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# Method for the isolation of biologically active monomeric immunoglobulin A from a plasma fraction

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**Abstract**

A purification method for immunoglobulin A (IgA) yielding monomeric IgA with a purity of over 97% has been developed. This procedure uses ethanol-precipitated plasma (Cohn fraction III precipitate) as the starting material and includes heparin-Sepharose adsorption, dextran sulfate and ammonium sulfate precipitation, hydroxyapatite chromatography, batch adsorption by an anion-exchange matrix and gel permeation. Additional protein G Sepharose treatment leads to an IgA preparation of greater than 99% purity. The isolated IgA presented with an IgA subclass distribution, equivalent to IgA in unfractionated plasma, and was biologically active, as was shown by its ability to down-modulate *Haemophilus influenzae*-b-induced IL-6 secretion of human monocytes.

*Keywords:* Immunoglobulins

## 1. Introduction

Over the past years, the role of IgA in the human immune response has gained increasing attention [1,2]. While secretory IgA, which is usually dimeric or polymeric, has been shown to constitute a primary defense system for protection against pathogens invading via mucosal tissues [1], the functions of monomeric serum IgA are less well understood [3]. Normal human serum contains 2.63 ± 1.13 mg/ml IgA, most of which is monomeric [4]. The concentration of polymeric IgA is 0.20–0.35 mg/ml [4]. IgA consists of two subclasses, IgA1 and IgA2 [5]. IgA2 is only present in small amounts in human serum and makes up 15.86 ± 7.98% of the total IgA [6].

In order to gain more insight into the precise mechanisms of the functions of monomeric serum IgA, experiments must be performed that require large amounts of a pure and largely monomeric IgA preparation. Methods for isolation of (usually small amounts of) IgA have been described [7,8], and the use of jacalin-affinity chromatography for the separation of IgA subclasses has also been reported [7]. Our aim, however, was to establish a method suitable for large-scale preparation of human serum IgA.

A common method for fractionation of plasma, with the goal of producing immunoglobulins, is

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ethanol precipitation according to the methods of Cohn et al. [9] and Oncley et al. [10]. One of the fractions obtained by this procedure, fraction III precipitate, contains the majority of serum IgA, but also a variety of other proteins (both native and partly aggregated) as well as lipidic material, and is therefore difficult to process. To obtain IgA in a reasonable yield and of high purity, a novel procedure which includes the sequential use of heparin-Sepharose chromatography, dextran sulfate and ammonium sulfate precipitation, hydroxyapatite chromatography, batch adsorption to an anion exchanger and gel permeation on Superdex S200 was developed.

## 2. Experimental

### 2.1. Materials

Cohn-Oncley fraction III precipitate originating from industrial plasma fractionation was kindly provided by R. Gloger, Immuno, Vienna, Austria. This fraction was obtained by extracting Cohn fraction II + III with phosphate–acetate buffer followed by ethanol precipitation and centrifugation. This procedure yielded a pellet (fraction III precipitate), that was used for the described purification of IgA.

Soy bean trypsin inhibitor (type I-S) and dextran sulfate (D-7037, av. mol. mass 5000) were obtained from Sigma (St. Louis, MO, USA). Heparin-Sepharose CL-4B and protein G Sepharose 4 Fast Flow were from Pharmacia Biotech (Uppsala, Sweden). Ammonium sulfate, buffer salts and Fractogel EMD TMAE 650(S) anion-exchanger gel were purchased from Merck (Darmstadt, Germany). Macro Prep Ceramic hydroxyapatite (20 $\mu$m) was obtained from Biorad (Richmond, CA, USA). Gel permeation columns Superdex 200 HR 10/30, Superdex 200 prep grade HR 16/60 and Superdex 200 prep grade HR 35/600 were purchased from Pharmacia-Biotech.

