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Semi-quantitative assessment of anti-insulin total IgG and IgG sub-classes in insulin-immunized patients using a highly sensitive immunochemical micromethod

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Summary. An immunochemical micromethod was designed to estimate total IgG and IgG sub-classes of anti-insulin antibodies in immunized diabetic patients. Insulin, immobilized on a solid phase, was allowed to react with serum samples containing anti-insulin antibodies. Bound anti-insulin IgG interacted with mouse monoclonal antibodies specific for total IgG or for each IgG isotype. The fixation of mouse monoclonal antibody was subsequently detected using a horseradish peroxidase-conjugated rabbit anti-mouse IgG in the presence of a chromogenic substrate. The test was standardized by an immunocapture assay utilizing coated rabbit anti-human IgG and known concentrations of purified human myelomatous proteins of each sub-class. Results of anti-insulin IgG and anti-insulin IgG sub-classes assay could therefore be expressed in ng equivalent myelomatous proteins per ml of serum. Analysis of serum samples from 24 insulin-immunized diabetic patients revealed a quasi absence of IgG2 anti-insulin antibodies and an increase of the relative abundance of the other three anti-insulin IgG isotypes. In our series, anti-insulin IgG1 was predominant, followed by IgG3 (in 17/24 patients) or IgG4 (in 7/24). Insulin immunization was deduced to be of polyclonal nature, the isotype pattern of which is not representative of the relative proportion of IgG sub-classes in whole normal serum.

Key words: Anti-insulin, IgG sub-classes, monoclonal antibodies, enzyme linked immunosorbant assay.

Owing to the availability of monoclonal reagents specific for human IgG isotypes, the profile of the humoral immune response in man becomes more and more documented. IgG2 is the major component of antibodies against bacterial polysaccharides [1], whereas IgG4 is predominant in immunization against coagulation factor VIII [2]. On the other hand, IgG1 and IgG3 are mostly responsible for anti-viral [3, 4] and anti-blood group responses [5, 6]. In most studies already reported, the distribution of IgG sub-classes carrying specific antibodies does not parallel that of isotypes in whole serum.

Since the physico-chemical and biological properties of the four human IgG isotypes are known to be different, the class and sub-class of antibody carrying immunoglobulins could be of clinical significance.

To our knowledge, the IgG sub-classes distribution of anti-insulin (AI) antibodies has never been characterized in man. Yet, animal experiments suggest that the sub-class of IgG may play an important role in determining the rate of immune complexes (IC) clearance in vivo. In the guinea pig, two IgG sub-classes can be identified and purified [7]: IgG1, which does not activate complement and is not cytophilic, and IgG2, which binds C1q and specific Fc receptors on macrophages. These two sub-classes were purified and utilized to form immune complexes with 123-I-insulin. In the rat, IgG2 IC rapidly disappeared from the plasma and were taken up by the liver and, to a much lower extent, by the spleen. By contrast, IC formed with IgG1 remained in plasma compartment and behaved as blood pooling agents [8].

The above considerations led us to design a method to identify AI IgG sub-classes and to estimate their plasma concentration in man in order to study the immunization pattern of diabetic patients and define a possible correlation between this pattern and clinical course.

The present paper reports the results obtained by analysis of 24 sera from immunized diabetic patients using a highly sensitive assay based on the principle of ELISA [9].

Materials and methods

Materials

The human sera analyzed by this ELISA method were collected from insulin-immunized diabetic patients, selected on the basis of their

reactivity in the radioimmunoassay [10] classically utilized to detect anti-insulin immunization.

Solid phase enzyme immunoassay was performed in polystyrene immunoplates (NUNC, N° 4-39454, Gibco, Paisley, Scotland) with 96 flat bottom wells. Bovine insulin utilized as target antigen was a generous gift from Novo Industri AS (Copenhagen, Denmark).

Immunological material comprised polyclonal and monoclonal reagents: rabbit immunoglobulin anti-human IgG (RAH-IgG, A090, Dakopatts, Glostrup, Denmark), peroxidase conjugated rabbit immunoglobulin anti-mouse immunoglobulin (HRP-RAM-Ig, DAKO P260), mouse monoclonal anti-IgG1 (BAM 15, clone NL 16), anti-IgG2 (BAM 10, clone GOM 1) and anti-IgG4 (BAM 16, clone GB 7b) obtained from Seward Laboratory (Bedford, UK), monoclonal antibodies specific of total IgG (γ-chain specific, MH 001) and for IgG3 (MH 013) obtained from Gamma (Neupré, Belgium). All monoclonals were mouse IgG1 isotypes.

