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SEPARATION AND PURIFICATION OF IMMUNOGLOBULINS M, A AND G FROM SMALL VOLUMES OF HUMAN SERA BY A CONTINUOUS, IN-LINE CHROMATOGRAPHIC PROCESS

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SUMMARY

A continuous, in-line chromatographic process for the separation of immunoglobulins M, A and G from small volumes of human sera is described. The process involves the sequential and uninterrupted application of molecular sieve, ion-exchange, immunoadsorbent-affinity and de-salting–concentrating chromatographic methodologies. From 1–3 ml of starting serum it yields 45–55%, by weight, of the three classes of immunoglobulins in 36 h. Each immunoglobulin fraction is free of contamination by immunoglobulins of the other two classes and retains full biological activity as measured by a sensitive immune lysis assay.

INTRODUCTION

Studies of human immunoglobulin A (IgA) and immunity to bacterial diseases have largely been restricted to its role on secretory surfaces (secretory IgA), where its dimeric form, coupled with secretory component and the conjoining J chain[1,2], facilitate its separation and identification. Understanding of its importance within the circulation (serum IgA), where it occurs primarily as a monomer, has been hindered by the technical difficulty of completely separating serum IgA from physicochemically similar IgG (ref. 3). The development of immunoadsorbent techniques to remove contaminating IgG and IgM has solved the problem of final purity[4], and we have recently reported that immune serum IgA, which does not activate complement, not only blocks the complement-mediated bacteriolytic activity of IgG and IgM against *Neisseria meningitidis*[4], but also may play a crucial role in the development of susceptibility to invasion by these organisms[5].

Extension of these data will require large-scale epidemiological studies of the prevalence and antigenic specificity of serum IgA. Purification of serum IgA for the initial studies, however, required three separate chromatographic steps which were cumbersome, time-consuming, poorly suited to small starting volumes of sera and which resulted in small yields of the purified final product. Therefore, we have de-

veloped a continuous, in-line chromatographic process involving sequential application of molecular sieve, ion-exchange, immunoadsorbent-affinity and de-salting–concentrating methods. The chromatographic process yields 45–55% (by weight) of the IgM, IgG and IgA from 1–3 ml of serum in 36 h, each free of contamination by immunoglobulins of the other classes, and without loss or alteration of biological activity. The theoretical basis and technical details of the procedure are reported below.

THEORETICAL

The mobile phase eluting from a molecular sieve gel consists of discrete compartments containing molecules of decreasing molecular size. If the difference in molecular size of any two target molecules is sufficiently great, complete separation of the compartments of the mobile phase containing them will be possible. The difference in molecular size of human IgM and IgA is sufficient for such complete resolution to be theoretically achievable. Human IgA and IgG, however, differ relatively little in molecular weight[1], and the compartments of the mobile phase containing them broadly overlap. Improved separation can be achieved, however, by filtering the overlapping compartments, as they are eluted, through a second chromatographic step which will trap or filter out one of the two components. Ion-exchange chromatography utilizes differences in the isoelectric points of amphoteric molecules such as immunoglobulins. Shunting of the compartment of the mobile phase containing an admixture of IgG and IgA through a suitably developed ion-exchange gel will allow complete binding of IgA to the gel, while permitting IgG to pass through. The bound IgA can then be eluted by altering its net charge by a change in pH and ionic strength.

Complete separation of IgA from IgG, though theoretically possible by ion-exchange chromatography alone, is not practically achievable, since a variable portion of the IgG will also bind to the gel. Bound IgG, along with traces of IgM which trail into the IgA compartment of the mobile phase and bind to the gel, will co-elute with IgA. By filtering the eluted IgA through an insoluble matrix to which goat anti-human IgM and IgG (μ and γ chain specific) have been immobilized by covalent coupling, it can be specifically cleansed of the other two contaminating immunoglobulins[4].

The IgG compartment of the molecular-sieve mobile phase is quite large. The portion nearer the elution volume also contains denatured Ig fragments (Fc and Fab) which activate complement and produce a prozone in assays of immune lysis[4]. These untoward contaminants can likewise be filtered from the mobile phase by passage over an ion-exchange gel, to which they bind.