### 2.2. Isolation of IgA from fraction III precipitate

A 58-g amount of fraction III precipitate originating from 1.7 l of human plasma was suspended in saline containing 10 $\mu$g/ml soybean trypsin inhibitor to obtain a final concentration of 40 g/l. After stirring for 16 h at 4°C, the suspension was centrifuged for 60 min at 18 900 *g* and the supernatant (the extract) was mixed with heparin–Sepharose CL-6B in a ratio of 1 ml gel per 20 mg total protein. The gel suspension was stirred overnight at 4°C and then filtered through an 85-$\mu$m nylon filter (Nytal, St. Gallen, Switzerland) to remove gel particles. The filtrate was treated with dextran sulfate (final concentration 8 mg/ml) and calcium chloride was added to yield a concentration of 0.5 *M*. The solution was stirred for 30 min at 25–30°C and then centrifuged (15 min, 4°C, 18 900 *g*). The pellet was removed and the supernatant dialyzed against saline using the Amicon cross-flow module S1Y30 (Amicon, Danvers, MA, USA). Then the dialysate was clarified by centrifugation and subjected to ammonium sulfate precipitation at 4°C (final concentration: 2 *M* ammonium sulfate) followed by stirring for 30 min. After centrifugation (20 min at 4°C and 1540 *g*) the supernatant was discarded and the pellet was resuspended in phosphate-buffered saline, pH 7.4 (PBS, containing 137 m*M* NaCl, 8.2 m*M* $Na_2HPO_4$, 1.8 m*M* $KH_2PO_4$, pH 7.4) and dialyzed against the same buffer. Following preclearing, the dialyzed material (corresponding to about 700 mg total protein content) was applied at room temperature to a hydroxyapatite column (50 ml gel bed, diameter 2.6 cm) equilibrated in PBS containing 0.02% (w/v) sodium azide at a flow-rate of 2 ml/min. The column was connected to a Pharmacia FPLC system equipped with an LCC-500 gradient controller, two P-500 pumps, a UV-M UV monitor with a 2-mm flow-through cell, and a Frac-200 fraction collector. Two buffer systems, PBS containing 0.02% (w/v) sodium azide, pH 7.4 (buffer A) and 0.5 *M* sodium phosphate containing 0.15 *M* NaCl and 0.02% (w/v) sodium azide, pH 6.8 (buffer B) were used. After application of the sample and washing with buffer A, a fraction containing IgA was eluted with 3% buffer B in buffer A. This eluate was dialyzed against 0.05 *M* sodium acetate–acetic acid, pH 5.0.

The dialyzed fraction was then subjected to anion exchange by a batch procedure as follows. Fractogel EMD TMAE 650(S) that was washed with and suspended (1:2, v/v) in 0.05 *M* sodium acetate–acetic acid, pH 5.0, was mixed with the sample

(about 5 mg total protein/ml gel suspension) and incubated with stirring overnight at 4°C. The suspension was filtered through a Buchner funnel and the gel was washed twice with 0.05 *M* sodium acetate–acetic acid, pH 5.0. The washed gel was then mixed with a buffer containing 50 m*M* sodium phosphate, 500 m*M* NaCl, pH 6.0, and agitated for 2 h at 4°C in order to elute an IgA-containing preparation. The gel suspension was filtered through a Buchner funnel and the filtrate was concentrated in an Amicon ultrafiltration cell equipped with an Amicon YM30 membrane to give a protein concentration of approximately 10 mg/ml.

The concentrated material was applied in 10-ml aliquots to a Superdex 200 HR 35/600 gel permeation column equilibrated with PBS containing 0.1% (w/v) sodium azide, pH 7.4, and passed through the column at a flow-rate of 2.5 ml/min. Fractions containing IgA were pooled and mixed with protein G Sepharose (about 100 mg protein/ml gel) that had been equilibrated in PBS containing 0.1% (w/v) sodium azide. After incubation overnight at 4°C the gel was removed by centrifugation and the purified IgA in the supernatant was dialyzed (dialysis tube molecular mass cut-off of 12–14 kDa, Spectropor, Spectrum, Los Angeles, CA, USA) against bi-distilled water and concentrated in a stirred ultrafiltration cell equipped with an Amicon YM30 membrane.

### *2.3. Protein determination*

Protein concentration was determined by the method of Lowry et al. [11].

