Circulating Immune Complexes and Platelet Thromboxane Synthesis in Patients with Insulin-dependent (Type I) Diabetes Mellitus

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SUMMARY

Platelets from diabetic subjects with circulating immune complexes (CIC) synthesized greater amounts of thromboxane than did platelets from CIC-negative patients or controls. In view of the known action of CIC on platelet function, a relationship between these two factors may be suggested in the initiation and progression of microangiopathy in diabetes. DIABETES *33*:728–731, August 1984.

There is evidence from several groups of an increased prevalence of circulating immune complexes (CIC) in diabetic patients.[1-3] It has also been suggested that CIC are pathogenetically related to chronic diabetic complications (reviewed in ref. 4). Whether CIC are directly involved in mediating vessel injury in diabetes is not well established, however.

Platelet abnormalities have been described in experimental[5] and human diabetes,[6-9] consisting in both increased platelet thromboxane synthesis and platelet aggregation. Studies of the mechanism of human platelet release reaction induced by immunologic stimuli, specifically CIC and IgG aggregates, have been made,[10-12] showing that antigen-antibody complexes may stimulate the synthesis and release of prostaglandins.

Preliminary data in diabetes suggest a possible interaction between CIC and platelets in the initiation and progression of microangiopathy.[13] Specifically, CIC appeared to be actively involved in accentuating ADP-induced platelet aggregation and ATP release, possibly by acting through prostaglandin pathways.

On the basis of these observations, we tested the hypothesis of an increased rate of platelet thromboxane synthesis in diabetic patients with CIC, as compared with patients without CIC or with controls.

Childhood prepubertal diabetic subjects with comparable glucose control, assessed by HbA_1 determination, and without complications, were selected for this study to minimize interfering factors on thromboxane synthesis due to age,[14] sex and hormone-influence,[15] hyperglycemia,[16] and angiopathy.[9]

MATERIALS AND METHODS

Participants. Twelve young diabetic patients (5 males and 7 females; mean age, 9.7 ± 2.2 yr) and 10 control subjects (5 males, 5 females) of equivalent age (9 ± 2 yr) with no history of diabetes mellitus or any diseases that might alter platelet aggregation were studied. Neither control nor diabetic subjects had taken aspirin or other medications except insulin for at least 2 wk before the study. All diabetic subjects were on insulin treatment (25 ± 5.6 U/day). The average duration of diabetes was 2.4 yr (range 1–5 yr).

None of the patients had clinical evidence of retinopathy, as assessed by routine ophthalmologic examinations and fluoroscein retinal angiography, or nephropathy (absence of proteinuria).

Blood was collected from the antecubital veins of subjects fasted overnight; 9 vol of blood were drawn into a plastic syringe containing 1 vol of trisodium citrate (0.126 M), and platelet-rich plasma (PRP) was prepared by centrifuging the citrated blood at 150 × *g* for 8 min at room temperature. Platelet counts were performed by phase-contrast microscopy. An additional sample of blood from each subject was obtained simultaneously, allowed to clot at room temperature for 2 h, and then centrifuged. Serum aliquots were stored at −30°C for CIC detection.

Reagents. The following were purchased from commercial sources: (5,6,8,9,12,14,15-^{3}H)-TXB_2, authentic TXB_2, and TXB_2 antibody from New England Nuclear Corp., Boston,

Presented in part at the 19th Annual Meeting of the EASD, Oslo, September 14–17, 1983.
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Received for publication 18 February 1983 and in revised form 4 January 1984.

Massachusetts; bovine thrombin from Behring Institute, L'Aquila, Italy; arachidonic acid (greater than 99% purity) from Sigma Co., St. Louis, Missouri; anti-C3 (Fab 2) from Cappel Laboratories, Cochranville, Pennsylvania; purified C1q, rabbit anti-human IgG, IgA, and IgM from Behringwerke, Marburg, West Germany; insulin antibodies from Calbiochem (La Jolla, California); and alkaline phosphatase (type VII S) and *p*-nitrophenil-phosphate disodium from Sigma.

