Albumin-directed antibodies in diabetes: demonstration of human serum albumin-directed IgM autoantibodies

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Summary. Sera of 406 individuals, 174 Type 1 (insulin-dependent) diabetic patients, 125 non-diabetic family members and 107 unrelated control subjects, were screened for the presence of antibodies against glycated albumin. In none of these sera could such antibodies be detected. However, antibodies directed towards monomeric, unmodified human serum albumin were detected in 13 sera. These albumin autoantibodies were of the IgM class, and occurred in sera from nondiabetic persons (0.9–1.6%) and with a five-fold higher frequency in

sera from diabetic patients (5.2%). The presence of albumin antibodies was neither related to the presence of diabetic late complications, islet cell antibodies, HLA-status nor duration of diabetes. The albumin antibodies were also found in sera from persons carrying antibodies against mumps (17%) or Epstein-Barr virus.

Key words: Albumin, autoantibodies, IgM, diabetes.

Covalent attachment of low molecular weight substances to proteins can induce formation of antibodies against these haptens. Such antibodies can cause clinically relevant complications. Examples of such substances are the penicillins or metabolites of phenacetin [1, 2]. Increased nonenzymatic glycosylation (glycation) of proteins in diabetes mellitus has also been considered to induce antibody formation against their respective glycated form [3–6]. However, to date the only case where this mechanism has been demonstrated to occur is that for the antibody causing agglutination of M-positive non-enzymatically glycosylated human erythrocytes [3]. In all other instances the antibodies were directed towards glycated proteins in which the ketoamine structure was reduced by chemical means.

The finding that antibodies against reduced glycated proteins occur in diabetic and nondiabetic person alike [6], and the fact that an enzymatic reduction of the ketoamine in vivo has not been demonstrated, renders it unlikely that these antibodies are of significance in the pathogenesis of diabetic complications.

Albumin is a suitable protein for investigating whether antibodies against its naturally occurring non-reduced glycated form occur in vivo for enhanced glycation of albumin in diabetes [7] as well as glycation-induced conformational changes of this protein are well established [8]. Therefore, we investigated whether ex-

cessive attachment of glucose to albumin in diabetic patients leads to the formation of autoantibodies against glycated albumin.

Subjects and methods

Sera were obtained from a total of 406 individuals, 174 Type 1 (insulin-dependent) diabetic patients treated at the outpatient clinics of the Kantonsspital Basel, and from their 125 first degree relatives. One hundred seven control sera were obtained from patients attending the clinics for reasons other than diabetes and from healthy blood donors.

Human albumin 'reinst' (ORHA, Behringwerke, Marburg, FRG) was used. Bovine serum albumin (BSA), fraction V, was from Fluka (Buchs, Switzerland) and other animal albumins, fraction V, were from Sigma (St. Louis, Mo, USA). Peroxidase conjugated goat-anti-human-Ig antibodies were obtained either from Sigma or Tago Inc. (Burlingame, Calif, USA). The nitrocellulose paper was obtained from L. Schleiffer AG (Zürich, Switzerland). All other chemicals were of commercial origin and of the highest purity available.

Dot assay

Proteins were dotted on nitrocellulose paper strips at a concentration of 1.25 mg/ml in Tris-buffered saline (TBS: 0.02 mol/l Tris-HCl, 0.15 mol/l NaCl, 0.01% sodium azide, pH 7.5). Two single successive microliters were applied per dot and air-dried. Human immunoglobulins (human plasma diluted 1:100 in TBS) were also dotted and

Fig. 1. Strip from dot-assay of a human albumin antibody positive serum (magnified). HA mono / sheep mono: monomeric human serum respectively sheep serum albumin; BSA: bovine serum albumin; gly HA: in vitro glycosylated monomeric human serum albumin; S1 1/100: human antibody serving as control of unimpaired dot assay

Table 1. Incidence of IgM albumin antibodies

Subject/condition	n	Positive	Percentage
Non-diabetic subjects			
Control	107	1	0.9
Family members of diabetic patients	125	2 ^a	1.6
Diabetic patient	174	9 ^{a, b}	5.2
Non-diabetic subjects, seropositive for:			
Mumps (IgM)	12	2	17
Cytomegalo virus (IgM)	12	0	0
Epstein-Barr virus (IgG)	36	1	2.8
Rubella (IgG)	6	0	0
Herpes zoster (IgG)	5	0	0

^a A non-diabetic family member found to be positive for albumin antibodies who developed diabetes during the study is excluded from the Table.