### *2.4. Radial immunodiffusion (RID)*

IgG and IgA were determined by RID according to Mancini et al. [12] with commercial RID plates from Immuno AG or with low level plates from Behringwerke (Marburg, Germany). IgA subclasses were determined using RID plates (IgA-Subclass-COMBI-NL) and standards from The Binding Site (Birmingham, UK). The immunoglobulin light-chain $\kappa/\lambda$ ratio was determined according to Sommer and Hohenwallner [13] with RID plates (M-Partigen) from Behringwerke. $\alpha_2$-Macroglobulin RID plates and standard serum were also purchased from Behringwerke.

### *2.5. SDS polyacrylamide gel electrophoresis (SDS–PAGE)*

SDS–PAGE was performed according to the system of Laemmli [14] with reagents from Biorad or ready-to-use gels from Novex (San Diego, CA, USA). Gels were stained with Coomassie blue.

### *2.6. Down-modulation of the Haemophilus influenzae-b-induced IL-6 release from human monocytes*

The procedure was performed exactly according to the protocol of Wolf et al. [15]. In brief, human mononuclear cells (MNC) were isolated from heparinized peripheral blood of healthy volunteers by buoyant density gradient centrifugation [16].

Monocyte monolayers were prepared from MNC by adherence to plastic tissue culture plates. For induction of IL-6 release, heat-inactivated encapsulated *Haemophilus influenzae*-b (Hib, strain Eagan, kindly provided by Dr. G. Zerlauth, Immuno AG) was added to the monocytes. IgA at a final concentration of 10 mg/ml was added where indicated. Monocyte cultures set up in the presence of Hib alone or in medium alone served as controls. After incubation for 24 h at 37°C in a $CO_2$ incubator, the cell supernatants were aspirated and centrifuged to remove contaminating cellular material.

IL-6 concentrations in monocyte supernatants were determined using commercially available ELISA kits (IL-6-EASIA, Medgenix Diagnostics, Fleurus, Belgium). Results are expressed as picograms per milliliter of IL-6.

## 3. Results and discussion

### *3.1. Isolation of IgA from fraction III precipitate*

Analysis of the starting material and the products of the various subsequent chromatography and precipitation procedures that lead to the preparation of

Table 1
Isolation of IgA from fraction III precipitate

| | Total protein (mg) | Total IgG (mg) | $\alpha_2$-Macro-globulin (mg) | IgA total (mg) | IgA percentage of protein | IgA yield (%) |
|---|---|---|---|---|---|---|
| NaCl extract | 14910 | 3164 | 2016 | 1434 | 9.6 | 100 |
| Heparin–Sepharose | 7592 | 2156 | 645 | 1125 | 14.8 | 78 |
| Ammonium sulfate | 5376 | 1105 | 522 | 1052 | 19.6 | 73 |
| Hydroxyapatite | 1561 | 640 | <55 | 990 | 63.4 | 69 |
| Fractogel | 502 | 50 | 12 | 465 | 92.6 | 32 |
| Superdex 200 | 326.4 | 15 | <0.1 | 318 | 97.4 | 22 |
| Protein G | 192.6 | <0.5 | <0.1 | 192 | 99.7 | 13 |

IgA-containing materials were analyzed by radial immunodiffusion for IgG, IgA and $\alpha_2$-macroglobulin and by the method of Lowry et al. [11] to determine the total protein content following each purification step.

highly purified monomeric serum IgA are shown in Table 1 (purity and yield), Fig. 1A–C (gel filtration analysis) and Fig. 2 (SDS–PAGE). As a first step [after extraction of the fraction III precipitate, which was used as a starting material (Fig. 1A and Fig. 2, lane 2)], a major part of the coagulation proteins and lipoproteins were removed by chromatography on heparin-Sepharose [17–19] and by precipitation with dextran sulfate [20]. (Heparin Sepharose also adsorbed two thirds of the $\alpha_2$-macroglobulin.) These purification steps also resulted in removal of most of the plasminogen and fibrinogen (not shown). In order to remove dextran sulfate, dialysis and an ammonium sulfate precipitation were performed.