The different buffers and solutions used to perform the ELISA assay were: *coating buffer* (Na2CO3 5.3 gr; NaHCO3 4.2 gr; NaN3 0.2 gr, H2O ad 1 L, pH 9.6); *washing buffer* (NaH2PO4 5.2 gr; K2HP04.3H2O 36.7 gr; NaCl 87.6 gr; H2O ad 10 L, pH 7.4) containing 0.5% Tween 20 (Merck, Darmstadt, FRG) and 2 g/l bovine serum albumin (BSA, fraction V, Sigma Chemical, St. Louis, Mo, USA). Washing buffer was the diluent of each antigen-antibody reaction. *Substrate solution* contained 75 mg ABTS (2.2'-azino-di-3-ethylbenzthiazolinosulfonat 6, from Boehringer, Mannheim, FRG) dissolved in 100 ml of substrate buffer (Na2HPO4.2H2O 20.5 gr, citric acid 8.9 gr, H2O ad 1 L, pH 5.6), containing 0.004% (v/v) H2O2. *Stopping solution* was 2 N H2SO4.

Optical density of chromogenic substrate was measured at 414 nm wavelength by using a multichannel spectrophotometer (Multiskan, Titertek, Flow Laboratories, Rockville, Md, USA).

Methods

ELISA tests: The ELISA tests for the class and sub-class characterization of IgG anti-insulin antibodies were sandwich assays realized as follows:

Class determination: Insulin/human serum/anti-IgG MAb/HRP-RAM-Ig/ABTS.

Sub-class determination: Insulin/humanserum/anti-IgG1,2,3,4 MAb/HRP-RAM-Ig/ABTS.

Seventy-five µl per well of bovine insulin dissolved in coating buffer at a concentration of 13.3 µg/ml were left overnight at 4 °C or alternatively 2 h at 37 °C. After three washings, the plates were filled with washing buffer and allowed to stand 30 min at room temperature in order to saturate the remaining polystyrene reactive sites.

Fifty µl of patients' sera previously diluted 1/5 were tested in serial two-fold dilutions from well 2 to 12 (1/5-1/5, 120) and incubated 2 h at 37 °C. After three washings, 50 µl of optimal dilutions of monoclonal reagents were allowed to react for 2 h at 37 °C. The excess of monoclonal reagent was eliminated by three further washings, and the specific antigen-antibody complex was revealed using peroxidase conjugated rabbit anti-mouse immunoglobulins at a 1/250 dilution as recommended by the manufacturer and confirmed by our own experience. After a 1-h incubation and four washings with tap water, the substrate solution was added (50 µl/well). The enzymic activity was stopped after 45 min by addition of 150 µl 2 N H2SO4.

Blank values (O. D. Blank) were obtained from column 1, which received all reagents but human serum or myelomatous protein.

Sera from 21 healthy blood donors were similarly tested and served as negative controls in order to determine the magnitude of optical density due to non specific binding.

Standardization of the assay was performed with purified myelomatous human IgG at known concentration in an immuno capture assay [11]:

RAH-IgG/myelom.IgG (1,2,3 or 4)/anti-hum IgG MAb (anti-IgG1,2,3 or 4)/HRP-RAM-Ig.

Table 1. Effect of insulin concentration in coating solution on coating efficiency. n = 12

Coating solution (µg insulin/well)	Coating efficiency (% insulin coated) mean ± SD (n = 12)	Amount of coated insulin (µg/well)
15	8.4 ± 3.2	1.26
10	9.7 ± 3.8	0.97
5	10.0 ± 3.1	0.50
1	13.5 ± 3.1	0.14
0.5	15.5 ± 3.2	0.08
0.1	23.7 ± 4.1	0.02
0.05	21.6 ± 3.1	0.01
0.01	14.3 ± 2.5	0.001