^b Two diabetic subjects were found seropositive for Hepatitis B surface/core antigens

Table 2. Genetic and immunologic characteristics of subjects positive for albumin antibodies

Patient initials	Sex	Duration of diabetes (years)	HLA				ICA*
			A	B	C	D	
MF	m	2	nd ^a	nd	nd	7/-	+
PH	f	31	1/ 2	40/41	-	-/6	-
LL	f	7	2/28	15/44	3/4	1/4	+
AN	f	10	1/ 2	15/ 7	-	2/3	-
BA	m	>7	nd	nd	nd	nd	-
RS	f	>3	nd	nd	nd	nd	nd
AA	m	13	2	35/15	0	4	+
WW	m	3	nd	nd	nd	nd	nd
CG	f	15	1/24	8	-	3/4	-
CC	m	nondiabetic	2/11	40/62	-	3/4	-
AE	m	nondiabetic	2/3	7/15	3	2/4	+
SF	m	0.5	3/2	7/ 8	-	4	-/+

^a nd: not determined

* ICA were determined and interpreted as described previously [9]

served as a positive control of the assay. The strips were blocked by gentle agitation in 5% bovine serum albumin in TBS for 15 h at room temperature. Subsequently each strip was incubated with 1 ml of diluted serum (1:100 in 5% BSA/TBS) for 5 h at room temperature. Diluted sera were then removed and the strips repeatedly washed with TBS for several hours. Peroxidase coupled antihuman antibodies (1:350 in TBS) were added and allowed to react for 4 h. TBS washes were repeated and the peroxidase reaction initiated by the addition of developer mix (prepared by adding 0.6 ml of the 0.3% chloronaphthol/methanol stock solution and 4 µl of 30% hydrogen superoxide to 10 ml TBS). A blue spot indicating a positive reaction became visible within 30 min. (It was noted that reactivity against albumin gradually decreased in some of the sera stored at -70 °C over a one year period. This has to be considered in studies using quantitative instead of qualitative methodology.)

Isolation of monomeric albumin

All commercial albumins were passed over Sephacryl S-200 in 0.5 mol/l ammonium acetate. Fractions containing monomeric albumin were pooled and lyophilized. Homogeneity of these fractions was assessed by SDS-PAGE and by HPLC gel filtration. The high molecular weight fraction containing polymerized albumin was also collected and lyophilized.

Preparation of glycosylated albumin

Albumin (50 mg/ml) was incubated in 0.05 mol/l phosphate buffer, pH 7.4, in the presence of glucose (50 mg/ml) and sodium azide (0.02%) for 10 days at 37 °C. Glycosylated albumin was isolated by affinity chromatography on Glycogel B (10 × 150 mm) with 0.25 mol/l ammonium acetate, pH 8.5, as application buffer. For buffer exchange the glycosylated albumin was passed over Trisacryl GF-05. The albumin was applied and the unbound fraction washed out with application buffer at a flow rate of 30 ml/h. Glycosylated albumin was eluted with 2 × 5 ml 0.2 mol/l sorbitol in the application buffer. Glycosylated albumin was subjected to gel filtration on Sephacryl S-200 as above and lyophilized.

Statistical analysis

The significance of the difference in the frequencies of occurrence of albumin autoantibodies was evaluated by Fisher's exact test.

Results

Applying the dot assay to sera of 406 persons, we found albumin directed antibodies in 13 cases. A typical result of a positive dot assay is shown in Figure 1. These antibodies reacted comparably well with isolated glycosylated monomeric albumin and with the purified monomeric, dimeric and polymeric albumin fractions. Therefore, these antibodies were not specifically directed against glycosylated albumin. The albumin autoantibodies were detected in sera from diabetic patients as well as from control subjects, but the frequency of their occurrence was five-fold higher in the diabetic patients ($p=0.0347$) than in the nondiabetic control subjects (Table 1).