The target immunoglobulins will be quite dilute in their recovered compartments; concentration must be carried out in such a way as to avoid alteration of their biological activities, such as aggregation of the Ig molecules or degradation of the IgM pentamers. This is best accomplished by avoiding sudden changes in ionic strength or pH. Of the several available methods for concentrating high-molecular-weight solutes, we have found treatment of the dilute eluates with a dry cross-linked dextran of low exclusion limit (de-salting–concentrating) to be the most suitable.

The IgM- and IgA-containing mobile phase compartments can be collected directly over the dry dextran gel, concentrated three-fold by the selective diffusion

of dissolved ions and water into the swelling gel and recovered by basket centrifugation, without alteration in the ionic composition of their buffered solvent. After passage through the ion-exchange gel, the volume of the IgG-containing compartment of the mobile phase is quite large, making dextran-gel concentrating less practicable; however, because of the relative stability of IgG, concentration by pressure ultrafiltration is a suitable alternative.

The theoretical basis of the process is schematized in Fig. 1. The mobile phase, eluting from the molecular sieve gel, is divided into four compartments (Fig. 1). The first compartment, containing only IgM, is collected directly over dry dextran gel, concentrated and recovered without further treatment. The second compartment, containing IgA and IgG, is shunted over an ion-exchange gel to remove IgA, and the IgG collected. Whilst the third compartment, containing primarily IgG with traces of trailing IgA and lower-molecular-weight components, is likewise shunted over an ion-exchange gel, the bound IgA is eluted from the first ion-exchange gel, filtered through immobilized anti-IgM and anti-IgG (affinity) and concentrated over dry dextran gel. The IgG from the second ion-exchange gel is combined with that from the first and concentrated by pressure ultrafiltration. The final compartment, containing primarily albumin, is discarded.

A representative molecular-sieve elution profile is included in Fig. 1, with the three immunoglobulin-containing volumes of the mobile phase indicated.

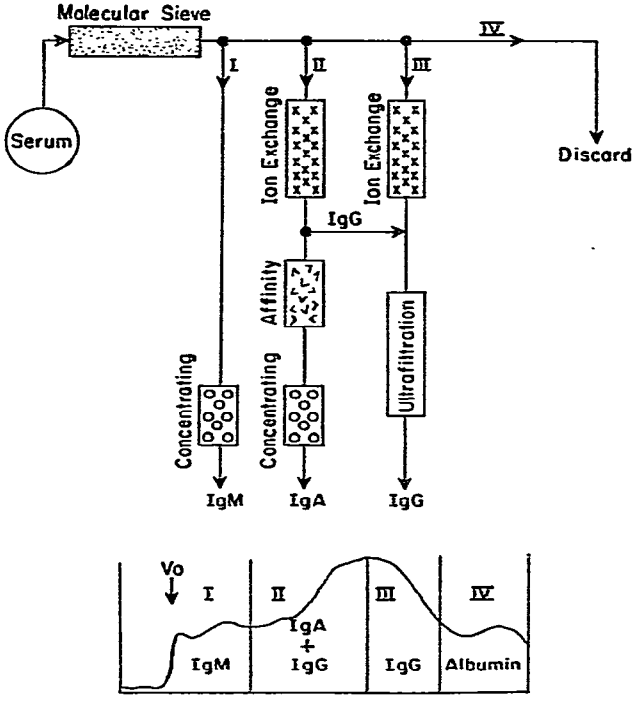

Fig. 1. Schematic of theoretical basis. The mobile phase eluting from the molecular sieve gel (bottom) is divided into four compartments, containing indicated immunoglobulins and albumin. Compartments I, II and III are shunted through subsequent chromatographic steps, as indicated by arrows, to effect final separation of IgM, IgA and IgG. Vo = Exclusion volume.

EXPERIMENTAL

Chromatography conditions

Molecular sieve. Separation of IgM from IgA and IgG is accomplished over 90 cm of a 3% acrylamide–4% agarose gel (AcA 34; L'Industrie Biologique Francaise, Gennevilliers, France)[5,6], equilibrated in 0.1 *M* Tris in 0.05 *M* NaCl of pH 8.2, packed into a 1.6 × 100 cm column (K16/100; Pharmacia, Uppsala, Sweden).

