Prevalence and persistence of C1q binding activity in healthy subjects

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SUMMARY Samples of serum from 885 normal healthy blood donors were tested for the presence of soluble immune complex-like material by a solid-phase C1q binding assay. The majority of donors (93%) had low or undetectable levels of C1q binding activity in their sera, but 6% had levels that were clearly outside the normal distribution. When these individuals were retested after several weeks half of them still had elevated levels of C1q binding activity.

Assays for the detection of immune complexes using human C1q adsorbed to the surface of plastic tubes (the solid-phase C1q assay) have been described by Svehag,¹ Farrel *et al.*,² Zubler *et al.*,³ and Hay *et al.*⁴ We used a modification of the solid-phase C1q binding assay with staphylococcal protein A as a probe to detect immune complex-like activity.

The aims of this study were to define a normal range for immune complex-like activity and, using the data obtained, to decide how to define a normal value clinically or statistically. We examined sera from 885 blood donors; a study of such magnitude has not previously been documented.

Material and methods

SERUM SAMPLES

Serum samples were obtained from 885 normal healthy blood donors attending the Blood Transfusion Centre, Royal Infirmary, Edinburgh. Samples were separated and stored initially at 4°C overnight and then at -40°C.

C1q

C1q was prepared from human serum by the rapid purification method of Yonemasu and Stroud.⁵ It was precipitated three times in the presence of chelating agents at low ionic strength, and the purified protein was stored at -40°C. The purity of the preparation was assessed by immunodiffusion, and trace amounts of IgG and IgM were present: <0.005 mg/ml IgG and <0.01 mg/ml IgM.

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AGGREGATED IgG

Normal human immunoglobulin (IgG), prepared for therapeutic use, was adjusted to 10 mg/ml in phosphate buffered saline (PBS), pH 7.2, and heated at 63°C for 15 minutes. Further purification of the heat-aggregated IgG was carried out by passing the suspension over Sepharose 6B (Pharmacia Fine Chemicals, Sweden) in PBS to remove 7S IgG and small aggregates.

The purity of the IgG was assessed by immunoelectrophoreses using a polyvalent anti-human serum, and trace amounts of albumin and IgM were present.

RADIOIODINATION OF PROTEIN A

Radioiodination of protein A was performed using a modification of the chloramine T method of Hunter and Greenwood.⁶ Fifty microlitres aqueous protein A (1 mg/ml) (Pharmacia Fine Chemicals, Sweden) was labelled with 1 mCi ¹²⁵I (10 µl) (Radiochemicals, Amersham) by the addition of 5 µl chloramine T (0.08 mg/ml) (BDH) in PBS. The reaction was stopped, after mixing for 1 minute, by the addition of 50 µl sodium metabisulphite (1 mg/ml) (BDH) in PBS. Of 5% bovine serum albumin in PBS 250 µl was added to the reaction mixture, and the iodinated protein was separated from free iodine by gel filtration on Sephadex G25.

C1q BINDING ASSAY

The assay was a modification of the method of Hay *et al.*⁴ One-millilitre volumes of C1q, containing 5 µg/ml in PBS, were incubated in polystyrene tubes (LP3, Luckham Ltd, Sussex) for 20-24 hours at 4°C, and the tubes were then washed three times to

remove any unbound C1q. Fifty-microlitre volumes of test sera were incubated with 100 μ l EDTA (0.2 M, adjusted to pH 7.5 with NaOH) at 37°C for 30 minutes to inactivate complement. The inactivated test sera (50 μ l in 950 μ l PBS) were allowed to bind to the solid-phase C1q by incubation in the C1q-coated tubes for 60 minutes at 37°C and for 30 minutes at 4°C. All samples were tested in duplicate. Unbound proteins were then removed by washing three times with PBS. Immune complexes bound to the C1q-coated tubes were detected by incubating the tubes with 25 ng radiolabelled protein A (125 I) in 1 ml PBS containing 0.05% Tween for 60 minutes at 37°C and for 30 minutes at 4°C. Unbound labelled reagent was removed by washing three times with PBS. The tubes were then counted in a gamma counter (Gamma, 3.33), and the average counts for a given sample were computed. These results were referred to a standard binding curve prepared by incubating serial dilutions of heat-aggregated human IgG in inactivated normal human serum. C1q binding activity was expressed as μ g/ml equivalents of heat-aggregated IgG.

