment of all samples to remove precipitated material. This is conveniently carried out either by filtration through a 0.2 μm membrane or by high-speed centrifugation. Set-ups for filtration of very small volumes (as low as 30 μl) are commercially available. Centrifugation works well for volumes in the hundreds of microlitres and greater.

An experiment to determine the size and composition of the soluble complexes formed on mixing divalent hapten with monoclonal antibody is readily carried out by HPLC analysis. Similar studies utilizing fractionated rabbit antibodies have been carried out previously using ultracentrifugation analysis [32–34]. In addition, because of the rapidity of the analysis (typically a sample takes 30 min) many variations of the initial mixture can be analysed. The HPLC is capable of isolating hundreds of micrograms of pure material, so the activity of various complexes can also be determined in functional assays.

Finally, HPLC can be used to assess the degree of binding to monosubstituted antigens, i.e. antibody mixed with hapten monosubstituted antigen will form soluble complexes of additive molecular weight. The combination of IgG with haptenated BSA is very informative as BSA (68 kDa), IgG (150 kDa) and the complex (around 300 kDa) are easily resolvable by available HPLC size-fractionation columns. Finally, if the hapten has an optical absorption distinct from the usual protein absorption (e.g. DNP at 360 nm), then dual monitors (at 280 and 360) can be used to determine the actual composition of complex mixtures of antigen and antibody.

Method: HPLC analysis of antibody complexes

Equipment

HPLC apparatus (injector, pump, UV monitor, Waters, Millipore Corp.).
Size-fractionation column (TSK-400, Bio-Rad, Richmond, CA).
Microcentrifuge (Eppendorf).

Materials

0.4 μm membrane filters.

Procedure

1 Wash the column with 2–4 column volumes of buffer or until the baseline of the effluent monitor stabilizes; depending on the sensitivity setting (high for very dilute samples) this may take more or less washing.
2 Mix the antibody with hapten (or monovalent antigen).
3 Remove particulates from a 30 to 100 μl aliquot either by centrifugation or by filtration.
4 Inject the sample; collect peaks if desired.
5 Continue washing until baseline of monitor returns to zero.

Notes and recommendations

A major problem with these columns is the relative ease of clogging should particulates be introduced. Accordingly, all samples must be free of particulates.

Applications to immunology

The complement fixing isotypes in mouse were initially determined by observing which aggregated myeloma proteins fixed complement [35]. Proteins were aggregated either chemically (by cross-linking) or physically (by heat aggregation). This means that only very strong fixation could be scored as positive and only a limited number of myeloma proteins could be examined. Such studies found that IgM, IgG2a and IgG2b fixed complement whereas IgG1 and IgE did not.

Such studies were always open to questions of *in vivo* appropriateness, since in the real complement cascade the initial trigger is the interaction of antibody with antigen and not simply aggregation of antibody. With the advent of large numbers of monoclonal antibodies of known specificity the variation of fixation with isotype was essentially confirmed. Still, some question remained of precise quantitative variation with isotype since, for example, a panel of DNP-specific immunoglobulins could include antibodies of very different affinities and possibly even some variation in their constant region sequences.

Application: complement fixation with different isotypes

The variation of fixation with isotype has been definitively answered by the use of 'heavy chain switch variant' hybridoma proteins which have different constant regions associated with the same combining site. Rare cells in the bulk culture of a 'parental' IgG1 anti-dansyl (DNS) hybridoma that express a different isotype on their surface can be isolated by the fluorescence activated cell sorter (FACS). When these cells are cloned and immunoglobulin isolated from their supernatant, this protein retains the same anti-DNS specificity in conjunction with a different (selected) isotype. This method is described in detail elsewhere [23 and Chapter 109 in this handbook]. Such proteins provide a unique system for the study of the relationship between isotype and immunoglobulin effector functions.

