PLATELET-AGGREGATING IMMUNE COMPLEXES IN IDIOPATHIC GLOMERULONEPHRITIS AND SLE

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Summary

Immune complex-like material with a molecular weight of greater than 500,000 Daltons was detected in the majority of sera from patients with idiopathic glomerulonephritis, as well as in systemic lupus. In the Raji cell and Clq binding assays the glomerulonephritis sera gave, in general, negative results. Patients with membranous nephropathy, minimal change disease and Henoch-Schönlein purpura also gave positive results in the platelet test, in agreement with other tests not dependent upon complement fixation by the complexes. The amount of platelet-aggregating material detected in vitro correlated closely with in vivo depletion of intraplatelet amines, judged by serotonin concentrations; which suggests that this material may play a major role in platelet activation within the circulation.

Introduction

In general, assays believed to detect immune complexes in patients’ sera are negative in patients with idiopathic glomerulonephritis [1]. Only in the nephritis of systemic lupus, in post-infectious nephritis, and in mesangiocapillary glomerulonephritis are positive results the rule. Since it is believed that most forms of glomerulonephritis result from the formation, circulation and deposition of soluble immune complexes within the kidney, this result is surprising. However, the most popular assays (binding to Raji cells and Clq binding) depend upon the complexes activating complement; and non-complement-fixing complexes have been much less studied. Rarely have several assays been used on the same sera and the results compared.

As part of the study of platelet involvement in glomerulonephritis [2] we undertook to investigate platelet-aggregating material (PAM) in the sera of patients with glomerulonephritis and with systemic lupus erythematosus (SLE). We used first the platelet aggregation test as described by Penttinen [3–5]. The results were compared with results on the same sera obtained with the Raji cell assay and the fluid phase Clq binding assay.
Methods

Patients were all attending the Renal Unit at Guy’s. All had had a renal biopsy except four children with a steroid-sensitive relapsing nephrotic syndrome, who have been included with those whose renal biopsies were classified as ‘minimal change’; and six children with classical acute Henoch-Schönlein purpura. Biopsies were taken with a Franklin-Vim-Silverman needle, processed for optical, immunofluorescent microscopy and embedded in plastic. Appearances were classified as described by Turner [6]. Systemic lupus erythematosus was diagnosed by at least four of the criteria of the American Rheumatism Association, and at least one observation of an elevated binding of double stranded DNA in the serum. Sera were obtained by allowing blood to clot at room temperature in glass tubes. The serum was separated within 4h, aliquoted and frozen at −70°C until required.

The platelet aggregation test was performed essentially as described [3–5]. For each run, 20ml of blood was taken from a healthy donor (usually the same individual) into 5ml of acid citrate-dextrose (ACD) and mixed by gentle inversion in a polycarbonate container. The platelet-rich plasma (PRP) was obtained by centrifugation at 136g for 20 minutes, and the supernatant PRP spun at 800g for six minutes to obtain a platelet button. This was resuspended in 2ml of calcium and magnesium-free phosphate buffered saline, pH 7.4. The platelet concentration was measured in a Coulter model ZF counter and the final concentration of platelets adjusted to 150,000/μl.

The platelet aggregating titre of each serum was determined in plastic microtitre plates (Flow Laboratories, Irvine, Scotland). To each well was added 50μl of platelet suspension, 25μl of buffer and doubling dilutions of 25μl serum. Plates were read after 18 hours at 4°C, fuzziness being taken as an indication of agglutination. Positive and negative controls were incorporated in each plate (anti-platelet antibody, aggregated human gamma globulin, normal human serum).

Intraplatelet serotonin concentration was measured spectrophotofluorimetrically as previously described [7]. Gel filtration chromatography was performed on 66 by 1.5cm sepharose CL6B columns, using phosphate buffered saline at pH 7.2 to elute 1.5ml of serum at 4°C. Portions of fractions were pooled and concentrated using diaflo PM 10 membranes.

The Raji cell assay [8] and Clq binding assay [9] were performed as previously described.

Results

The PAT could detect approximately 5μg/ml of aggregated human gamma globulin (or equivalent amounts in serum). The titre was not affected by addition of 1U/ml of the thrombin antagonist hirudin, or by purified monoclonal IgM anti-IgG antibody up to 1mg/ml. The addition of pure Clq in concentrations of 50μg/ml reduced the titre of positive sera by two dilutions, but larger amounts of Clq up to 200μg/ml did not further inhibit (results not shown). The PAM eluted from the sepharose column with an apparent molecular weight of greater than 500,000 in
Seharose CL/6B fractions

Figure 1. Elution patterns of sera from three patients with glomerulonephritis and one patient with SLE, chromatographed on seharose CL6B columns. The continuous line indicates the pattern of protein elution (E_{280}). Unless otherwise indicated fractions were negative in the platelet aggregation test. + = positive 1/1; ++ = positive 1/2; +++ = positive 1/4; ++++ = positive 1/8 in unconcentrated fractions. Note the absence of positivity at the elution point of monomer IgG.

