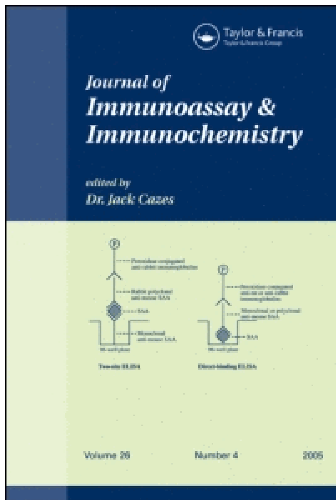
This article was downloaded by:
On: *16 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Detection of Human Anti-Annexin Autoantibodies by Enzyme Immunoassays

M. Kraus[a]; J. Römisch[a]; B. Bastian[b]; E. -P. Pques[a]; A. A. Hartmann[b]
[a] Forschungslaboratorien der Behringwerke AG, Marburg, FRG [b] Universitätsklinik und Poliklinik für Hautkrankheiten, Würzburg, FRG

To cite this Article Kraus, M. , Römisch, J. , Bastian, B. , Pques, E. -P. and Hartmann, A. A.(1992) 'Detection of Human Anti-Annexin Autoantibodies by Enzyme Immunoassays', Journal of Immunoassay and Immunochemistry, 13: 3, 411 — 439
To link to this Article: DOI: 10.1080/15321819208021241
URL: http://dx.doi.org/10.1080/15321819208021241

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF IMMUNOASSAY, 13(3), 411-439 (1992)

DETECTION OF HUMAN ANTI-ANNEXIN AUTOANTIBODIES BY ENZYME IMMUNOASSAYS.

M. Kraus*, J. Römisch*, B. Bastian+, E.-P. Pâques*, and A. A. Hartmann+

*Forschungslaboratorien der Behringwerke AG, D-3550 Marburg, FRG; +Universitätsklinik und Poliklinik für Hautkrankheiten, D-8700 Würzburg, FRG.

Downloaded At: 11:35 16 January 2011

ABSTRACT

Annexins belong to a family of proteins characterized by calcium-dependent binding to the cytoskeleton and phospholipid surfaces. Basing on these properties annexins are discussed to be involved in the regulation of cytodynamic, anticoagulatory and antiinflammatory processes. Since autoantibodies against annexin I had been detected in patients suffering from inflammatory or autoimmune diseases, an impact on the pathophysiological outcome was assumed. Therefore we developed solid phase, enzyme-linked immunoassays for the quantitative determination of autoantibodies directed against six members of the annexin family. Some preliminary results

Copyright © 1992 by Marcel Dekker, Inc.

obtained from sera of patients with malignant melanoma show a quite frequent presence of such autoantibodies. These data suggest that autoantibodies are generated against all annexins. Furthermore, in the individual patient autoantibodies of the IgG-type are monospecific, while about 1/4 of the IgM-type are directed against several annexins. These observations imply that for investigation of anti-annexin autoantibodies in inflammatory and autoimmune diseases as well as cancer all members of the annexin family have to be taken into consideration.

(KEYWORDS: autoantibodies - annexins I to VI - malignant melanoma - ELISA)

INTRODUCTION

Downloaded At: 11:35 16 January 2011

During the last decade a family of structurally related calcium-binding proteins has been intensively investigated. Due to their ability to bind to cytoskeletal elements like actin/spectrin as well as negatively charged phospholipids/membranes in a calcium-dependent manner, they were termed calpactins or lipocortins (1-3). Since a variety of biological functions were ascribed to these proteins, they were renamed annexins (4). Their real physiological importance is yet unknown, however, they are discussed to be involved in cytodynamic/cell growth-differentiation or anticoagulatory processes (5-9). Since annexins, six of which are also physicochemically well characterized, lack a leader sequence, they are mainly intracellularly localized. By covering the inner surface of the plasma membrane they inhibit the degradation of phospholipids by phospholipase

A_2, which enzymatically releases arachidonic acid (10,11). The metabolites of this fatty acid like prostaglandins, leukotrienes and thromboxanes support inflammatory processes in a well-known fashion.