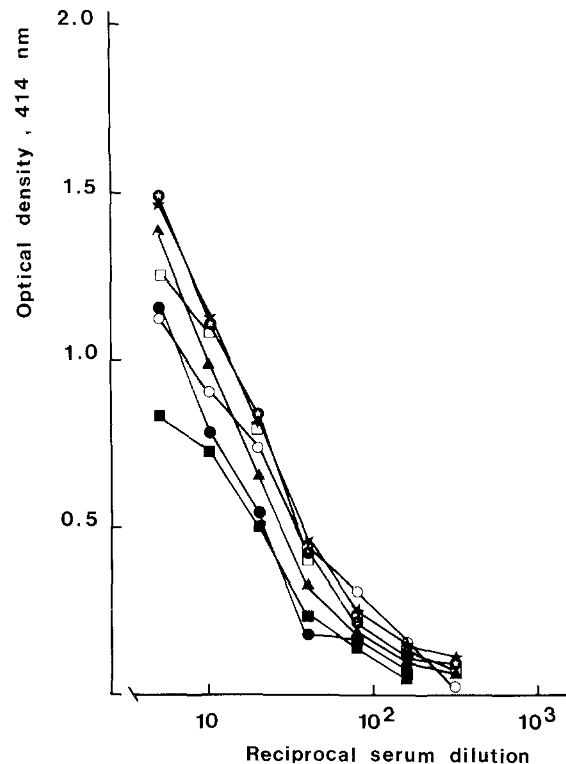


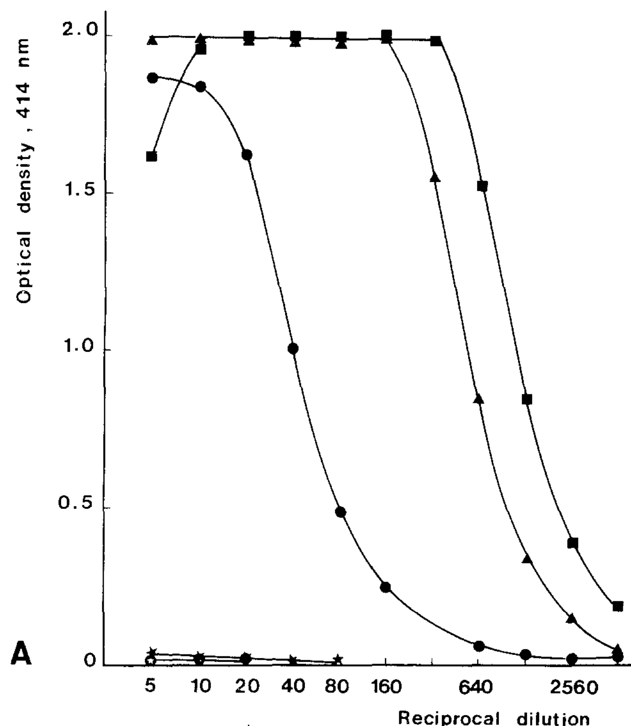
Fig. 1. ELISA curves obtained for total IgG anti-insulin at different insulin coating concentrations: (✪) 15 µg/well, (✶) 10 µg/well, (▲) 5 µg/well, (□) 1 µg/well, (○) 0.5 µg/well, (●) 0.1 µg/well (■) 0.05 µg/well

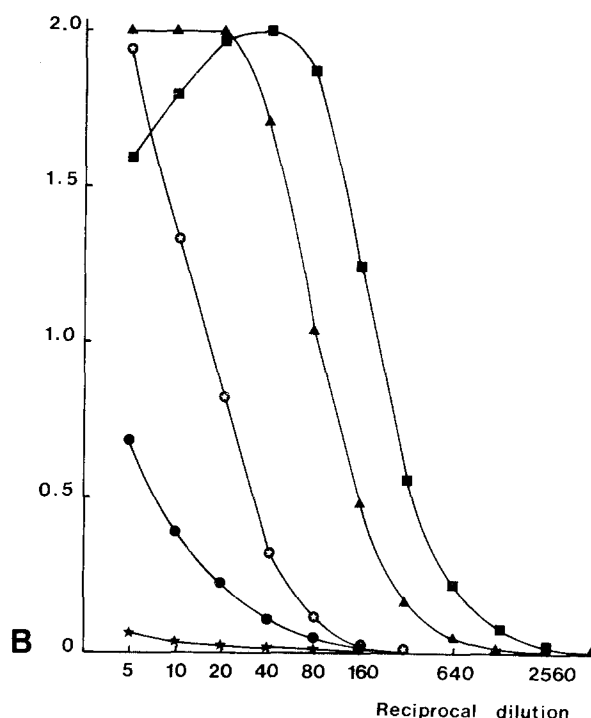
In this assay, plates previously coated with 75 µl of the optimal dilution of rabbit Ig anti-human IgG (γ-chain specific), received, 50 µl of 11 two-fold dilutions of each purified IgG isotype at a known initial concentration (2.000 - 2 ng/ml). The subsequent steps of the standardization assay were identical to those above mentionned.