To remove most of the remaining $\alpha_2$-macroglobulin, hydroxyapatite chromatography was required (note the reduction of the 180 kDa band, which corresponds to $\alpha_2$-macroglobulin, in Fig. 2, lane 3, and the decrease in $\alpha_2$-macroglobulin determined by RID in Table 1). The gel permeation analysis shows peaks mainly in the molecular mass range of IgA and IgG (Fig. 1B). Fractogel EMD TMAE 650(S) adsorption further increased the IgA content of the isolate from 63% to 93% IgA (Table 1 and Fig. 2, lane 4). After testing different conditions for adsorption and elution, conditions were chosen which, although resulting in a reduced yield of IgA, would afford the highest possible enrichment of IgA (Table 1).

Gel permeation on Superdex 200 HR 35/600, which was applied subsequently, removed all hitherto remaining traces of $\alpha_2$-macroglobulin (Table 1) and other high-molecular-mass proteins and aggregates. Surprisingly, IgG and IgA (identified by SDS–PAGE of the respective peaks followed by blotting and immune detection, data not shown) were also separated from each other, although they differ only slightly in molecular mass (IgA: 162 kDa, IgG: 153 kDa, according to Heremans [4]).

One possible reason for the separation of IgG and IgA that was obtained is that the IgA might have covalently or non-covalently dimerized during the purification process. This would lead to its elution from the gel permeation column faster than IgG. Covalent dimerization, however, could be ruled out, as SDS–PAGE analysis of the respective IgA peak under non-reducing conditions (see Fig. 3C) showed an apparent IgA molecular mass similar to that of IgG. In order to rule out the possibility that this IgA peak consisted of non-covalently bound IgA dimers, a mixture of IgA and IgG was treated with 1% (w/v) SDS and run on the Superdex 200 HR 10/30 column with a PBS buffer containing 0.1% (w/v) SDS. Under these conditions, a non-covalently linked IgA dimer should dissociate and IgA and IgG should coelute. However, IgA again characteristically eluted earlier than IgG, demonstrating that the IgA material did indeed contain IgA in monomeric form (data not shown). Therefore mechanisms other than mere separation by differences in molecular mass must account for the observed fractionation. Possible mechanisms are hydrophobic and/or electrostatic interactions with Superdex 200, as was recently described for other gel permeation matrices [21].

The IgA preparation isolated from Cohn III precipitate by the procedure described contained at least 97% monomeric IgA (Table 1). The yield of IgA was usually greater than 20% of the IgA present in

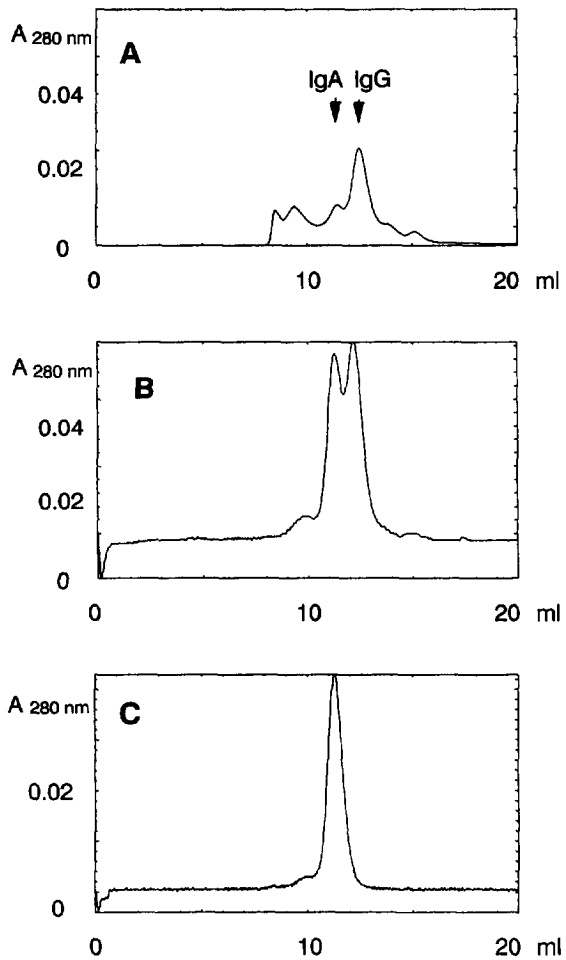


Fig. 1. Analysis of IgA-containing fractions by gel permeation on an analytical Superdex 200 HR 10/30 column. Conditions: column buffer, PBS containing 0.1% (w/v) sodium azide, pH 7.4; flow-rate, 0.5 ml/min; sample, 25 $\mu$l (containing 40–250 $\mu$g total protein). (A) Starting material (fraction III precipitate extract); (B) IgA-containing material eluted from the hydroxyapatite column; (C) IgA after protein G Sepharose treatment.