Results

SENSITIVITY OF THE C1q BINDING ASSAY FOR THE DETECTION OF HEAT-AGGREGATED IgG
Staphylococcal protein A was used to detect various concentrations of heat-aggregated IgG bound to solid-phase C1q. In all experiments, the C1q concentration used when coating the polystyrene tubes was 5 μ g/ml. The minimal concentration of heat-aggregated IgG that could be detected was 1.0 μ g/ml. Tubes not coated with C1q showed negligible binding of the iodinated protein A preparation. There was also very little binding of unaggregated IgG or of the 7S monomeric fraction from the Sepharose 6B separation column (Fig. 1). The values shown are the mean $\pm$ 1 standard deviation of 10 determinations.

Fig. 1 C1q binding activity of heat-aggregated IgG. Values shown are the mean $\pm$ 1 SD of 10 determinations.

REPRODUCIBILITY OF THE C1q BINDING ASSAY
The reproducibility of the assay was examined by testing consecutively aliquots of increasing concentrations of heat-aggregated IgG diluted in normal human serum. Serum from a patient with systemic lupus erythematosus known to contain immune complexes was also tested on 10 separate occasions (Table).

Reproducibility of the C1q binding assay

Heat-agg. IgG (μ g/ml)	No. of determinations	Mean (cpm)	1 SD	%CV
500	10	13701	832	6
250	10	12827	978	8
125	10	11567	1153	10
62	10	10193	1125	11
31	10	8418	1140	14
16	10	7005	545	8
8	10	5665	525	9
4	10	4721	557	12
2	10	3914	449	11
1	10	3670	285	8
Patient serum	10	6318	305	5

CV = coefficient of variation.

The coefficient of variation in C1q binding of increasing amounts of heat-aggregated IgG was <15%, and the mean percent difference for 10 replicates of a known positive sample was 5%.

EFFECT OF STORAGE AND FREEZE-THAWING ON SERUM SAMPLES

Sera from 10 normal healthy donors were stored in multiple aliquots of 1 ml at 4°C overnight or at -40°C. The effects of storage overnight at 4°C and of repeated freeze-thawing cycles were evaluated.

Data analysis using Student's paired *t* test showed that there was no significant increase in C1q binding activity when serum samples were stored overnight at 4°C compared to fresh serum samples ($0.20 < P < 0.40$). There was, however, significantly higher C1q binding activity after one freeze-thaw cycle ($0.05 < P < 0.025$) and even greater significance after two freeze-thaw cycles ($0.05 < P < 0.01$).

ESTIMATION OF A NORMAL RANGE

Using the modified C1q binding assay, we examined the sera from 885 normal healthy individuals. Analysis of the C1q binding activity data showed that there was a continuous range of results, the majority of blood donors (94%) having low levels of activity within a narrow band (Fig. 2).

Since the data obtained from these normal individuals cannot be described statistically by Gaussian or log-Gaussian curves, non-parametric methods for estimating a normal range were applied. The method of percentile estimates was used to

Fig. 2 Distribution of C1q binding activity in 885 normal blood donors.

define the normal range or limits.⁷ Estimation of the 95th percentile (ie, upper 5th percentile) has shown that 6% of 885 donors show abnormal C1q binding activity.

The 885 blood donations were collected over 11 separate blood donor sessions. Those donors whose serum showed abnormal C1q binding activity were recalled after one month and again six to nine months after the original donation had been collected. The retestings of these individuals indicated that one-half of them still had elevated levels of binding after one month and one-quarter of them still showed increased C1q binding activity after six to nine months (Fig. 3).

Fig. 3 C1q binding activity in normal individuals followed for several months. The individuals who showed positive binding initially (●—●) were retested after 1 month (●---●) and after six to nine months (X—X).

RELATION OF AGE AND SEX TO C1q BINDING ACTIVITY

There was no correlation between either the donor's age or sex to C1q binding activity (data not shown).

