ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN — POLONIA
VOL. XLIII, 9 SECTIO D 1988

Zakład Biologii. Akademia Medyczna w Plewen
Kierownik: prof. dr Lilia I. Novoselska

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Determination and Identification of Elastin-antielastin Circulating Immune Complexes (CIC) in Serum from Normal and Atherosclerotic Subjects

Oznaczanie i rozpoznawanie elastin-antielastin kompleksów odpornościowych krążących w surowicy osób zdrowych i chorych na miażdżycę

Определение и распознавание элестин-антиэластин иммунизованных комплексов циркулирующих в сыворотке крови здоровых людей и больных атероматозом

There are several known methods for determination of circulating immune complexes (CIC) (4–7). Most of them are based on the precipitation of CIC with polyethyleneglycol (PEG). The routine techniques (7) are useful for the crude quantification of CIC, but they fail to discriminate between CIC and aggregated immunoglobulins, as well as to identify the specific antigen incorporated in CIC. Some progress has been achieved when the CIC have been purified by adsorption on rheumatoid factor (6) or by binding to Clq antibodies (4, 12). Some others (5) use sequential PEG precipitation and then purification of CIC on protein A containing *Staphylococci*. Usually these methods use electrophoresis in SDS-containing poloyacrylamide gels and subsequent autoradiography of the previously labelled with radionuclides antigens and antibodies in order to determine the specifity of the precipitated CIC.

In the present study we describe a new method for isolation and identification of elastin-antielastin CIC, based on the sequential PEG precipitation (5). Then the precipitated CIC are dissociated and an enzyme-linked immunosorbent assay (ELISA) for detection and quantification of elastin peptides, incorporated in CIC, is carried out. By this method we have investigated sera from normal and atherosclerotic subjects trying to find elastin-antielastin CIC and to determine some of their characteristics in relation to age and atherosclerosis.

MATERIAL AND METHODS

Antigen — Insoluble elastin was isolated according to Barnard et al. (1) from the aorta of a 30 year old person (killed in accident), without macro-or microscopically detectable changes

along the intima and media. It was used for the preparation of soluble α-elastin according to Patridge et al. (10).

Immune sera — Young male sheep and male rabbits were immunized with the human aortic α-elastin as previously described (2). The IgG fraction was isolated by salting out with $(NH_4)_2SO_4$ at 35% saturation and subsequent fractionation on a column of CL-Sepharose 6B. The sheep antielastin IgG were conjugated to horse-radish peroxidase (Type VI of Sigma, St. Louis, USA) according to Wilson and Nakane (13).

Investigated sera — Human sera were obtained from healthly subjects of different ages, grouped as follows: I — 1–7 year old (40 cases); II — 18–20 year old (102 cases); III — 30–40 year old (40 cases); IV — 41–50 year old (40 cases); V — 51–60 year old (40 cases); VI — 61–75 year old (40 cases). All persons had no clinical or laboratory signs of atherosclerosis. The next group (VII) consisted of patients with clinically manifested atherosclerosis, at the age between 50 and 75 (88 cases). All of them showed signs of brain or heart ischaemia, as proved by clinical and paraclinical methods (abnormal neurological status, ischaemic type of ECG, stenocardia, ischaemic brain incidents, etc.), and dislipidaemia (serum cholesterol — over 9.0 mmol/l LDL — over 6.0 g/l, HDL — less than 1.5 g/l). Most of them were diagnozed as generalized atherosclerosis.