Patients with SLE, mesangiocapillary glomerulonephritis, membranous and IgA nephropathy and polyarteritis (Figure 1). No material with a molecular weight consistent with monomer IgG antibody or thrombin was detected, even in 20-fold concentrated fractions. In contrast, all the Raji cell positive material eluted at the monomer IgG position in eight SLE patients.

The platelet aggregating titres obtained in 33 normal subjects, 114 patients with various forms of glomerulonephritis, and 55 patients with systemic lupus and nephritis (50 with and 5 without clinical nephritis) are shown in Figure 2 compared with the results in the same sera using the Raji cell and Clq binding assays. Figure 3 demonstrates the correlation between the amount of PAT and the degree of depletion of intraplatelet serotonin, believed to indicate in vivo platelet activation [7, 10].
Figure 2 (a)

Figure 2 (b)
Figure 2. Results of (a) platelet aggregating titres, (b) Clq binding assays, and (c) Raji cell assays in sera from patients with glomerulonephritis. The results are expressed as (a) maximum dilution of serum to show positive in the platelet aggregation test, (b) binding of Clq, and (c) equivalent concentration (µg/ml) of aggregated IgG. The dotted lines indicate the upper limit of normal for the Clq and Raji cell assays; in the case of platelet aggregation test more than 90% of normal sera showed a titre of 1/2 or 1. Histological groups: Min. Ch. = minimal change nephropathy; Mes. Pro. = mesangial proliferative glomerulonephritis; Foc. Pro. = focal proliferative glomerulonephritis; FSGS = focal and segmental glomerulosclerosis (hyalinosis); Membr. = membranous nephropathy; MCGN = mesangiocapillary glomerulonephritis; AGN = acute post-infectious nephritis, including shunt nephritis; HSP = Henoch-Schönlein purpura nephritis; PAN = polyarteritis nodosa; SLE = systemic lupus erythematosus.

Figure 3. Results of the platelet aggregation test (horizontal axis) on serum, and intraplatelet serotonin of platelets taken at the same time as the serum sample (vertical axis). The normal range for the latter is 300–980ng/10^9 platelets. Data are shown for (a) 124 sera from patients with SLE nephritis, and (b) 86 samples from patients with various forms of glomerulonephritis.
Discussion

In our hands the PAT proved simple, reliable and sensitive. It appears to detect immune complexes in the sera of the majority of patients with glomerulonephritis, since the PAM detected is neither thrombin, antiplatelet antibody, nor low molecular weight platelet-activating factor (PAF) [11]. In contrast, the Raji cell test detected material in only the patients with SLE, and the Clq binding test only in patients with SLE, post-infectious nephritis, and in MCGN; this is as previously reported [1]. Although the PAT as performed is unphysiological (being performed in calcium- and magnesium-free buffer in which platelets can agglutinate but not undergo release) the good correlation between the in vitro PAT and the in vivo depletion of intraplatelet serotonin (Figure 3) suggests that this material may be an important — perhaps the dominant — source of platelet activation in the circulation.

The finding of high molecular weight PAM in membranous nephropathy is in disagreement with other previously reported studies, and our own data using Clq binding and Raji cell assays [1]; but is in agreement with the finding that most patients with membranous nephropathy have circulating cryoglobulins [12] with the results of other immune complex assays not dependent upon complement fixation by the complex [13] and a recent report using the PAT [14]. This suggests that the apparent absence of circulating immune complexes in membranous nephropathy cannot be cited as evidence for in situ immune complex formation within the glomeruli; it appears likely that non-complement-fixing complexes are abundant in the circulation of patients with membranous nephropathy.

This also appears to be true in both Henoch-Schönlein purpura and in minimal change nephropathy. Again, non-complement dependent assays using rheumatoid factor agree with the results presented here [15, 16].

We suggest that more than one method should be used to study immune complexes in glomerulonephritis, and that one of the assays used should be an assay which is independent of complement fixation by the complex. The platelet aggregating test appears to fulfill the criteria of reproducibility and sensitivity for this, although rheumatoid factor assays [15, 16] and K cell assays [17] would also be suitable. The Raji cell assay detects material other than immune complexes in SLE, and fails to detect the complexes in a number of types of glomerulonephritis. It is therefore not a suitable technique for this purpose.