Ion exchange. Diethylaminoethyl derivatized cross-linked dextran gel (DEAE-Sephadex A-50, Pharmacia), developed in the same 0.1 *M* Tris–0.05 *M* NaCl buffer as the AcA 34 gel (starting buffer), and packed into two 80-ml columns (K16/40, Pharmacia) is used to absorb IgA and Ig fragments from the second and third compartments of the molecular-sieve mobile phase, respectively.

Elution of IgA from the first ion-exchange column is accomplished with a 0.4 *M* phosphate buffer (Sørensen's) of pH 5.3 (ref. 4).

Immunoadsorbent affinity. IgA is purified of contaminating IgM and IgG by passage of the first ion-exchange eluate through 9.5 ml of cyanogen bromide-activated agarose gel (CNBr-activated Sepharose 4B, Pharmacia), to which goat anti-human IgM and IgG has been covalently coupled[4,5]. Prior to coupling, the goat γ-globulin fraction was separated from μ- and γ-chain-specific anti-human antisera (Hyland, Division of Travenol Lab., Costa Mesa, Calif., U.S.A.) by precipitation with 30% saturated ammonium sulfate (SAS), followed by a second precipitation of the resuspended γ-globulin (distilled water) with 45% SAS (ref. 7). After exhaustive dialysis against 0.1 *M* $NaHCO_3$ in 0.5 *M* NaCl of pH 7.5, the γ-globulin fractions were separately coupled to CNBr-activated Sepharose according to the manufacturer's procedure (Pharmacia), combined in a ratio of one part anti-IgM to four parts anti-IgG, packed into a 9.5-ml column (K9/15, Pharmacia) and equilibrated in 0.4 *M* Sørensen's phosphate buffer of pH 5.3.

The immunoabsorbent is regenerated by elution of bound molecules with 0.2 *M* glycine–hydrochloric acid of pH 2.8, followed by equilibration in Sørensen's buffer.

De-salting–concentrating. Fractions containing pure IgM and IgA are concentrated by treatment with dry cross-linked dextran (Sephadex G-25 Coarse, Pharmacia[8]. Aliquots (6 ml) of the respective fractions are collected directly over 3.0 g each of dry Sephadex gel (water regain 2.5 ± 0.2 g/g) retained in syringes fitted with porous plastic frits. The concentrated immunoglobulins are recovered by basket centrifugation[8] of the swollen gel at 3000 *g* for 15 min. The concentrated IgM is tested without further treatment. The pH of the IgA fraction is adjusted to 7.4 with 0.1 *M* NaOH prior to testing.

IgG-containing fractions are separately collected and concentrated by pressure ultrafiltration (Diaflo X M 100A; Amicon, Lexington, Mass., U.S.A.).

Interconnection of chromatography apparatus (Fig. 2)

The separate modalities (molecular sieve, ion-exchange, immunoadsorbent and concentrating) are connected through a series of four-way valves such that the mobile phase eluting from the molecular sieve gel can be monitored by its optical density at 254 nm and shunted through appropriate subsequent chromatographic steps without interruption or change in the ionic composition of its buffer solution (to effect separation of pure IgM and IgG). IgA is then eluted from the ion-exchange gel by

a one-step change in ionic strength and pH, filtered through the immunoabsorbent and concentrated. To permit simplified switching from starting to eluting buffers, separate reservoirs of Tris–NaCl and Sørensen's buffer are ganged together and connected through four-way valves to the loading port of each of the ion-exchange columns.

IgG and IgA from the ion-exchange gels are monitored by optical density at 254 nm, using a separate UV absorptiometer (fraction collector and UV monitors; LKB, Bromma, Sweden).

The entire separation–purification process is carried out at 4° and completed within 36 h to minimize degradation of the immunoglobulins. To facilitate accurate switching, the entire process is developed at a constant speed, controlled by a variable-speed peristalic pump.