Since annexin I synthesis was shown to be inducible by glucocorticoids in some cell-types, these proteins are discussed to be the mediators of the steroid antiinflammatory action (12,13). Favouring this theory it can be deduced that the development of autoantibodies (AA) against annexins could have a decisive impact on the pathophysiological outcome of inflammatory and/or autoimmune diseases.

Development of AA as the cause or as consequence of disorders is a well-known fact. Secondary AA in diseases like systemic lupus erythematodes (anti-DNA AA), chronic arthritis ('rheuma factor', anti-IgG) or the Sjögren syndrome (anti-cytoplasmatic AA) are used as diagnostic tools (14). Their presence can lead to side-effects with consequences for therapy; e.g. formation of immune complexes in the renal tubuli may cause glomerulonephritis.

As early as 1981 Hirata et al. reported the detection of anti-lipomodulin (annexin I) AA in patients suffering from rheumatoid arthritis or dermatomyositis (15). Such AA were able to prevent the lipomodulin-mediated inhibition of the arachidonic acid release from phospholipids by phospholipase A_2 in vitro. Since AA

Downloaded At: 11:35 16 January 2011

against annexin I obviously neutralize its potential regulatory action, it seems to be worthwhile to investigate, whether AA against the other members of the annexin family exist in patients suffering from inflammatory and autoimmune diseases or cancer.

For this reason we report the development of solid phase, enzyme-linked immunoassays for the quantitative determination of AA directed against six members of the annexin family. Furthermore, we present data from healthy blood donors as well as patients suffering from malignant melanoma, sera of which were investigated for anti-annexin I to VI AA.

Downloaded At: 11:35 16 January 2011

MATERIAL AND METHODS

Normal Subjects.

Sera and plasma of 114 apparently healthy blood donors were investigated for antibodies against the various annexins.

Patients With Malignant Melanoma.

32 patients with different clinical stages of malignant melanoma were also studied for the presence of antibodies against annexins. 16 patients with stage I disease (localized disease, absence of sonogrphic and radiologic

evidence of tumor in regional lymph nodes), 22 patients with stage III disease (histopathological documentation of distant metastasis). All blood samples investigated were taken before treatment. The samples were processed immediately and the sera were frozen at -40°C.

Reagents.

Microtiter plates (Immuno module, type B) were from Nunc (Denmark); CNBr-activated Sepharose from Pharmacia (Uppsala, Sweden); gelfiltration media were from Serva (Heidelberg, FRG); goat anti-rabbit IgG/HRP conjugate was from Dianova, (Hamburg, FRG); rheuma factor control sera, murine monoclonal and rabbit antibodies, anti-human IgG- and IgM- horseradish peroxidase (HRP) conjugate, HRP substrate solution (o-phenylendiamin), washing solution for microtitration plates and bovine serum albumin (BSA) were from Behringwerke AG (Marburg, FRG); all other reagents were from Sigma (Deisenhofen, FRG).

Human Annexins.

The annexins I to VI were purified from human placenta according to Römisch and Heimburger (9). Annexins I and II were obtained as a mixture in a ratio of 2:1, while the other lipocortins were pure as revealed by sodium dodecyl polyacrylamide gelelectrophoresis.

Downloaded At: 11:35 16 January 2011

Development of ELISA Assay for Anti-Annexin Antibodies.

Antigen concentration and coating conditions: Antigen concentration and coating conditions were optimized in order to achieve a maximum ratio between the signals from sera known to have high anti-annexin AA titres and those from normal blood donors. The purified annexins were coated separately onto wells of microtiter plates at various concentrations. The determination of the signal after sample incubation and detection of bound human IgG and IgM was carried out as described under 'standard ELISA procedure' further below. The coating conditions were optimized using buffers with different pH (range 5.5 to 9.5). Also various proteins commonly applied as blocking agents were tested in regard to minimization of non-specific binding of human IgG- and IgM-type antibodies.