Rabbit anti-human IgG, IgA, and IgM and insulin antibodies were coupled to alkaline phosphatase type VII S by the method of Voller et al.[17]

p-Nitrophenil-phosphate disodium was used as substrate and dissolved in 1 M diethanolamine HCl buffer, pH 9.8, at a concentration of 1 mg/ml.

Thromboxane B_2 assay. Thromboxane B_2 levels were measured in samples of PRP under conditions similar to those used for aggregation studies;[9] 500-μl aliquots of PRP were incubated in an aggregometer together with 50 μl of thrombin (5 U/ml) or arachidonic acid (final aggregometer concentration, 1.0 mM).

Exactly 60 s after addition of arachidonic acid and 4 min after the addition of thrombin, 550 μl of 100% ethyl alcohol was added and the precipitated protein removed by centrifugation for 2 min.[9]

Thromboxane content was determined in appropriate dilutions of supernatant with a previously described radioimmunoassay.[9]

The platelet count in the PRP was not standardized, but all the TXB_2 levels were normalized to a platelet count of 10^8 platelets/ml.

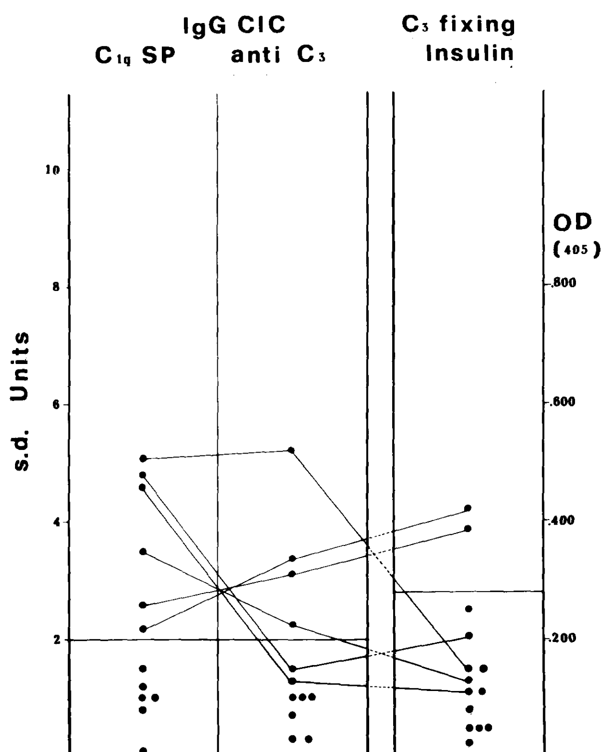

FIGURE 1. Levels of circulating immune complexes (CIC) containing IgG and insulin, as detected by solid-phase C1q binding and anti-C3 EIA.

CIC detection. IgA-, IgM-, and IgG-CIC were detected by an anti-C3 EIA that has been recently described;[3] briefly, wells of microtest plates (Falcon, Becton-Dickinson and Co., Oxnard, California) were coated with anti-C3 (Fab 2) and used as solid phase. Sera were diluted 1:20 in borate-buffered saline (0.1 M, pH 7.5) that contained 1% BSA and 0.05% Tween 20, and were incubated for 2 h at 37°C. After washing, IgA-, IgM-, and IgG-anti-C3–bound immune complexes were determined by adding alkaline phosphatase-labeled anti-human IgA, IgM, and IgG.

IgG-CIC were also detected by the solid-phase C1q-binding enzyme immunoassay adapted from Hay et al.[18] as previously described.[19]

Briefly, wells of microtest plates were coated with C1q (10 μg/ml in PBS 0.05 M, pH 7.2); unreacted sites were blocked by incubation with 2% BSA-PBS. Fifty microliters of test serum were mixed with 100 μl of EDTA (0.2 M, pH 7.4) and incubated at 37°C for 30 min.

After incubation, tubes were cooled on an ice bath, and 50 μl of EDTA-treated sera supplemented with 150 μl PBS were incubated in the C1q-coated wells. After washing, wells were reacted with 2% BSA-PBS containing alkaline phosphatase-labeled rabbit anti-human IgG.