The organization of the interconnected chromatographic steps is schematized in Fig. 2. Because of its superficial complexity, the system was humorously dubbed The Goldberg Variation No. 2, after Rube, or GV-2.

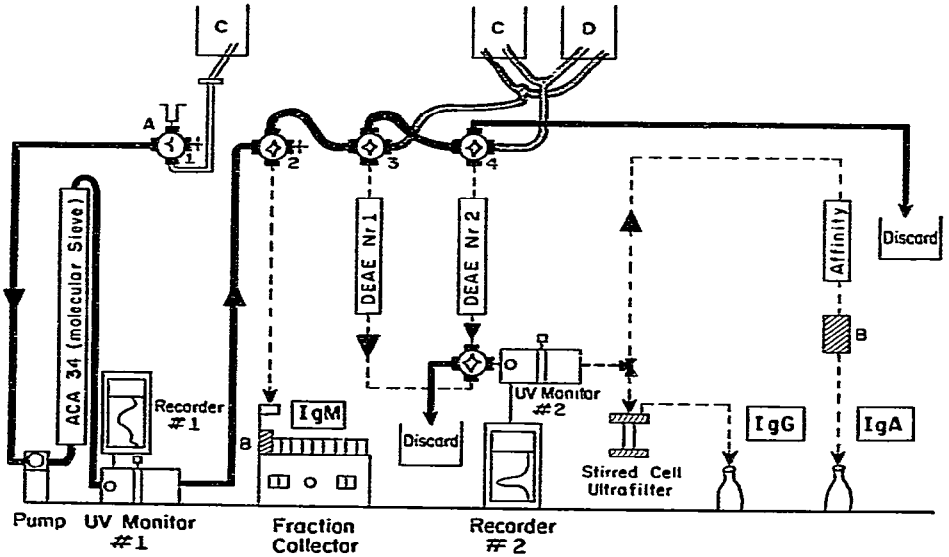

Fig. 2. Schematic of interconnection of chromatography apparatus. A = Loading port; B = concentrating chromatography (dry Sephadex G-25); C = Tris–NaCl buffer; D = Sørensen's buffer; 1–4 = four-way valves used to control flow of mobile phase and elution buffers. Solid black line indicates uninterrupted flow of molecular-sieve (AcA 34) mobile phase; interrupted lines indicate flow of shunted mobile phase through subsequent chromatographic steps. Open parallel lines indicate buffer feed into the molecular sieve gel, stippled parallel lines into the ion-exchange (DEAE) gel.

Calibrating the system

The integrity of the separation process depends upon accurate calibration of the molecular-sieve step. In order to maximize separation of the IgM compartment from the following IgA compartment, zone broadening through the molecular sieve gel must be minimized. For the AcA 34 gel the height equivalent to the theoretical plate, a measure of zone broadening, is optimal at a flow-rate of 3 ml/cm²·h (ref.

9). The peristaltic pump is therefore adjusted to maintain a constant flow at this rate.

Determining the size of the IgM compartment is of critical importance and best accomplished using lapine serum, since lapine IgA is slightly retarded relative to IgG, allowing for a clear determination of the volume of the IgM compartment. For the conditions described above, the IgM compartment consists of the initial 20–22 ml of the mobile phase.

Precise timing of the second and third switches is less critical. The second compartment of the mobile phase, containing IgA and IgG, can be considered to be the next 32–35 ml, while the third compartment, containing IgG, traces of IgA and low-molecular-weight fragments, consists of the following 32–40 ml.

Procedure

The unadjusted serum (1–3 ml) is introduced through a loading port (A, Fig. 2), connected to a reservoir of Tris–NaCl buffer (C) through valve No. 1, and chromatographed through the AcA gel. The eluting mobile phase is monitored and the initial 20 ml (exclusion volume), containing IgM, collected into syringes of dry Sephadex G-25 placed directly in the fraction collector (B). The mobile phase is then shunted through valves 2 and 3 to pass through the first DEAE column. When 34 ml of the mobile phase, corresponding to the center of the major 7S peak, has entered the first DEAE column, it is switched to the second DEAE column, through valves 3 and 4, until 35 ml has entered this column, and then switched (valve No. 4) to flow into discard.