Semi-quantitative estimation: Optical density values of standards and samples were plotted on a graph as a function of the logarithm of dilution. From the calibration curves obtained with known amounts of purified isotypes, a concentration unit (Cu) was defined as the Ig concentration giving a ΔOD = 0.2 (ΔOD = OD sample - OD Blank) in the test conditions. This Cu was measured for each isotype/anti-isotype system individually so that the following formula could be applied to each sub-class assay:

$C = Cu \times Dil. (\Delta O.D.\ 0.2)$

where C = concentration of anti-insulin immunoglobulin G1 (G2, G3 or G4) in ng per ml of serum; cu = concentration of myelomatous protein (in ng/ml) giving a ΔO. D. = 0.2, Dil. = dilution of the tested serum sample giving ΔO. D. = 0.2





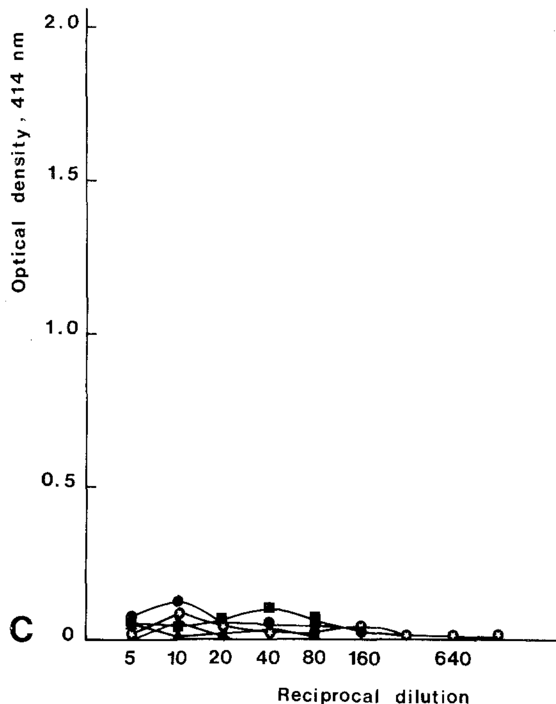


Fig. 2 A–C Total anti-insulin IgG and anti-insulin IgG sub-classes pattern in two insulin immunized patients (**A** and **B**) and in a blood donor (**C**): (■) total IgG, (▲) IgG1, (★) IgG2, (●) IgG3, (✪) IgG4

Results

Coating conditions were optimized to obtain maximal sensitivity for the detection of anti-insulin antibodies. At first, efficiency of coating was improved by raising the pH of coating solutions from 7.4 to 9.6. On the other hand, using HPLC-prepared 125-I Tyr A 14-bovine insulin, we measured the percentage of insulin really adsorbed from various insulin concentrations in the coating solution (0.67 to 200 µg/well). Coating efficiency was the highest (23.7%) with 0.1 µg insulin per well (1.33 µg/ml) (Table 1), a result consistent with that of Nell et al. [12].

As illustrated in Figure 1, ELISA curves obtained with the same AI serum and varying amounts of coated antigen exhibited highest optical density values - and consequently, the best sensitivity - in the range of 6.67–13.3 µg/ml coated insulin per well. An insulin concentration of 13.3 µg/ml in coating buffer was therefore used through all assays.

Optimal dilutions of both monoclonal and polyclonal reagents were established in series of standard three-way chequerboard titrations in order to determine the greatest dilution of each reagent still detecting the smallest concentrations of target antigens. We deduced that monoclonal anti-IgG, anti-IgG1, anti-IgG2, anti-IgG3 and anti-IgG4 could be respectively utilized at dilutions: 1/4,000, 1/1,000, 1/500, 1/2,000 and 1/4,000.

Similarly, optimal dilution of the polyclonal RAH-IgG utilized for coating in the standardization assay was found to be 1/4,000.

It must be mentioned that we encountered an important problem of cross-reaction between rabbit anti-human IgG and mouse monoclonal antibodies. This made it necessary to further purify RAH-IgG by affinity