Downloaded At: 11:35 16 January 2011

Sample and conjugate buffer: In order to determine the best diluent for testing samples anti-annexin autoantibody (AA) binding capacity of positive samples and of a normal serum pool were compared using Tris-buffered saline (TBS) with or without various additions of BSA, NaCl or (R)Tween 20 (polyoxyethylenesorbitan monolaurate). The composition achieving the best discrimination between the positive and negative samples was selected and used throughout the study.

Influence of rheuma factor: The influence of rheuma factor (human IgM anti-human IgG), which might lead to false positive results for IgM in samples with high anti-annexin AA titres of the IgG-type, was examined as follows. Serum samples with known high IgG-AA titres against the annexins I to VI or standard serum with low titre were mixed with equal volumes of rheuma factor control sera (125 IU/ml; Behringwerke, Marburg, FRG) and were diluted (1:2) with sample buffer. Additionally samples with low IgG- and IgM-anti-annexin AA titres (IgG-/IgM-), and exclusively either IgG-type (IgG+/IgM-) or IgM-type (IgG-/IgM+) positive anti-annexin AA titres were used. The signals for IgG and IgM determined with the standard ELISA procedure were compared.

Downloaded At: 11:35 16 January 2011

Standard ELISA Procedure.

Microtiter plates were coated each with 125 µl of purified annexins III to VI separately or the mixture of annexin I/II at a concentration of 5 µg/ml in 0.01 M Na-acetate buffer, pH 5.5. After incubation over night at room temperature in a moist chamber, the plates were washed three-times with TBS. Then each well was again filled with 1% BSA-solution and incubated for at least 1 hour at room temperature. The plates were finally washed four-times with TBS. Samples were diluted 1:10 in dilution

medium (TBS, containing 3% BSA (w/v), 0.5 % (R)Tween 20 (w/v)). 100 μl of each diluted sample were incubated for 1 hour at room temperature in parallel in two annexin coated wells. In order to control non-specific binding a pool of sera with low signal was used as reference. The microtiter plates were washed three times using Enzygnost(R) washing buffer (Behringwerke AG, Marburg, FRG) and the wells were incubated each with 100 μl anti-human IgG- or IgM-POD conjugate in dilution medium (containing only 1% BSA) for 1 hour at room temperature. Subsequently, the microtiter plates were washed again and 100 μl substrate solution per well (Enzygnost(R) POD substrate solution, Behringwerke AG, Marburg, FRG) were added. After 30 min incubation at room temperature the reaction was terminated with 100 μl 0.5 N H_2SO_4. The absorbance values at 490 nm (reference: 630 nm) were obtained with a MR 5000 ELISA plate reader (Dynatech, Guernesey, UK). The results were expressed in absorbance values or in values related to the 95%-interval of the distribution of the absorbances as determined in healthy blood donors (rel. abs.).

Downloaded At: 11:35 16 January 2011

Validation of the Assay.

The validity of the assay in the presence of serum was tested by adding different concentrations of polyclonal

antibodies against each annexin to serum. The polyclonal antibodies had been raised in rabbits using purified annexins and were purified using protein A sepharose and immunoaffinity chromatography on annexin coupled CNBr-Sepharose. The assay was carried out according to the standard procedure except for using goat-anti rabbit-IgG/HRP conjugate (final dilution 1:3000 according to the indications of the manufacturer) for detection. The linearity of the assay and the validity of the sample diluent were followed mixing rabbit anti-annexin antibodies as well as positive sera of human origin (1:2) with either sample diluent or human serum prior to anti-annexin AA determination in the ELISA. In a further set of experiments increasing concentrations of the different annexins (up to 2 g/l) were added to solutions containing polyclonal antibodies (1 g/l) specific for the respective antigen. Furthermore, undiluted human sera with high titres of AA directed against the definite annexins were spiked with the purified annexins (up to 1 g/l). The samples were incubated for 1 h at 37°C and the competitive effect was followed applying the standard ELISA procedure. At last polyclonal antibodies specific for the respective annexin were added to human anti-annexin AA positive sera up to final concentrations of 0.4 g/l with respect to the undiluted serum. The competitive effect was determined in the standard ELISA procedure.