IgG is then washed from the two DEAE columns with Tris–NaCl buffer from the separate, ganged reservoir (C) through valves 3 and 4; monitored through the second UV absorptiometer, combined and concentrated by ultrafiltration in a stirred cell.

IgA is eluted from the first DEAE column by switching the ganged reservoirs so that Sørensen's buffer (D) flows through valve No. 3. The eluted IgA is monitored through the second absorptiometer, filtered, without interruption of the effluent stream, through the affinity-immunoadsorbent column and collected over dry Sephadex (B).

Regeneration

The entire system can be readily regenerated. The AcA gel is washed thoroughly with Tris–NaCl buffer; the DEAE gel, after desorption with Sørensen's buffer, is re-equilibrated with Tris–NaCl buffer by batch washing and repacked in the columns. Bound molecules are desorbed from the immunoadsorbent gel with glycine–hydrochloric acid buffer and re-equilibrated in Sørensen's buffer without repacking. Syringes are washed, dried and recharged with dry Sephadex. After re-connecting the components, the system is ready for a subsequent run.

Serology

Quantitation of immunoglobulins. The purified immunoglobulins were quantified by the Mancini method of quantitative radial immunodiffusion against heavy-chain specific antisera (RID; Behring Diagnostics, Sommerville, N.J., U.S.A.)[5].

Biological activity. The biologic integrity of the recovered immunoglobulins

was determined by assaying the ability of IgM and IgG to initiate, and of IgA to block, immune lysis. The immunoglobulins were purified from a 3.0-ml pool of seven 7–13 day convalescent sera from patients recovering from group C meningococcal disease, and tested against a serogroup homologous strain of *N. meningitidis*. Details of the test procedures have been reported[4].

Aggregation of the purified immunoglobulins was estimated by assaying anti-complementary activity in a standard complement-fixation test[10].

RESULTS

Recovery of immunoglobulins

None of the sera were hemolyzed, lipemic or floculated, conditions which would interfere with their chromatography and require clarification by centrifugation. Completion of a single separation procedure required 36 h, regeneration of the system another 36 h.

Average yields of purified immunoglobulins, by weight, from six different sera or serum pools were: IgM, 45.5%; IgG, 52%; and IgA, 45% (Table I). The initial volume of serum applied did not influence the results, nor did the concentration of individual immunoglobulin classes within a given serum.

TABLE I

RECOVERY OF IMMUNOGLOBULIN CLASSES BY WEIGHT

Sera	*Vol. applied (ml)*	*IgM/vol.**	*IgG/vol.**	*IgA/vol.**
1	1.8	28.5/4.2	43.3/2.2	18.4/1.0
2	2.8	40.0/2.8	74.0/2.8	65.0/5.0
3	3.0	70.0/4.8	74.0/2.9	60.0/4.7
4	3.0	Not collected	34.5/2.8	50.2/9.1
5	3.2	40.0/7.7	40.0/2.5	39.2/9.3
Pool	3.0	49.0/5.6	46.4/6.0	37.8/8.1
Average		45.5	52.0	45.0

* Weight percentage recovered per volume (ml) of final product.

Recovery of IgM was primarily influenced by the efficiency of the concentration step; over-loading the absorptive capacity of the dry Sephadex gel necessitated repeated application with diminished yields. Purity of the IgM fraction was affected by the volume of the first compartment of the mobile phase. When the IgM compartment was taken as $\geqslant$25 ml, contamination with IgA occurred; when the IgM compartment was taken as $\leqslant$20 ml, there was no contamination with IgA. IgA contaminating the first (IgM) and third (IgG) compartments of the mobile phase was lost, resulting in diminished yields and emphasizing the importance of accurately determining the shunted volumes of the mobile phase. Although higher yields were obtained (70%, 74% and 60% of IgM, IgG and IgA, by weight, respectively; Table I), the average values given represent realistic and practical expectations.