Although we failed to find autoantibodies specific against glycosylated albumin, we detected autoantibodies

against unmodified albumin. All the albumin antibodies detected were of the IgM class as evidenced by their reactivity with antihuman-IgM but not with antihuman-IgA, E or G. The antibodies reacted only with human albumin and showed no crossreactivity with sheep, rabbit, pig or bovine serum albumin. Reaction between dotted human albumin and albumin antibodies present in the sera could be blocked by preincubating the sera overnight with human albumin prior to dot-assay. In the most reactive sera complete blocking could be achieved by preincubation of the 1:100 diluted sera with 10 mg/ml of albumin. The reaction between human albumin and human albumin antibodies was not blocked by preincubation with animal albumin up to the concentration of 25 mg/ml.

The presence of albumin antibodies seems not to be related to diabetic complications, as none of eight diabetic patients showing severe late complications of diabetes carried albumin directed antibodies, and all albumin antibody positive individuals were free of such complications. Duration of diabetes could also not be associated with the presence of these albumin antibodies (Table 2).

The genetic (HLA) or immunologic (ICA) status seems, in addition, unrelated to the expression of albumin antibodies (Table 2). The HLA D 3/4 and the islet cell antibody frequencies in the albumin antibody carrying subjects were comparable to those of the general local diabetic population [9].

The temporal relationship between the appearance of albumin directed antibodies and islet cell antibodies was evaluated retrospectively. A tendency of the albumin antibodies to precede occurrence of the islet cell antibodies was noted. In 2 of our subjects (AE and SF) albumin antibodies were present before ICA occurred. In all other instances, subjects either carried albumin antibodies only, or both albumin antibodies and ICA were present in all available samples. In this context it is of note that during the course of this study one of the nondiabetic albumin antibody positive individuals developed islet cell antibodies and then diabetes mellitus.

Because the appearance of islet cell antibodies is often connected with the destruction of B cells, and viral damage is considered to be a possible cause of this destruction [11], we also studied sera from persons seropositive for viral infections. Interestingly, the highest frequency of albumin antibodies was found in the sera containing mumps-virus-IgM antibodies (Table 1).

Discussion

The fact that we did not detect antibodies specific for glycosylated albumin in our experiments argues against the hypothesis that excessive attachment of glucose renders albumin antigenic. This finding may seem surprising in view of the reported occurrence of glycation-induced an-

tibodies against a new epitope in the case of the M-positive blood group antigen [3].

Glycation of proteins is a continuous and physiological process. In order to prevent excessive formation of autoantibodies by the haptene mechanism, autoantibodies may get formed only if a potentially antigenic region is modified. In glycosylated albumin the principal site of glucose attachment is Lys-525, an amino acid residue outside the antigenic regions of albumin [11].

The occurrence of antibodies against albumin and the five-fold increased incidence of these IgM-albumin antibodies in a diabetic population is striking (Table 1). These antibodies with specificity toward native monomeric albumin and consequently also reacting with polymerized albumin, are different from the antibodies directed specifically towards polymerized albumin. The latter ones found in patients having hepatitis or otherwise affected livers [12] lack the reactivity towards monomeric albumin of the albumin antibodies described in this paper.

Albumin antibody expression seems to be a specific event and not an accompanying phenomenon to another autoimmune process. Five diabetic patients with high titres of both nuclear antibodies and thyroid antibodies, and 20 patients with thyroid disease and known titres of antibodies against various cell constituents studied, carried no albumin antibodies.

It remains to be established why albumin as an endogenous substance ordinarily in contact with the immune system becomes antigenic. It is known that an association between albumin and a viral protein occurs during infection with the hepatitis B virus [13]. A mechanism through which albumin could become antigenic might therefore consist of its association with an otherwise antigenic region, provided e.g. by a viral particle.

If this hypothesis explaining how viral damage to B cells renders albumin antigenic proves valid, albumin antibodies would represent a marker of a viral etiology of diabetes mellitus.

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