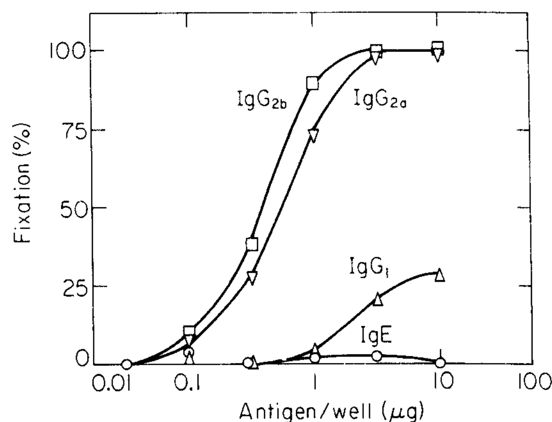

Fig. 40.3. Complement fixation as a function of isotype with the anti-dansyl variant family. The association of an identical combining site with different constant region isotypes demonstrates clearly the order of efficiency of complement fixation in the mouse.

Table 40.1. Complement fixation for anti-dansyl isotype variants

		Degree of lysis (supernatant c.p.m.)				
Ag	Dilution (μg)	No antibody	IgG2b	IgG2a	IgG1	IgE
1	30	17349	5665	4943	17434	17135
2	10	18137	15	221	13385	16953
3	3	18356	76	159	13034	16274
4	1	18025	165	451	14619	15948
5	0.3	17890	1920	4944	17979	18392
6	0.1	18784	11311	12952	18260	18268
7	0.03	18325	16701	16762	18400	18027

Water alone	100% Lysis	20273
Buffer alone	Background lysis	336
Complement alone	Two C_H50 units	18533
Antibody + C	Spontaneous fixation	
IgG2b		18458
IgG2a		17956
IgG1		18363
IgE		18583

Following such a procedure, a 'family' of IgCH variant anti-DNA antibodies has been constructed which includes IgG1, IgG2b, IgG2a and IgE. Dansyl–BSA was produced by the procedure described above for labelling proteins with small haptens. Complement consumption assays were performed as described above. The protocol is presented in the next section. The results are presented in Table 40.1 and the plots are shown in Fig. 40.3.

Complement fixation as a function of isotype

Equipment

Centrifuge (CU-5000, IEC) with plate carriers (Cooke Microtiter Systems, Dynatech Labs Inc., Alexandria, VA).
Pipettors (Pipettman, Gilson).
Gamma counter with ^{51}Cr window (Gamma 4000, Beckman).

Materials

Immunoglobulins (IgG1 (27–44), IgG2b (27–35), IgG2a (27–13), IgE (27–74); antigen (DNS_{20}–BSA).
0.5 ml solutions of each of the four proteins at 400 μg/ml in gel-HBS.
0.5 ml solutions of dilutions of antigen (4 mg/ml and seven threefold dilutions).
Two millilitre complement source (appropriate dilution of titred guinea-pig serum) to give two C_H50 units per 25 μl.
Two millilitres of 2% solution of ^{51}Cr activated SRBC.

Procedure

1 Aliquot antibody solutions (25 μl each) into microtitre plate: 2 × 8 wells each; also 2 wells separated for spontaneous antibody fixation rate.
2 Aliquot antigen dilution (25 μl/each) into wells: 10 × 8 (this includes 2 × 8 with no antibody for antigen fixation control).

3 Aliquot 25 μl gel-HBS into the wells with antibody alone; also aliquot 50 μl into 4 wells for complement-only fixation rate; aliquot 75 μl into 4 wells for red cells only (spontaneous lysis control).
4 Aliquot 25 μl complement solution into all wells except for spontaneous lysis control wells.
5 Aliquot 75 μl distilled water into 4 wells for 100% lysis control.
6 Incubate for 45 min at 37°C; add 50 μl of red cell solution; incubate for a further 45 min.
7 Pellet cells by centrifuging plate in a refrigerated centrifuge equipped with plate carriers; harvest and count 50 μl aliquots of supernatant from each well on a gamma counter set for ^{51}Cr.