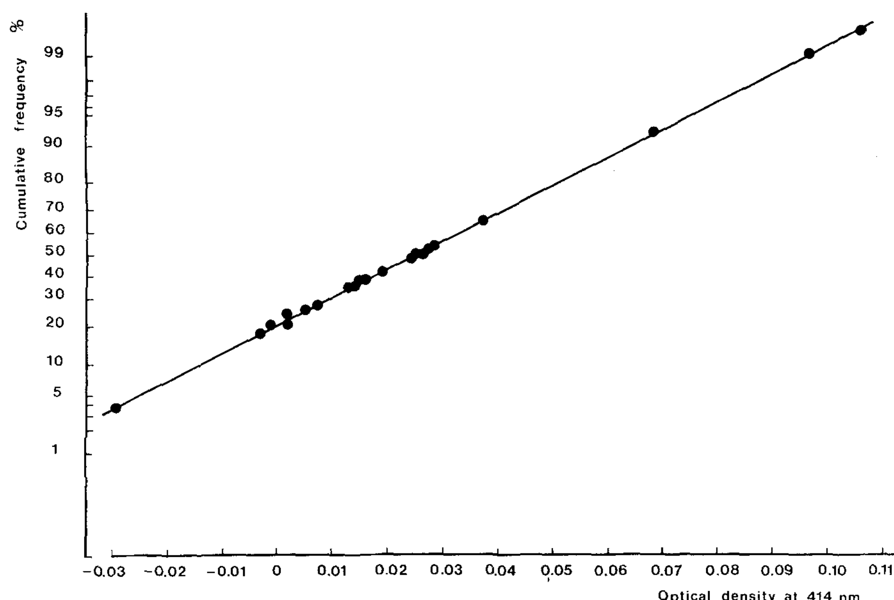


Fig. 3. Gausso-arithmetic distribution of O. D. at 414 nm of 21 normal sera at 1/5 dilution

Table 2. Inter and intra assay reproductibility: coefficient of variation at the level of ΔOD = 0.2. $n = 8$ (patient 2 was chosen for his high level of AI-IgG3, and patient 3 for his high level of IgG4)

Patient No.	IgG	Intra assay	Inter assay
Patient 2	IgG1	23.2%	26.3%
	IgG3	16.6%	11.4%
	IgGT	19.4%	30.3%
Patient 3	IgG1	41.9%	10.3%
	IgG3	22.2%	12.5%
	IgG4	19.2%	15.5%
	IgGT	21.6%	15.7%

chromatography with mouse IgG covalently coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden).

Figure 2 A, B illustrates typical patterns obtained when testing serum samples of insulin-immunized diabetic patients: in the two cases shown, IgG1 was predominant, whereas the second most abundant subclasses were respectively IgG3 (2A) and IgG4 (2B). Figure 2 C shows the pattern obtained with the serum collected from a non-diabetic healthy blood donor. In this instance, no IgG material was detected, demonstrating the specificity of the test. Assay of 21 negative sera from blood donors allowed us to calculate that the threshold of non-specific Ig binding, expressed as the mean O. D. value (mean ± 2 S. D.), in each Ag-Ab system was always lesser than 0.2: for IgG1 (0.039 ± 0.134), IgG3 (0.047 ± 0.127), IgG4 (0.029 ± 0.133) and total IgG (0.038 ± 0.135). As illustrated by Figure 3, blank values displayed a Gaussian distribution.

The standard curves obtained with serial dilutions of myelomatous proteins are shown in Figure 4. Satisfactory standard curves were obtained for IgG1, IgG3 and IgG4, but not for IgG2. The strong cross-reaction between RAH-IgG and monoclonal anti-IgG2 persisted after affinity chromatography and was also encountered with the other anti-IgG2 monoclonal antibodies we tested: anti-IgG2 from Seward (BAM 14, clone GOM 2) and from Bio-Yeda (6010-1, Rehovot, Israel). Because of this persistent cross-reaction, IgG2 were detectable in the serum of patients but not quantifiable by the immuno capture assay. On the contrary, the sensitivity limit of the assays for IgG1, IgG3, IgG4 and total IgG was respectively: 8.9 ng/ml, 12.4 ng/ml, 6.3 ng/ml and 4.5 ng/ml. The lack of accuracy in IgG2 measurements is one of the reasons why the presently described test was qualified as semi-quantitative.

Inter and intra assay reproducibility was assessed with two different sera (Table 2). At the threshold of ΔO. D. = 0.2, the average coefficient of variation was 20.4% (range from 10.3 to 41.9%), and this rather high value provides a second reason for the assay to be considered as semi-quantitative.

The results obtained with a series of 24 insulin immunized diabetic patients are shown in Table 3. They are expressed in ng equivalent myelomatous protein per ml of serum according to the formula described in the method section. In this table, "zero" indicates that O. D. values obtained with a given serum were not significantly different from blanks, and "traces" (TR) means that O. D. in the presence of serum sample was lower than 0.2 even at initial serum dilution.

All patients presented IgG1 AI antibodies and, in all cases, IgG1 was the predominant anti-insulin IgG subclass. In our series, the mean concentration of IgG1 AI was found equal to 3,980 ± 4,334 ng equivalent myelomatous protein per ml of serum (mean ± S. D.). On the other hand, IgG2 AI was absent or present in trace amount only. Measureable levels of AI IgG3 and IgG4 were found: (541 ± 625) and (286 ± 452) ng equivalent myeloma protein per ml respectively.