IgA was invariably free of contamination by immunoglobulins of the other two classes ($<$0.8 mg/dl by low-concentration RID plates); purity of the IgG fraction was dependent upon the capacity of the DEAE gel. Filtering the IgG–IgA compartment through 80 ml of DEAE gel resulted in complete removal of IgA.

Biological activity

All three immunoglobulin fractions were anti-complementary at dilutions of 1:2, but not 1:4. (1:2 dilutions contained 19.9, 178.2 and 16.5 mg/dl of IgM, IgG and IgA, respectively. Complement-fixation tests kindly performed by Miss B. L. Brandt.) Anti-complementarity most likely resulted from slight aggregation of the immunoglobulins and was insufficient to affect immune lysis.

IgM and IgG were functionally intact as evidenced by their ability to initiate immune lysis. In Fig. 3 the bactericidal activity of a pool of convalescent case sera is compared with that of the IgM, IgG and IgA fractions separated therefrom. As expected, IgM was bactericidal at a titre greater than, and IgG at a titre less than, that of the whole serum[4]. Scant bactericidal activity was present in the IgA fraction, in keeping with its degree of anti-complementarity.

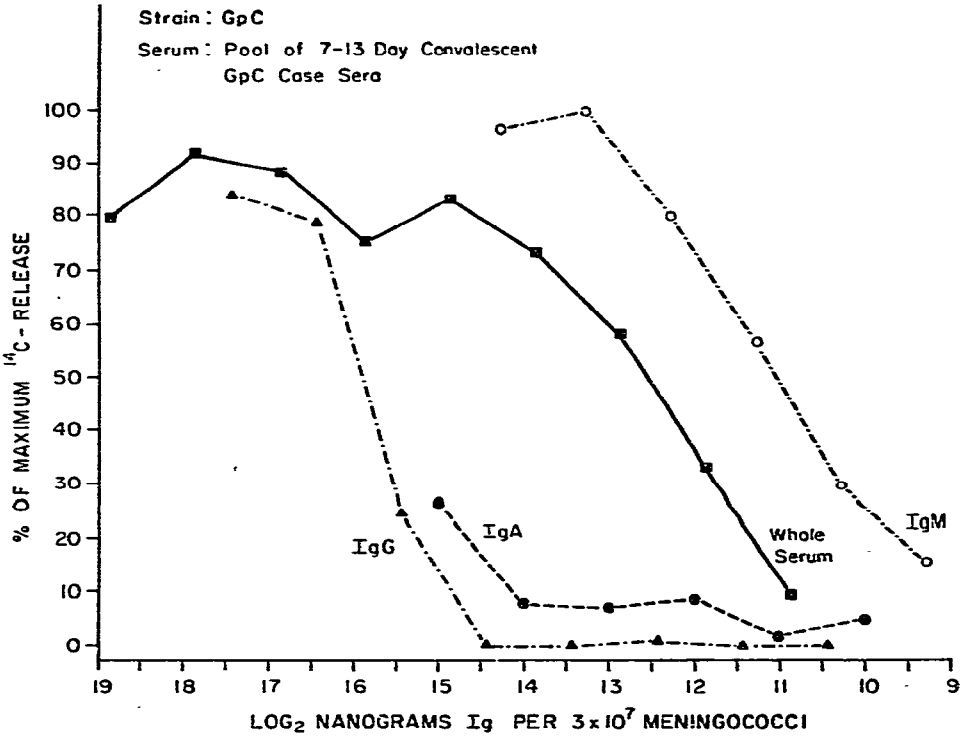

Fig. 3. Immune lysis of group C homologous strain of *N. meningitidis* by human case sera (■—■) and IgM (○—·—○), IgA (●—●) and IgG (▲—·—▲) separated therefrom[4].

IgA was functionally intact as evidenced by its ability to inhibit immune lysis initiated by IgM and IgG (Fig. 4)[4]. The greater inhibitory capacity of IgA for IgG-initiated than IgM-initiated immune lysis has been previously reported[4].