RELATION OF AUTOANTIBODY LEVEL TO C1q BINDING ACTIVITY

A preliminary study of 20 serum samples with elevated C1q binding activity showed that there was no relation between raised C1q binding and auto-antibody levels.

Discussion

In this study we have defined a normal range and demonstrated the presence of detectable amounts of circulating immune complex-like material in the sera of some of the normal individuals. There has been an obvious lack of information on 'normals' in the existing literature on immune complex studies; no large studies have attempted to define a normal range or to investigate the prevalence or persistence of abnormal levels in a normal population.

We used a modification of the solid-phase C1q binding assay, with staphylococcal protein A as a probe in the detection of C1q binding activity. There were several variations from existing assays in the procedure. Hay *et al.*⁴ used more C1q per assay tube, inactivated the test sera at 56°C, and incubated the sera with iodinated protein A overnight at 4°C. The sensitivity of the assay used in our study was such that 1.0 µg/ml or less of heat-aggregated IgG could be detected. The advantages of being able to detect such small quantities of immune complex-like material were important in our large study, especially when attempting to follow the persistence of low levels of binding in some individuals. The reproducibility of the assay was good, and variability among replicates was not sufficient to cause high C1q binding among some of the normal sera. The possibility that the elevated C1q binding results observed could be explained by the overnight storage at 4°C was investigated. Storage at 4°C overnight had no apparent effect on C1q binding activity, although repeat freeze-thawing cycles induced C1q binding. These observations confirm the results of Rossen *et al.*,⁸ which also showed that more than one freeze-thaw cycle considerably altered the reproducibility of the C1q binding activity in some sera.

Statistical analysis of the C1q binding data in normal individuals was by non-parametric methods. These can be applied to estimate a normal range, regardless of the underlying form of the statistical population from which the data were obtained. Any 'bumps' in the distribution curve of normal indi-

viduals could be accounted for by either technical variation or perhaps by the presence of subgroups in the normal population.

There could be many biological explanations for the occurrence of low levels of C1q binding activity in the normal population. The low levels of immune complex-like activity may be due to the variation expected within a normal population. Not all immune complexes may be damaging, since our sensitive methods of detection have shown low levels of C1q binding in people with no apparent disease. Perhaps some normal individuals show elevated C1q binding activity as they encounter and react to various environmental antigens.⁹ Post-prandial immune complexes, which are usually cleared by the macrophages of the reticuloendothelial system, may be deposited in the other tissues if the macrophage function is blocked in some other way. There may be a relation between the detection of food antibodies, for example, milk proteins, and complexes in normals. It is also important to recognise that the incidence of positive sera detected in assays depends to some extent on the population selected as controls. In this study we selected as normal controls volunteer blood donors who were able to affirm that they had not recently been ill. Future experience with other apparently normal but less vigorously controlled populations, for example, hospital laboratory staff, may produce a higher value on the upper limit for 'normal' C1q binding activity and therefore a lower incidence of abnormal levels. Theofilopoulos *et al.*¹⁰ reported detectable amounts of immune complexes in normals (19%) and suggested that the discrepancy between the number of positive sera detected in two different 'normal' populations was due to the selection of the donors, that is, staff v normal blood donors.

Further investigations into the 'normality' of our normal blood donors with low C1q binding levels were carried out. It was noted that one of the 'positive' donors had allergies to dust and pollen, two donors had minor cold infections at the time of retesting, two had high rubella antibody titres, and one donor had nursed Q-fever. Another interesting observation was that the original study took place in the late autumn and winter when contact with cold and 'flu viruses was common. The second retesting of the 'positive' sera took place some six to nine months later when the incidence of colds and 'flu should have decreased, and in fact the levels of C1q binding activity had almost reverted to normal. There was no apparent correlation between age, sex, and C1q binding activity or between autoantibody levels and C1q binding.

There are many problems in defining the normal range. The sensitivity and reproducibility of the assay are important, as is storage of the serum samples under test. The selection of the population of 'normals' used in a study and the statistical analysis of the data should also be considered. The data from this study suggest that care should be taken in accepting and using the term 'normal'. The low levels of immune complex-like activity are perhaps universal, and errors of clinical interpretation may arise from a failure to recognise the variability in a normal population.

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