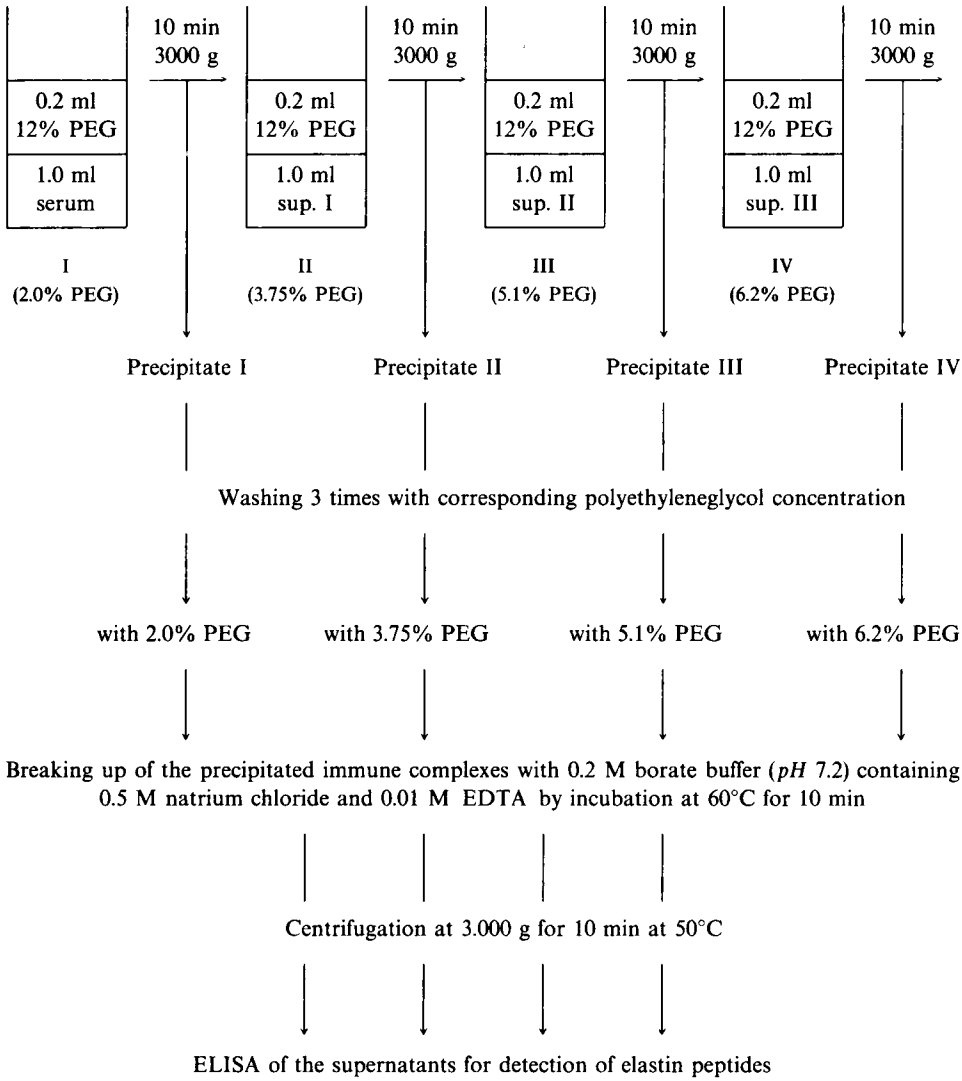
Determination of elastin-antielastin CIC — The principle scheme of the newly developed method is presented in Table 1. The CIC in the investigated human sera were precipitated with rising concentrations of PEG 6000 (Merck, F.R.G.) according to Eskinazi (5). In order to make sure that elastin-antielastin CIC are really precipitated and not just the elastin peptides from the serum, we made a parallel precipitation of an α-elastin solution (1 μg human aortic α-elastin, dissolved in 1 ml standard human albumin solution — standard of Difco Lab., USA). This control sample went through the same steps of the sequential PEG precipitation and further analysis.

The precipitated CIC were then dissociated by incubation for 10 min at 60°C in 0.2 M borate buffer, *pH* 7.2 containing 0.5 M NaCl and 0.01 M EDTA. The samples were then centrifuged at 3.000 g for 10 min and the supernatants, diluted 1 : 2 in distilled water, and tested for the presence of elastin peptides by ELISA. The following reagents were used: rabbit immune serum towards human aortic α-elastin (IgG fraction); human aortic α-elastin as reference antigen; immunoconjugate — sheep antielastin IgG, conjugated with horse-radish peroxidase (type VI of Sigma Chemical Co., USA). The immunoconjugate was diluted 1 : 200 in dilluting buffer — phosphate buffered saline (PBS), *pH* 7.4, containing 1% bovine serum albumin and 0.05% Tween 20; substrate solution — *o*-phenylenediamine (0.4 mg/ml in 0.05 M citrate buffer, *pH* 5.0) and 0.01% H_2O_2.