Table 3. Anti-insulin (AI) total IgG and IgG sub-classes concentration in the serum of 24 insulin immunized diabetic patients. Results are expressed in ng equivalent myelomatous proteins per ml of serum, and in relative abundance

Patient no.	AI IgG1		AI IgG2	AI IgG3		AI IgG4		Sum of AI IgG sub-cl	Total AI IgG
	ng/ml	%	%	ng/ml	%	ng/ml	%	ng/ml	ng/ml
1	554	72	0	69	9	151	19	774	1119
2	1393	59	Tr	949	41	Tr	0	2342	5610
3	6078	91	0	249	4	330	5	6657	14576
4	2653	94	0	165	6	0	0	2818	7395
5	2311	70	Tr	865	26	109	4	3285	5610
6	2013	83	0	321	13	100	4	2434	3881
7	1753	71	Tr	454	19	245	10	2452	4256
8	3925	86	0	300	6	362	8	4587	5236
9	1753	76	0	509	22	41	2	2303	4888
10	7308	92	0	656	8	Tr	0	7964	9310
11	2593	83	Tr	233	8	301	9	3127	3330
12	19221	89	Tr	2275	11	0	0	21496	23931
13	1330	71	Tr	169	9	379	20	1878	1694
14	11319	78	Tr	2495	17	757	5	14571	16942
15	5056	68	0	533	7	1815	25	7404	5482
16	10563	83	Tr	753	6	1442	11	12758	12559
17	1213	67	0	414	23	173	10	1800	1656
18	1009	93	0	0	0	79	7	1088	2339
19	2778	92	0	198	7	34	1	3010	2339
20	1132	94	Tr	0	0	69	6	1201	2748
21	3418	81	Tr	599	14	186	5	4203	3013
22	2778	98	Tr	67	2	0	0	2845	5482
23	2653	80	0	414	13	229	7	3296	1858
24	714	66	0	300	28	69	6	1083	975

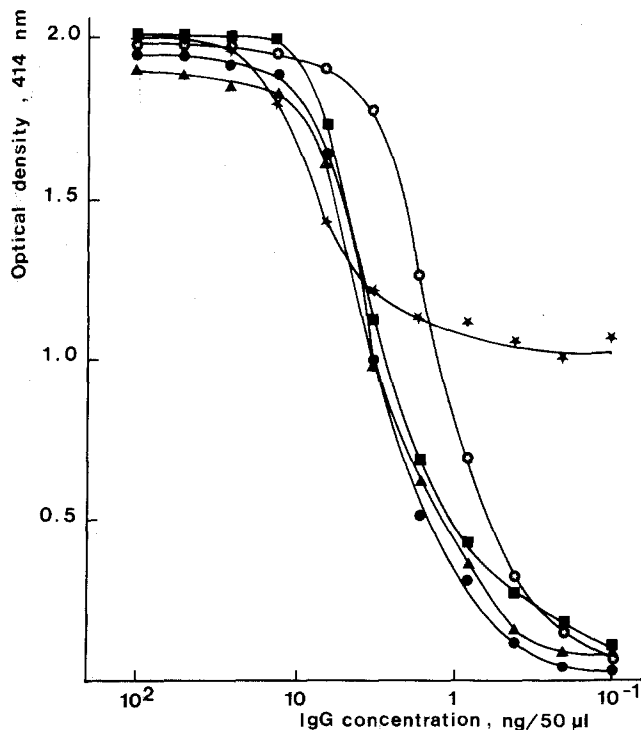


Fig. 4. Standardization ELISA curves obtained with purified total IgG and myelomatous proteins of each IgG isotype: (■) total IgG, (▲) IgG1, (★) IgG2, (●) IgG3, (✪) IgG4. $n = 18$ replicates

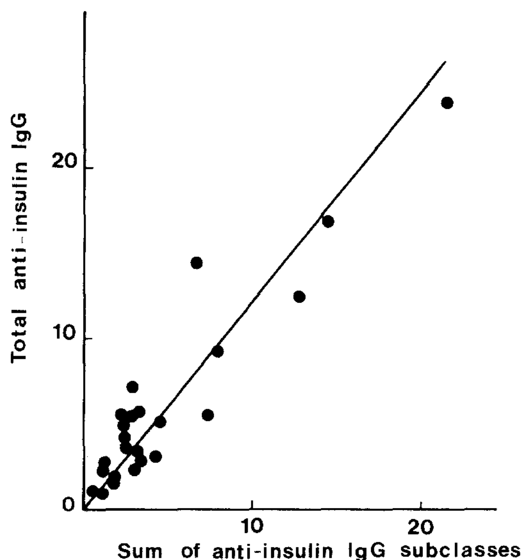


Fig. 5. Correlation between total anti-insulin IgG (Y) and the sum of anti-insulin IgG sub-classes (X): $Y = 1.051\ X$, $r2 = 0.928$, $n = 24$