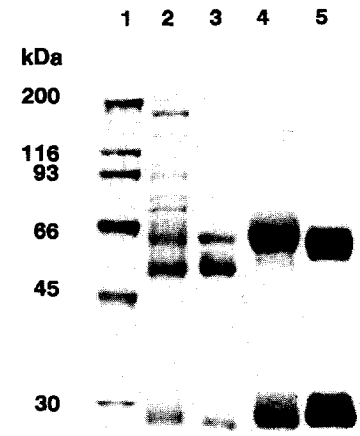


Fig. 2. SDS–PAGE analysis of reduced samples of an IgA isolation on a commercially available 10% TRIS–glycine gel (Novex). Lanes 1 to 5 represent the following: 1 = SDS–PAGE standards; 2 = starting material (NaCl extract of fraction III precipitate); 3 = material after hydroxyapatite chromatography; 4 = material after ion-exchange chromatography; 5 = material after gel permeation on Superdex 200.

the starting material. The traces of IgG remaining in the preparation appeared to consist, at least partly, of non-covalently linked IgG dimers, since no IgG monomer peak or shoulder was observed and IgG dimers were found to elute at the same volumes as IgA monomers (data not shown). IgG dimers can be formed in small amounts by an idiotype–anti-idiotype reaction in pools of plasma, as was described recently [22].

The remaining IgG could be removed by adsorption to protein G Sepharose (Table 1). Protein G was preferred over protein A, as protein A was reported to react weakly with IgA from myeloma, colostrum and serum [23], and such an interaction could result in a decreased yield of IgA. Moreover, human IgG3 does not bind to protein A [24] and thus would not be removed. In contrast, in buffers containing less than 1 *M* NaCl, protein G was described as binding only IgG (including IgG3) [25]. The final product was a monomeric IgA isolate with a purity of at least 99%. It should be noted that on prolonged storage in soluble form, IgA dimers (up to 8%) may be formed from the monomeric IgA (the dimers can be seen in Fig. 1C at an elution volume of about 10 ml), as has also been observed with IgG preparations [22,26].

### *3.2. Characterization of the isolated IgA*

IgA subclass distribution and immunoglobulin light chain ratio of the isolated IgA were analyzed by RID, and the results (Table 2) were found to be comparable to the results obtained by analyzing