The polysterene plates were first coated with 100 l of rabbit antielastin IgG (10 kg/ml in 0.05 M carbonate buffer, *pH* 9.6) by incubation for 3 h at 37°C and overnight at 4°C). Before use the plates were washed three times with the diluting buffer to remove unbound protein. The wells were filled with 100 μl of the serum samples to be tested and were incubated for 30 min at 30°C. After washing thoroughly with the diluting buffer the plates were then washed 3 times with the diluting buffer and the incubation with the substrate solution was carried out for 1 h at 20°C in a dark chamber. The reaction was stopped by adding 50 μl of 8 NaH_2SO_4. The extent of the reaction was measured spectrophotometrically at 492 nm after transferring the reaction mixture into test tubes, containing 850 μl of distilled water, using the immunoconjugate control as a reference. The statistical analysis was carried out according to Sepetlliev (11).

The following controls were used: a) substrate control — only substrate solution was added to the polystyrene wells coated with the rabbit antielastin IgG; b) immunoconjugate control — the immunoconjugate was added directly to the wells coated with the rabbit antielastin IgG, and then incubation with substrate solution was carried out. This control served as a reference for the extinction values of the tested samples; c) controls of the rabbit antielastin IgG — the rabbit antielastin IgG were replaced with normal rabbit IgG; d) controls of the specificity of the assay — the tested samples were replaced with: standard human albumin solution (Difco Lab., USA), standard collagen preparations (type VI, VIII and X of Sigma, USA), saline extracts from the organs of the same

Table 1. Scheme of the method



person whose aorta was used for the preparation of α-elastin, and from human foetus (20th week of gestation) with concentration 1 mg protein per 1 ml; e) control of the precipitation of the "free" circulating elastin-derived peptides from the serum samples and with the supernatants obtained after each step of the sequential PEG precipitation, diluted 1 : 2 in the diluting buffer; f) positive control — the tested samples were replaced with human aortic elastin (1 μg per 1 ml of the diluting buffer).

The investigation was carried out in three series acomplished in 3 subsequent weeks and the variation of the extinction values, obtained for each tested sample, was less than 7%. Each sample was tested in triplicate and the variation of the extinction values was less than 5%. The first series of the

investigation were carried out "blind" — the experimentators and the technical assistants did not know which sample belonged to the group of the healthy subjects and which one belonged to the group of the atherosclerotic patients. We measured the extinction values of all the tested samples and the controls against the immunoconjugate control, and thus, the "noise" of the reaction was very low — less than 0.01 as expressed in extinction values.

RESULTS

Firstly we wanted to ensure that we really precipitate the elastin-antielastin CIC and not just elastin-derived peptides circulating in the serum, found in an earlier investigation of ours (2), as well as some other authors (9). For this purpose we used the parallel control of human aortic α-elastin in standard human albumin solution. If the elastin peptides themselves (or the albumin bound elastin peptides) were precipitated by the PEG concentrations used, then high extinction values in the subsequent immuno-enzyme analysis should be expected. However, the control samples showed very low extinction values (0.005 ± 0.003). The presented figures are mean ±SEM from 10 tested samples of the control. We confirm these results by the investigation of the native serum samples of some of the patients with highest levels of circulating elastin-derived peptides and then the comparative testing of the supernatants after each step of PEG precipitation. If the elastin peptides were precipitated then a significant decrease of the extinction values of the supernatants should be expected. But the decrease was less than 5% of the values of the native serum. Thus, we concluded that the elastin peptides found in the dissociated resuspended precipitates had really been incorporated in CIC.