It is interesting to note that AI IgG3 were more abundant than IgG4 in 17 out of 24 patients (i. e. in 70% of cases), whereas an inverse pattern was observed in 7/24 patients (30%). Some patients lacked IgG3 AI (patients No 18, 20) or IgG4 AI (patients No 2, 4, 10, 12, 22).

For each patient, the sum of IgG1, 3 and 4 was calculated and taken as 100% value in order to assess the

relative abundance of each IgG sub-class implicated in AI immune response. On the average, the relative participation of anti-insulin IgG sub-class was respectively 81% for IgG1, 12% for IgG3 and 7% for IgG4. (Table 3). As shown by Figure 5, we observed a correlation ($r2 = 0.928$) between the sum of IgG sub-classes (calculated) and total IgG as experimentally determined in an assay utilizing anti-γ chain reagent.

Discussion

Immunosorbent assays have already been applied to the detection of anti-insulin IgG antibodies using either enzyme conjugated anti-human IgG reagents [13-15] or 125-I-protein A [10, 16].

In order to detect individually each IgG sub-class, we engaged monoclonal antibodies specific for each isotype in a four step procedure. This method offers the advantage of increasing the test sensitivity and specificity. Indeed, Moudallal et al. [17] demonstrated that multiplying the number of immunological layers of sandwich tests results in a significant enhancement of sensitivity. Furthermore, owing to the properties of anti-isotypic monoclonal antibodies, only one epitope is recognized so that no cross-reaction can occur.

This ELISA assay allows a specific detection of AI IgG antibodies, since no reaction was observed with normal sera.

The assay of AI IgG sub-classes described in this report should be regarded as semi-quantitative rather than quantitative for several reasons. We know that optical densities are related to the amount of human IgG bound to immobilized insulin, but we do not know to what extent these detectable IgGs represent exhaustively or not all circulating AI antibodies. Several parameters, some of them being hardly controllable, may interfere and prevent full detection. The serum of insulin immunized diabetic patients contains free antibodies, but also insulin-anti-insulin immune complexes. Some of these complexes probably react with coated insulin, and others partially or totally escape detection. Pretreatment of serum with acid (pH 2.8), charcoal, chaotropic agents or a combination of them could have been added as a preliminary step but, to our knowledge, these extraction methods [18] dissociate mainly immune complexes involving weak affinity antibodies and introduce an error in re-establishing the original volume. Therefore, the amount of detectable human anti-insulin IgG sub-classes depends not only upon the proportion of free antibodies but also upon the potential dissociation of immune complexes.

An additional potential difficulty of this assay stems from the fact that several anti-insulin sub-classes are present in most sera. Thus, each sub-class competes with the others for binding to the immobilized ligand. As a consequence, the amount of insulin available for binding will be influenced by the proportion of each isotype, the others acting as competitors. It is therefore conceivable that in hyperimmunized patients, a poorly represented anti-insulin IgG sub-class will be partially or even fully masked by the most abundant one.

Another point is the choice of appropriate standards. Since human specific anti-insulin IgG isotypes have not yet been purified, we used an immuno capture assay with RAH-IgG coated plates and human myelomatous protein of each sub-class. Since the cross-reaction between anti-IgG2 monoclonals and coated RAH-IgG persisted even after affinity purification, standardization of IgG2 sub-class was unfortunately not possible. Nevertheless, we can conclude from our data that this isotype was generally absent or present in amount not exceeding 0.2 μg/ml in the present series of immunized patients.

Despite the fact that the method is semi-quantitative, it is interesting to note that the sum of sub-classes was correlated to total anti-insulin IgG.

The relative abundance of anti-insulin IgG sub-classes differs from their proportion in normal serum, particularly with regard to IgG2 which are lacking. Some immunogenic proteins and glycoproteins are known to preferentially stimulate IgG1 and IgG3 sub-classes rather than IgG2 and IgG4. This is, for example, the case for most blood group antigens and especially the Rhesus (D) antigen. On the other hand, bacterial wall polysaccharides have been described to elicit an almost pure IgG2 humoral response. Imbalanced repartition of specific IgG antibodies is therefore not necessarily surprising. It may be speculated that insulin induces a specific pattern of response characterized by a lack of IgG2 production.