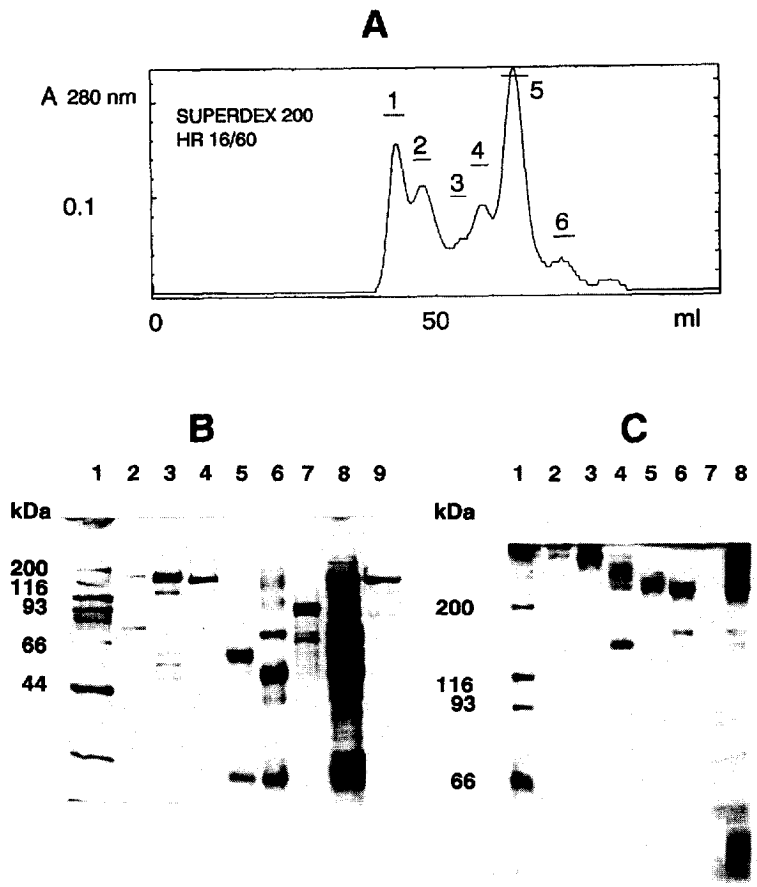


Fig. 3. (A) Separation of a NaCl extract of fraction III precipitate (clarified by ammonium sulfate precipitation) on a Superdex 200 HR 16/60 column. Conditions: column buffer, PBS containing 0.1% (w/v) sodium azide, pH 7.4; flow-rate, 0.5 ml/min; sample volume, 2 ml. Six peak fractions were collected and further analyzed. (B and C) SDS–PAGE analysis of the peaks obtained from the Superdex 200 separation shown in A. Reduced samples (B) or non-reduced samples (C) were run on a 10% (B) and 8% (C) polyacrylamide gel according to the method of Laemmli [14]. Lanes 1–9 represent the following: 1 = SDS–PAGE standards; 2 = peak 1; 3 = peak 2; 4 = peak 3; 5 = peak 4 (IgA); 6 = peak 5 (IgG); 7 = peak 6 from the gel permeation chromatography step shown in A; 8 = starting material; 9 $\alpha 2$-macroglobulin (Sigma) (only shown in B).

Table 2
Characterization of isolated serum IgA

| | IgA1 (percentage of total IgA) | $\kappa/\lambda$ ratio |
|---|---|---|
| Purified IgA | 79 | 0.94 |
| Human plasma (pool) | 81 | 0.99 |

IgA subclass and Ig light chain concentrations were measured by radial immunodiffusion with commercially available RID plates. Concentrations of $\kappa$ and $\lambda$ Ig light chains were determined as units/ml, and the $\kappa/\lambda$ ratio was then calculated according to the protocol provided by the supplier of the RID plates.

pooled human plasma from more than 1000 donors. IgA subclass distribution and light chain ratio were also comparable to values reported earlier for serum IgA [6,9,27].

### 3.3. Biological activity of the isolated IgA

Biological activity of the isolated IgA was tested in two independent experiments by its ability to decrease Hib-induced IL-6 release by monocytes [15]. Substantial levels of IL-6 production (17 070–17 593 pg/ml) were induced in monocytes stimu-

lated with Hib, while resting monocytes produced only very low levels of this cytokine (18–87 pg/ml of IL-6). The induction of IL-6 secretion in Hib-activated monocytes was substantially inhibited to 864–7280 pg/ml IL-6 when IgA (at a concentration of 10 mg/ml) was added, demonstrating that the purified monomeric IgA was indeed biologically active and exhibited activities comparable to those described recently [15].

### 3.4. Fast purification of IgA

The finding that IgG and IgA could be separated from each other by gel permeation on Superdex 200 was subsequently used to purify IgA quickly from a complex mixture. A saline extract of fraction III precipitate was treated with 2 *M* ammonium sulfate and the precipitated material was dissolved and run directly on a Superdex 200 HR 16/60 gel permeation column. Six peaks (one of them a shoulder) were obtained (Fig. 3A). The material fractionated in the maximum of peak 4 was analyzed on SDS–PAGE and consisted of rather pure IgA (shown in Fig. 3B, lane 5 and Fig. 3C, lane 5). The molecular masses found were in accordance with recently reported data [7,28,29]. Moreover, by blotting the bands onto nitrocellulose and probing with anti-IgA reagents, the bands were identified as IgA (data not shown).

This rapid IgA purification procedure is, however, only practicable for isolating small amounts of IgA, since the starting material, although centrifuged and filtered, was opaque, and a small but distinct fibrin-like precipitate formed on prolonged storage. Both of these factors would, in the case of large-scale or repeated isolation, lead to clogging and/or destruction of the column. However, when combined with adequate column cleaning, Superdex 200 can be used for quick purification of small amounts of IgA.