CONCLUSIONS

The GV-2 system clearly demonstrates the feasibility and advantages of a continuous, in-line chromatographic process for the separation and purification of human

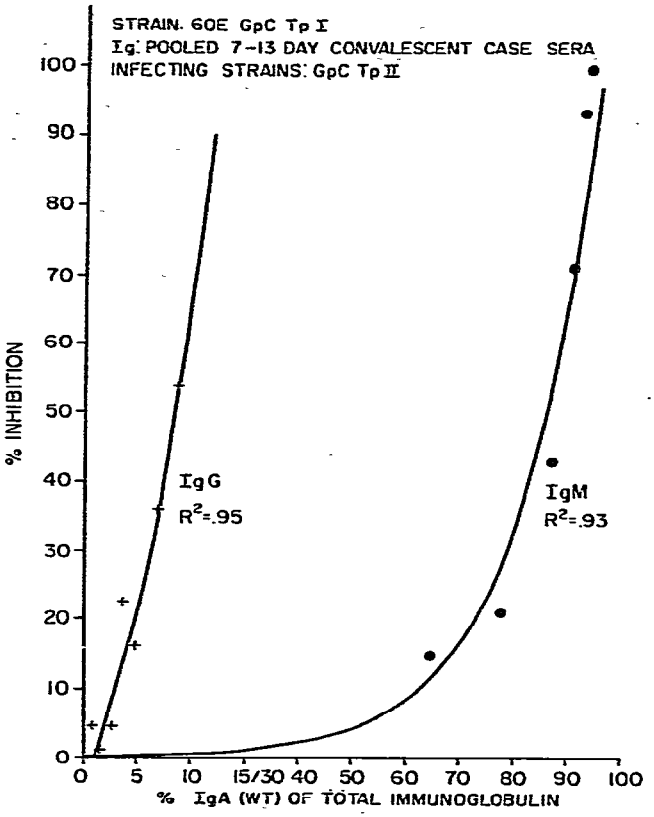

Fig. 4. Inhibition of immune lysis initiated by IgG (+—+) and IgM (●—●) by IgA separated from pool of human case sera, against $1 \cdot 10^7$ group C homologous *N. meningitidis* organisms. Correlation coefficients: $R^2 = 0.95$ and 0.93, respectively[4].

IgM, IgG and IgA from modest volumes of sera. Our major interest is in IgA; therefore, the system was devised to maximize its yield. The average yield of 45% is *ca.* four-fold greater than that previously achieved with discontinuous methods[4], while recovery of IgM and IgG compares favorably with single-step techniques. Furthermore, the purified and functionally active immunoglobulins are simultaneously available from the same starting sera, without waste, and dissolved in buffers suitable for most immunological assays. Because of the speed of the process and the short regeneration time, multiple sera can be fractionated rapidly for comparative testing. Yields from as little as 1 ml of sera are more than adequate for assays of immune lysis and other microtechniques.

The process is made possible by the chromatographic properties of the AcA 34 gel, which permit complete separation of the IgM and IgA compartments of the mobile phase[5,6,9], the use of buffers which are compatible with multiple chromatographic modalities and the development of immunoadsorbent filters which are stable for many months, can be regenerated and re-used. In addition the basic Tris–NaCl buffer minimizes self-aggregation of immunoglobulins and is suitable for most immunological assays.

The GV-2 system lends itself to further refinements and flexible applications. The use of an ion-exchange gel whose degree of swelling is less dependent on pH and ionic composition, such as DEAE-agarose, might obviate the most time-consuming step in the regeneration of the system: washing and repacking the ion-exchange gel. The capacity of the system can be scaled up or down, as needed, by the use of larger volumes of each chromatography gel, and the entire system can be semi-automated by the use of electronic valves and timing devices, such as an LKB Ultragrad Gradient Maker[11].

For applications in which IgM recovery is of greater importance, the size of the IgM compartment can be extended and contaminating IgA removed by filtration through an anti-IgA immunoadsorbent column. When IgG is the most desired product, the order of the chromatographic steps may be altered, with ion-exchange chromatography preceding molecular sieve. Further increases in IgG recovery might then be achieved by substituting a stronger anion exchanger such as diethyl(2-hydroxypropyl)aminoethyl-derivatized dextran (QAE-Sephadex, Pharmacia).

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