The specificity of the reaction was tested by the negative controls indicated in "Materials and Methods". The standard human albumin solution gave a colour reaction equivalent to that of the immunoconjugate control. The same occurred with the standard collagen precipitations and with the control with normal rabbit IgG. The controls with saline extracts from different human organs gave a very weak colour reaction — extinction values of 0.008 ± 0.002 (values are mean ±SEM, $n = 10$). It is lower than the accepted "noise" of the immuno-enzyme assay and may be due to contamination with elastin peptides from the serum. Due to those results we considered the optimized ELISA version to be specific enough for the purposes of the investigation.

We did not find any elastin-antielastin CIC in the sera of all the healthy subjects up to the age of 60 (I–V groups). Such complexes could be detected only in the sera from atherosclerotic patients (group VII) and from the old healthy persons over 60 (group VI), but there were differences between these 2 groups (Table 2).

The table shows the number of persons who had elastin-antielastin CIC in the serum and the PEG concentrations by which the latter were precipitated. The

Table 2

Investigated groups	Total number	Number of persons, in whose sera we have found elastin-antelastin CIC, precipitated by the following PEG concentrations:				Mean extinction values (±SEM), obtained from ELISA for the detection of the elastin peptides
		2.0	3.7	5.1	6.2	
61–75 year old healthy persons	40	15	29	3	3	0.015 ±0.002
Atherosclerotic patients	88	3	–	38	52	0.063 ±0.022

mean extinction values (±SEM) obtained from the ELISA of the resuspended precipitates are also presented. These values were read against the immunoconjugate control. Thus we considered the specific colour reaction, proportional to the quantity of the "immunologically active" elastin peptides, incorporated in elastin-antielastin CIC.

When comparing the group of the atherosclerotic patients (VII) with that of the old healthy persons (VI) the following main differences were observed. Firstly, the elastin-antielastin CIC from the serum of atherosclerotic patients were precipitated mainly by the higher PEG concentrations — 5.1 and 6.2%, while those in the serum of old healthy subjects were precipitated by the lower PEG concentrations — 2.0 and 3.7%. Secondly, the mean quantity of elastin peptides incorporated in the precipitated CIC was fourfold greater among the atherosclerotic patients in comparison with the old healthy persons. This quantity varied from one atherosclerotic patient to another, but the main tendency to be above the values characteristic of normal subjects over 60 was preserved. This variation, however, was independent of the patients'age, and thus they were not divided into age groups.

DISCUSSION

The new method enables us to determine two important features of the precipitated elastin-antielastin CIC. Firstly, the concentration of PEG required for the precipitation of certain CIC, according to (5) corresponds to their size. Our preliminary investigations with artificial elastin-antielastin CIC confirmed his conclusion. Thus, the small-sized CIC are precipitated by the greater PEG concentrations. This can be related to the greater colloidal stability of the smaller CIC. This is one of the greatest advantages of the sequential PEG precipitation, designed by (5), and we use it successfully in our method. On the other hand, the use of higher PEG concentrations (up to 6.2%) gives the oportunity to precipitate CIC of very small dimensions. The sequential PEG precipitation itself fractionates CIC of different sizes.

The second significant feature of the precipitated CIC is the quantity of the specific antigen incorporated in the precipitated CIC. The identification of the CIC via the specific antigen has other great advantages. In this way we avoid the necessity to purify the precipitated CIC on *Staphylococcus aureus* or on rheumatoid factor, as we set a parallel control by which we provide the reliability of the investigation and the immunoglobuline aggregates are totally excluded, as we determine only the specific antigen. Thus we overcome the disadvantages of the other methods for determination of CIC. On the other hand, the immunoenzyme assay is easier to perform and cheaper and quicker than the radiolabelling of the dissociated immune complexes with subsequent SDS polyacrylamide electrophoresis and autoradiography of the electrophoregrams.

In our previous investigations we found circulating elastin derived peptides (2) and antielastin antibodies (3) in the serum of all the tested normal and atherosclerotic subjects. It was logical to suppose that under these conditions elastin-antielastin immune complexes may form, especially among the atherosclerotic patients with high levels of elastin peptides in the serum. Furthermore, Ivanovskii and Zota (8) found increased levels of CIC among atherosclerotic subjects, but without determination of these CIC. Thus we tested sera from healthy subjects of different age and from atherosclerotic patients for the presence of elastin-antielastin CIC. The absence of such complexes in the serum of the healthy persons up to 60, of course, does not mean that the circulating elastin-derived peptides and the antielastin antibodies do not form immune complexes. Probably, those complexes are practically insoluble (due to the equivalent quantities of the antigen and the specific antibody) and that is why they cannot be detected in their serum.