In classical conditions, patients are challenged with antigen for a short period of time (vaccination, transfusion, infection). In insulin-treated patients, the antigen is daily administered so that the clearance rate of anti-insulin IgG sub-class probably reflects that of immune complexes rather than that of free circulating antibodies.

Aside from insulin-dependent diabetes, haemophilia is another disease in the course of which a protein is frequently administered. It is worth noting that the profile of anti-factor VIII immunization also shows an abnormal IgG distribution, since it is characterized by a higher than expected abundance of IgG4 [2, 19]. However, we must keep in mind that coagulation factor VIII is a glycoprotein whereas insulin does not contain any saccharidic components.

Additional investigations will be undertaken in order to determine whether conclusions drawn from the analysis of these 24 sera can be generalized to all insulin immunized patients and to establish potential correlation between anti-insulin IgG sub-class pattern and clinical course.

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References

1. Siber GR, Schur PH, Aisenberg AC, Weitzman SA, Schiffman G (1980) Correlation between serum IgG2 concentrations and the antibody response to bacterial polysaccharide antigens. N Engl J Med 303: 178-182
2. Robboy SJ, Lewis EJ, Schur PH, Colmal RW (1970) Circulating anticoagulants to factor VIII. Immunochemical studies and clinical response to factor VIII concentrates. Am J Med 49: 742-752
3. Linde GA (1985) Sub-class distribution of rubella virus specific immunoglobulin G. J Clin Microbiol 21: 117-121
4. Sundqvist VA, Linde A, Wahren B (1984) Virus specific immunoglobulin G sub-classes in herpes simplex and varicella zoster virus infections. J Clin Microbiol 20: 94-98
5. Hardman JT, Beck ML (1981) Hemagglutination in capillaries: correlation with blood group specificity and IgG sub-class. Transfusion 21: 343-346
6. Mattila PS, Seppala IJT, Eklund J, Makela O (1985) Quantitation of immunoglobulin classes and sub-classes in anti-Rh (D) antibodies. Vox Sang 48: 350-356
7. Yagi Y, Maier P, Pressman D (1962) Two different anti-insulin antibodies in guinea pig antisera. J Immunol 89: 442-451
8. Sodoyez J-C, Sodoyez-Goffaux F, Arquilla ER, McDougall B, De Vos CJ (1985) Effect of IgG subclass on the metabolic fate of insulin immune complexes in passively immunized rats. Diabetes Res Clin Pract Suppl 1: S525 (Abstract)
9. Engvall E, Perlmann P (1972) Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen coated tubes. J Immunol 109: 129-135
10. Sakata S, Imai T, Kamikubo K, Nagai K, Okuyama M, Sato H, Kashiwamata S, Miura K (1983) A solid phase radioimmunoassay for the detection of anti-insulin antibodies. Endocrinol Jpn 30: 23-28
11. Husby S, Jensenius JC, Svehag SE (1985) ELISA quantitation of IgG subclass antibodies to dietary antigens. J Immunol Meth 82: 321-331
12. Nell LJ, Virta VJ, Thomas JW (1985) Application of a rapid enzyme-linked immunosorbent microassay (ELISA) to study human anti-insulin antibody. Diabetes 34: 60-66
13. Wilkin T, Nicholson S, Casey C (1985) A micro enzyme-linked immunosorbent assay for insulin antibodies in serum. J Immunol Meth 76: 185-194
14. Telford ME, Wisdom GB (1979) Enzyme-immunoassay of anti-(bovine insulin) antibodies. Biochem Soc Trans 7: 527-529
15. Kato K, Haruyama Y, Hamaguchi Y, Ishikawa E (1978) Comparison of three enzyme-linked procedures for the quantitative determination of guinea pig anti-porcine insulin antibody. J Biochem 84: 93-102
16. Hamilton RG, Rendell M, Adkinson NF Jr (1980) Serological analysis of human IgG and IgE anti-insulin antibodies by solid-phase radioimmunoassays. J Lab Clin Med 96: 1022-1036
17. Moudallal ZA, Altschuh D, Briand JP, Van Regenmortel MHV (1984) Comparative sensitivity of different ELISA procedures for detecting monoclonal antibodies. J Immunol Meth 68: 35-43
18. Dixon K (1974) Measurement of antibodies to insulin in serum. Clin Chem 20: 1275-1281
19. Andersen BR, Terry WD (1968) Gamma G4-globulin antibody causing inhibition of clotting factor VIII. Nature 217: 174-175

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