Variation in fixation within an isotype (IgG1)

Recently, studies with a large number of hapten-specific IgG1 hybridoma proteins have shown that certain IgG1 antibodies do fix complement [13–15], albeit at a level considerably lower than for IgG2a or IgG2b isotypes. In particular, many IgG1 antibodies can fix mouse complement to a greater extent than rabbit or guinea-pig complement [36]. This finding complicates any explanation of isotype triggering specificity based simply on the amino acid sequence of the various isotypes.

Complement consumption assays have been carried out with a series of IgG1 monoclonal antibodies that bind DNP, DNS, NIP, and eosin. The antigens were made as described above for BSA with DNS-Cl and DNP-Fl; NIP–BSA was prepared by reaction with NIP–succinimide ester (Biosearch) and eosin–BSA was prepared by reaction with eosin (Molecular Probes, Junction City, OR). Fixation curves over a wide range of antigen concentration demonstrate (Fig. 40.4) that certain IgG1 antibodies do fix complement. As noted in the previous section, the 27–44 anti-DNS IgG1 antibody also fixed at a moderate rate. Thus it is now clear that some IgG1 antibodies fix complement considerably better than others (but all much more poorly than IgG2a).

Blocking by anti-allotypes

It is established that the C_H2 domain of IgG antibodies is important in the initiation of complement fixation; indeed, the C1q binding site is in this domain [5]. The spacial organization of various surface determinants defined by monoclonal anti-allotype antibodies [37] has been extensively investigated by binding to C_H fragments and by cross-blocking experiments [38]. In order to investigate the relationship of these determinants to the C1q binding site, a complement-fixing antibody is pre-treated with one of these anti-allotype antibodies prior to the complement fixation assay. In this manner the surface determinants that are sufficiently nearby to perturb triggering of fixation can be found.

Antibodies that react with localized determinants in the C_H domains of IgG2a immunoglobulin have been produced and characterized previously [39,40]. An IgG2a monoclonal antibody specific for eosin was purified by ion-exchange chromatography (see Chapter 31 in this handbook). Eosin–OVA was prepared as described above. In the standard complement consumption assay, the anti-eosin antibody fixed complement as a typical IgG2a. Next, different anti-C_H determinant antibodies were added to the wells in the first-step incubation and complement assays were performed. Controls were included to determine complement fixation by the anti-C_H antibodies (which were all of the IgG1 class). The results show (Table 40.2) that certain anti-C_H determinant

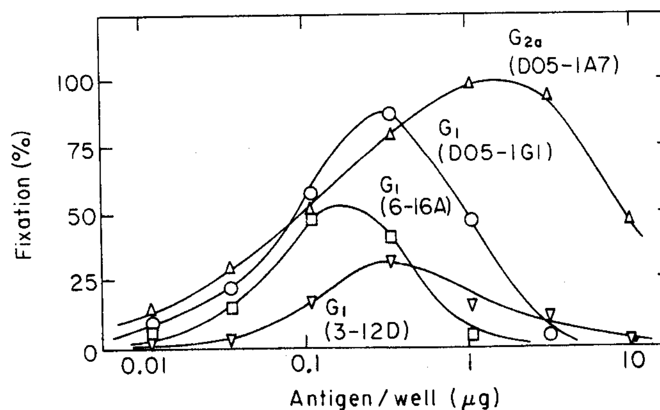

Fig. 40.4. Fixation of complement by several DNP-specific IgG1 antibodies. A typical DNP-specific IgG2a antibody is included for comparison.

Table 40.2. Blocking of fixation by anti-allotype antibodies

Antibody	Location	Effect on fixation
4.7	Hinge	Enhances
2.9	Upper C_H2	Blocks
BG1	,,	,,
1A7	Mid C_H2	Blocks
3.1	,,	No effect
19.8	Lower C_H2	Blocks
20.1	Lower C_H2	No effect
5.7	C_H3	No effect

antibodies blocked complement fixation very effectively and the determinants these antibodies recognize are all in the C_H2 domain.