After adding the substrate, plates were incubated again and optical density (OD) read in a Titertek Multiscan (Flow Laboratories, McLean, Virginia).

All tests were assessed in duplicate and results expressed as SD units according to the formula X − M/SD, in which X was the absorbance of the test specimen, M was the mean absorbance of a basic set of 50 blood donor sera, and SD their standard deviation. Results were considered positive for CIC when the SD unit was above the value of the 2SD unit.

The anti-C3 EIA was used also to define the presence of CIC reacting with insulin antibodies.[3] The anti-C3–coated plates were incubated with twofold dilutions of test sera, and alkaline phosphatase-conjugated insulin antibodies were used as complexed insulin detectors. Positivity was defined for values above the mean of the controls (9 healthy subjects and 26 diabetic subjects on oral hypoglycemic agents) plus 3SD.

HbA_1 was determined by a commercial microchromatographic method (Helena Laboratories, Beaumont, Texas).

Statistics. Statistical analysis of the results was carried out with the Student's *t* test and with the Spearman rank correlation test.

RESULTS

Immune complex assays. Sera were found to be positive for CIC of the IgG class by at least one method in 50% of patients (Figure 1). Fifty percent of patients were positive for CIC by the solid-phase C1q binding and 33.3% were positive by the anti-C3 EIA. CIC of the IgM class were not detected, whereas only one patient showed IgA-CIC. C3-fixing material reacting with labeled anti-insulin antibodies was found to be present in two of the CIC-positive sera (Figure 1). None of the control subjects exhibited CIC.

No significant correlation was demonstrable between titers of the solid-phase C1q binding test and the anti-C3 assay; such discrepancy may reflect the fact that each assay detects different determinants of the immune complex.

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TABLE 1
Thromboxane B_2 (TXB_2) synthesis in diabetic subjects with and without CIC (mean ± SD)

Subjects	N	HbA_1 (%)	TXB_2-thr ng/10^8 platelets	TXB_2-AA ng/10^8 platelets
Diabetic subjects	12	8.5 ± 1.2	121 ± 57*	171 ± 84*
CIC positive	6	8.5 ± 1.3	155 ± 64†	220 ± 97§
CIC positive (anti-C3 EIA only)	4	8.2 ± 0.9	190 ± 44‡	263 ± 91‖
CIC negative	6	8.5 ± 1.3	88 ± 20	122 ± 22
Normal subjects	10	5.8 ± 0.8	107 ± 26	129 ± 34

*P = NS versus normal.
†$P < 0.05$ versus CIC-negative diabetic; P = NS versus normal.
‡$P < 0.001$ versus CIC-negative diabetic or versus normal.
§$P < 0.025$ versus CIC-negative diabetic or versus normal.
‖$P < 0.01$ versus CIC-negative diabetic or versus normal.

Thromboxane B_2 assay. After the addition of arachidonic acid (1 mM at 60 s), the amount of TXB_2 in PRP from patients positive for CIC by at least one method (220 ± 97 ng/10^8 platelets) was significantly higher than that in PRP from CIC-negative patients (122 ± 22 ng/10^8 platelets, $P < 0.025$) or controls (129 ± 34 ng/10^8 platelets, $P < 0.025$) (Table 1).

Similar behavior was observed after the addition of thrombin (5 U). The amount of TXB_2 was 155 ± 64 ng/10^8 platelets in PRP from CIC-positive patients and 88 ± 20 ng/10^8 platelets in PRP from CIC-negative patients ($P < 0.05$). Values obtained in PRP from controls were 107 ± 26 ng/10^8 platelets; the difference, however, was not significant.

The highest levels of TXB_2 were observed in the four patients exhibiting CIC, as detected by the anti-C3 EIA (263 ± 91 ng/10^8 platelets after arachidonic acid and 190 ± 44 ng/10^8 platelets after thrombin) (Table 1), and, specifically, in the two patients in which insulin-containing CIC were found (Table 2).