## 4. Conclusion

A procedure for large-scale purification of IgA from an ethanol fraction of human plasma was developed, yielding pure and monomeric IgA. This product is comparable to human serum IgA with respect to IgA subclass distribution and is also biologically active. Availability of large quantities of monomeric IgA will facilitate studies to further determine the biological functions of this Ig isotype and may also provide a product to be employed in therapy.

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## References

[1] R.M. Goldblum, J. Clin. Immunol., 10 (1990) 64S.
[2] J.M. Griffiss, Ann. N.Y. Acad. Sci., 409 (1983) 697.
[3] P.S. Hiemstra, A. Gorter, M.E. Stuurman, L.A. Van Es and M.R. Daha, Eur. J. Immunol., 17 (1987) 321.
[4] J.F. Heremans, The Antigens, Vol. 2, Academic Press, New York, 1974, p. 365.
[5] H.M. Grey, C.A. Abel and B. Zimmerman, Ann. N.Y. Acad. Sci., 190 (1971) 37.
[6] M.E. Conley, A. Arbeter and S.D. Douglas, Mol. Immunol., 20 (1983) 977.
[7] L.M. Loomes, W.W. Stewart, R.L. Mazengera, B.W. Senior and M.A. Kerr, J. Immunol. Methods, 141 (1991) 209.
[8] H.C. Morton, J.D. Atkin, R.J. Owens and J.M. Woof, J. Immunol., 151 (1993) 4743.
[9] E.J. Cohn, L.E. Strong, W.L. Hughes, Jr., D.J. Mulford, J.N. Ashworth, M. Melin and H.L. Taylor, J. Am. Chem. Soc., 68 (1946) 459.
[10] J.L. Oncley, M. Melin, D.A. Richert, J.W. Cameron and P.M. Gross, Jr., J. Am. Chem. Soc., 71 (1949) 541.
[11] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[12] G. Mancini, A.O. Carbonara and J.F. Heremans, Immunochemistry, 2 (1965) 235.
[13] R. Sommer and W. Hohenwallner, Laboratoriumsblätter, 29 (1979) 27.
[14] U.K. Laemmli, Nature, 227 (1970) 680.
[15] H.M. Wolf, M.B. Fischer, H. Pühringer, A. Samstag, E. Vogel and M.M. Eibl, Blood, 83 (1994) 1278.
[16] A. Bøyum, Scand. J. Clin. Lab. Invest., 21 (Suppl. 97) (1968) 77.
[17] M. Miller-Andersson, H. Borg and L.-O. Andersson, Thromb. Res., 5 (1974) 439.
[18] L.-O. Andersson, H. Borg and M. Miller-Andersson, Thromb. Res., 7 (1975) 451.
[19] M. Miller-Andersson, P.S. Gaffney and M.J. Segatchian, Thromb. Res., 20 (1980) 109.
[20] A. Van Dalen, H.G. Seijen and M. Gruber, Biochim. Biophys. Acta, 147 (1967) 421.
[21] M. Herold, International Laboratory, March issue (1993) 34.

[22] D.L. Tankersley, Immunol. Rev. 139 (1994) 159.
[23] J. Balint, Jr., Y. Ikeda, T. Nagai and D.S. Terman, Immunol. Commun., 11 (1982) 283.
[24] J.W. Goding, J. Immunol. Methods, 20 (1978) 241.
[25] J.B. Pilcher, V.C.W. Tsang, W. Zhou, C.M. Black and C. Sidman, J. Immunol. Methods, 136 (1991) 279.
[26] M. Svenson, M.B. Hansen and K. Bendtzen, J. Clin. Invest., 92 (1993) 2533.
[27] S. Barandun, A. Morell, F. Skvaril and A. Oberdorfer, Blood, 47 (1976) 79.
[28] J.P. Vaerman, A. Langendries and C. Vander Maelen, Immunol. Invest., 24 (1995) 631.
[29] S. Fasler, F. Skvaril and H.U. Lutz, Anal. Biochem., 174 (1988) 593.