Fixation by monovalent antigen after prior antibody cross-linking

The molecular events involved in the initiation of the complement cascade are still not well understood. Monoclonal antibodies specific for simple haptens provide tools for further investigation of these events, especially in conjunction with monosubstituted antigens and anti-C_H domain antibodies. It should be possible to distinguish between antigen binding and aggregation of immunoglobulin as the essential event in triggering complement fixation. The complement consumption assay described above provides a sensitive read-out system for the determination of such triggering.

The IgG2a monoclonal antibody specific for eosin mentioned above was employed in this study. Multisubstituted eosin–OVA was prepared as described above. $Eosin_1$–OVA was prepared as described above for preparation of monosubstituted antigens. An IgG1 monoclonal antibody that binds to the C_H region outside the C_H2 domain (and, therefore, does not block complement fixation) was used to aggregate the IgG2a antibody. Thus assays could be carried out either with normal or pre-aggregated anti-eosin antibody in association either with $eosin_1$–OVA or $eosin_{10}$–OVA. The results are presented in Fig. 40.5.

The results show that: (1) anti-eosin antibody did not fix complement to any extent; (2) anti-eosin antibody together with $eosin_{10}$–OVA fixed complement very efficiently; (3) anti-eosin antibody together with $eosin_1$–OVA did not fix complement to any extent; (4) anti-eosin antibody pre-aggregated with anti-C_H antibody did not spontaneously fix complement; (5) such pre-aggregated antibody still fixed complement quite well with $eosin_{10}$–OVA; and, finally, (6) pre-aggregated antibody did fix complement when combined with $eosin_1$–OVA.

Buffers and media

Gelatin HEPES-buffered saline (gel-HBS)

	1 × (per litre)	10 × (per l)
0.01 M-HEPES	2.38 g	23.8 g
0.15 M-NaCl	8.8 g	87.5 g
0.15 mM-$CaCl_2$	16.7 mg	0.167 g
0.50 mM-$MgCl_2$	102 mg	1.02 g
0.1% Gelatin	1.0 g	

Adjust pH to 7.40 with 1 M-NaOH.
Immediately prior to use dilute 10 × stock, add gelatin and adjust pH.
Dissolve gelatin in a 37–60 C water bath.
Chill prior to use in an ice bath.

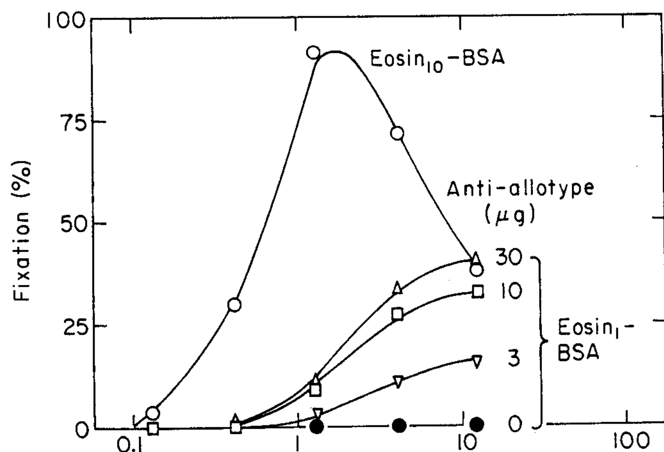

Fig. 40.5. Complement fixation by pre-aggregated antibody using monovalent antigen. IgG2a anti-eosin antibody fixes complement very efficiently with polyvalent antigen, but not at all with monosubstituted antigen. However, pre-aggregated anti-eosin antibody (aggregated by anti-allotype antibody) does fix complement upon addition of monovalent antigen. The pre-aggregated antibody does not fix complement measurably in the absence of antigen.

Borate buffer (SRBC coupling)

50 mM-$Na_2B_4O_7$.
75 mM-KCl.
Adjust pH to 9.5 with 0.3 M-KOH.

Protein coupling buffer

0.2% Potassium carbonate.

HPLC buffer

0.05 M-sodium phosphate buffer.
0.1 M-sodium sulphate.
pH 6.8.
Filter through 0.4 μm membrane prior to use on HPLC.

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