DISCUSSION

It has been previously demonstrated that platelets from insulin-dependent diabetic subjects synthesize increased amounts of thromboxane.[5,16] Whether the platelet abnormalities are related to diabetes per se or due to its complications has not been well established, however. Indeed, a significant increase in thromboxane synthetase activity has been observed only in diabetic subjects with complications[7] and, more recently, our observations point to the presence of vascular atherosclerotic lesions as a factor leading to platelet abnormalities in diabetes.[9]

In this study, we have shown that platelets obtained from young, noncomplicated, insulin-dependent diabetic patients with CIC synthesized greater quantities of thromboxane either after thrombin or after arachidonic acid than did platelets from CIC-negative patients or controls.

In view of the known action of CIC on platelets, a prior in vivo platelet activation by CIC may be suggested as a putative cause of this enhanced TXB_2 synthesis.

Immune complexes and aggregated IgG, in fact, are known to induce platelet aggregation via the Fc receptor for IgG,[20] and this behavior has been used as a screening method for the detection of CIC. It has also been shown by Colwell and colleagues[13] that soluble CIC purified from sera of diabetic subjects are capable of accentuating in vitro ADP-induced aggregation and ADP release in platelets from normal subjects, probably through prostaglandin synthesis and thromboxane release.

In this study, a solid-phase anti-C3[3,19] as the recognition unit for complement-fixing CIC was used together with the C1q solid-phase binding assay, enabling us to detect immune complexes also activated via the alternative pathway.

The highest values of TXB_2 synthesis were obtained in platelets from patients with CIC as detected by the anti-C3 EIA, indicating that CIC that fix complement by the alternative pathway may play a peculiar role in enhancing thrombin- or arachidonate-induced platelet activation. Although human platelets, in contrast with rabbit platelets, do not exhibit C3b receptors,[21] C3-bound platelets have been found frequently,[22,23] probably as part of an immune complex, the IgG attaching to the platelet via its Fc receptor. It has been shown, also, that zymosan-induced human platelet stimulation requires the alternative pathway of complement

TABLE 2
Clinical and laboratory findings in CIC-positive and CIC-negative patients

Patients	Age/sex	Duration of diabetes	HbA_1 (%)	Circulating immune complexes containing (SD units) IgG (C1q)	IgG (anti C_3)	Insulin (anti C_3)	TXB_2-thr ng/10^8 platelets	TXB_2-AA ng/10^8 platelets
1	11/F	3	6.9	5.15	5.2	neg	160	196
2	12/M	4	8.3	3.48	2.2	neg	145	183
3	6/F	2	8.8	2.1	3.36	pos	217	377
4	11/F	3	10.7	4.87	neg	neg	81	142
5	9/M	1	8.8	2.57	3.1	pos	238	295
6	8/F	2	7.9	4.6	neg	neg	89	126
7	12/F	4	10	neg	neg	neg	68	108
8	11/M	3	6.3	neg	neg	neg	70	147
9	11/M	2	8	neg	neg	neg	70	87
10	6/F	1	9.3	neg	neg	neg	109	140
11	12/F	2	8.8	neg	neg	neg	105	126
12	8/M	2	8.9	neg	neg	neg	103	123
Normal subjects 13–22	9 ± 2 (5F/5M)	—	5.8 ± 0.8	neg	neg	neg	107 ± 26	129 ± 34

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activation,[24,25] and recent studies also showed that arachidonate-mediated release of thromboxane was significantly enhanced in the presence of complement.[26]

Sera from two patients were found to be positive for C3-fixing material reacting with labeled anti-insulin antibodies that we assume to be C3-fixing insulin–anti-insulin complexes. It has been speculated that insulin–anti-insulin immune complexes may be implicated in late diabetic complications,[4] but our recent findings[3] failed to support this contention. In this study, however, positivity for insulin-containing CIC was found to be associated with the highest rates of TXB_2 synthesis. If confirmed, the role of insulin–anti-insulin complexes in platelet activation might be potentially relevant.

In conclusion, both platelet abnormalities and CIC have been thought to play a role in the pathogenesis of the late diabetic complications. In this report, we present data concerning a possible relationship between these two factors.

ACKNOWLEDGMENTS

We wish to thank Stefano Taormina for his help in preparing the illustrative material for this paper, and Lilya Colonna Romano for secretarial assistance.

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