The elastin-antielastin CIC found in the serum from atherosclerotic patients are small-sized (precipitated by the higher PEG concentrations) and with high elastin content. Such complexes are formed when there is a great antigen excess and unequally small quantity of specific antibodies. This correlates well with the data from our previous investigations where we find high levels of circulating elastin-derived peptides (2) and low levels of antielastin antibodies (3) in the serum of atherosclerotic subjects. This imbalance between the quantities of the elastin peptides and the specific antibodies appear in the serum of the old healthy subjects over 60 as well, but it is not expressed in such a degree. That may explain why we find elastin-antielastin CIC of greater dimensions and with lower elastin content among healthy persons over 60. We cannot exclude the possibility that those healthy subjects at the age between 61 and 75 have some minor atherosclerotic lesions that cannot be detected by the routine clinical or paraclinical methods and furthermore, that in the group of the atherosclerotic patients we have included persons with grossly manifested atherosclerosis. But the attempt was to find out whether our method for detection and identifica-

tion of elastin-antielastin CIC can distinguish the "practically normal" from "pathological" turnover of the elastic structures.

Our method can also be applied for the investigation of CIC, containing some known antigen, that is not precipitated by PEG and that can be detected by a specific ELISA. It provides some information of the size of the precipitated CIC it can be used for prognostic purpose as the detection of small-sized CIC may predict the appearance of some severe complications (e.g. glomerulopaties, arthropaties, etc.). On the other hand, this method is easier, cheaper and quicker than most of the contemporary techniques, which makes it suitable for clinical practice.

Acknowledgement. We would like to thank Dr Z. Gancheva and Dr A. Chernov from the Institute of Infectious and Parasitic Diseases, Sofia, for their help in the purification of the immune sera and preparation of the immunoconjugate for ELISA.

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Otrzymano 1988.04.04.

STRESZCZENIE

Opisano nową metodę oznaczania i rozpoznawania elastin-antielastin (CIC) w surowicy ludzkiej. CIC są izolowane metodą kolejnego wytrącania przy pomocy wzrastających stężeń roztworów PEG. CIC są następnie rozdzielane i wykonywana jest próba z ELISA, służąca do wykrywania i oceny ilościowej białek elastin połączonych (wbudowanych w) z CIC.

Przy pomocy tej metody przebadano osoby zdrowe w różnym wieku i grupę osób chorych na miażdżycę. Obecność elastin-antielastin (CIC) wykryto tylko w surowicy osób chorych na miażdżycę i u osób zdrowych powyżej 60 roku życia. Stwierdzono istotne różnice głównych cech wytrąconych CIC, ich rozmiary i zawartość elastinu u obu porównywanych grup pacjentów. Opisana metoda może być stosowana do wykrywania i rozpoznawania każdego rodzaju CIC zawierających jakiś znany antygen, który może być wykryty i oznaczony ilościowo przy pomocy próby z ELISA.

РЕЗЮМЕ

В данной работе представлено метод обозначения и определения эластин-антиэластин (CIC) в сыворотке людей. CIC изолировано методом очередного осаждения при помощи возрастающей концентрации растворов PEG. CIC разделено и проведено пробу с ELISA, которая дает возможность определить и количественно оценить белки эластин связанных (вкомпонированных в) с CIC.

При помощи этого метода осмотрено здоровых людей разного возраста и группу людей больных атероматозом. Эластин-антиэластин CIC определено только в сыворотке больных атероматозом и у людей старшего возраста (с 60 года жизни). Определено существенные разницы главных осажденных черт CIC, их размеры и содержание эластина в двух сопоставленных групп пациентов. Описанный метод можно применять при разоблачении и определении всякого рода CIC содержащих любой известный антиген, который может быть количественно разоблачен и определен при помощи пробы ELISA.