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References

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Open Discussion

STRUVENBERG (Chairman) May I start by asking whether you have found any correlation between activity of SLE and positivity of your test?

CAMERON A correlation yes, but a very poor one. I think that that is a difficult question to answer because it is very easy to doctor the data in such a fashion that you can produce beautiful graphs. If you take a group of patients who are already on intensive immunosuppression, then by and large you get no correlation. I think that there is a correlation between this material and this emerges best in the serial studies which, as I mentioned, we have, but have not shown to you this morning. So the answer is yes, if you study the acute patients serially; and no, if you just take a population of lupus patients ‘off the street’!

BROWN (Sheffield) I wonder if you’ve ever looked histologically at your platelet buttons, either by immunofluorescence or immunoperoxidase?

CAMERON No, but we’re looking at platelet-bound immunoglobulin at the moment by a rather elegant immunoperoxidase method described by Leporvlier in France, but we haven’t got any results from this yet. It is well documented in the literature (by at least half a dozen techniques) that in lupus there is a large amount of platelet-bound immunoglobulin. To my knowledge there is no data yet on glomerulonephritis, and that we hope to provide within the next month or two.

HOUWERT (Utrecht) In your group of SLE patients you had only positive results in your Raji cell assay and the other groups were negative?

CAMERON Yes, there were a few positive.

HOUWERT Did you look for lymphocytotoxins in these groups of patients, because you stated that the Raji cell positives are not immune complexes.

CAMERON Yes, this paper was on the platelet-aggregating material and not on the Raji cell assay, on which I could give another paper. Basically the answer is
yes, we have looked at the lymphocytotoxins; and the material is not Raji-cytotoxic, nor is it peripheral blood lymphocytotoxic. However this does not mean it cannot be a non-cytotoxic cell-fixing antibody for example against fetal antigens. Certainly the Raji-positive material has a molecular weight of around 160000 in the majority of 10 patients with lupus, and only one of those has high molecular weight material reacting in the Raji cell assay. If this causes some of you some surprise, I would suggest that you go back and look at the ultracentrifugation pattern published by Woodruffe and Theofilopoulos in 1977 in their paper in Kidney International, but which received no comment there or from other people. This showed precisely the same thing, i.e. material eluting in sera from nephritis and in lupus with a molecular weight consistent with monomer IgG and not with a larger molecular weight complex.

REES (London) You alluded to the problem of variability of the source of platelets. Can I ask two questions in relation to this. The first is how much variability is there in healthy subjects between the source of platelets? And secondly, in those that are not healthy, those that have the diseases which you have studied if you use their own platelets?

CAMERON We have used lupus platelets in this system. There are many problems with this, as you can imagine. The system dilutes out plasma, so it is essentially in buffer. Certain lupus platelets will aggregate in this system, even though they are almost devoid of platelet amines. This is of some interest and shows probably that the test is independent of intraplatelet amine. So far as the first question is concerned there is a variation of at most about 2 dilutions between individuals, but of course 2 dilutions on a log 2 graph is a large difference in terms of derived concentration. So we’ve tended to use one or two donors, working within a blood transfusion department of course you use pooled platelets. In using this as a screening test for immune complexes, (if these are immune complexes!) then it wouldn’t be a tremendous problem. You shift a small number of patients from negativity to positivity and vice versa. But it is not a negligible problem, and that’s why I put it on the slide.

BONE (Liverpool) Have you measured this activity in patients with other diseases and have you considered whether this might be one of the known acute phase reactants you are picking up?

CAMERON Well, I think if it is one of the acute phase reactants it has to be one with a molecular weight of 500000 to 900000, and if so I would like to know what it is, because most of the acute phase reactants are smaller than that. Yes, we have measured PAM in other diseases, and in other immune complex-associated diseases such as lupus without nephritis, (which I mentioned) and rheumatoid arthritis (which I didn’t). We do find these to be positive, and other people have used the PAT in other diseases such as transplant patients, where, fascinatingly, it is very strongly positive. That we are going to look into.

EGIDO (Madrid) In a model of serum sickness in rats we have observed a desensitisation of platelets to PAF (platelet activating factor) along the disease. In this sense, have you performed the platelet aggregating test on patients’ platelets compared to platelet pools?

CAMERON No we haven’t. We would like to get into this field (i.e. PAF as a platelet-stimulating agent in vitro) but we haven’t done it yet!