Downloaded At: 11:35 16 January 2011

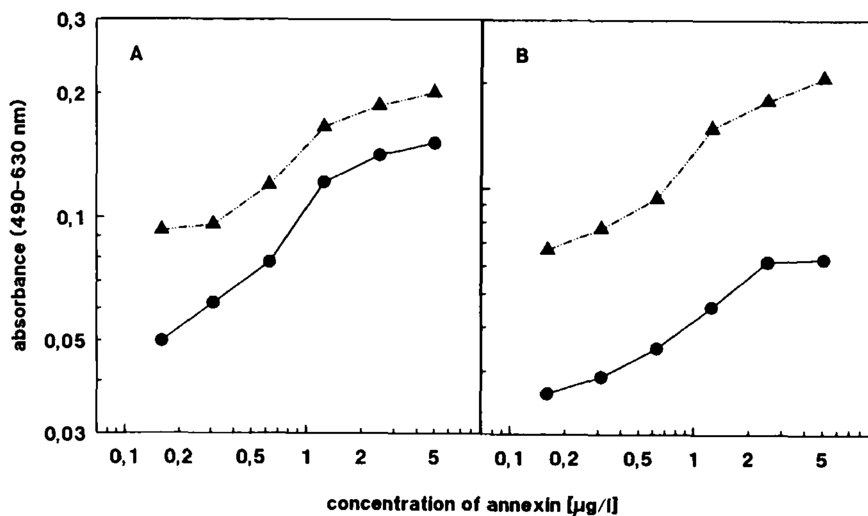


FIGURE 1

Optimization of coating conditions for determination of human anti-annexin autoantibodies. Microtitration plates were coated with increasing concentrations of human annexin V and the absorbance determined with anti-human IgG/HRP conjugate was followed in normal (—●—) and pathological serum (--▲--) (A). Same as in (A) instead using annexin IV for coating and anti-human IgM/HRP for detection (B).

Downloaded At: 11:35 16 January 2011

RESULTS

Development of the ELISA Assay

Antigen concentration and coating conditions: Anti-annexin AA positive sera were used to study the binding capacities of AA for the corresponding annexins at varying antigen concentrations and coating conditions. For comparison a serum pool with low binding activities

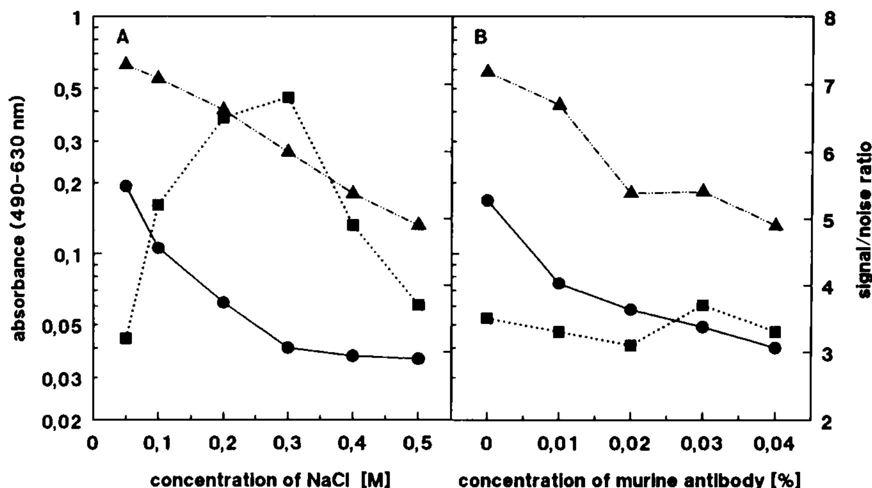


FIGURE 2

Optimization of signal/noise ratio and suppression of non-specific binding in the anti-annexin AA ELISA. Influence of NaCl concentration in the sample diluent as evidenced by determination of IgG anti-annexin I/II titres in normal and pathological serum. (—●—) absorbance obtained with normal serum; (--▲--) absorbance obtained with pathological serum; (···■···) signal/noise ratio as calculated from absorbance of pathological serum divided through absorbance from normal serum.

Downloaded At: 11:35 16 January 2011

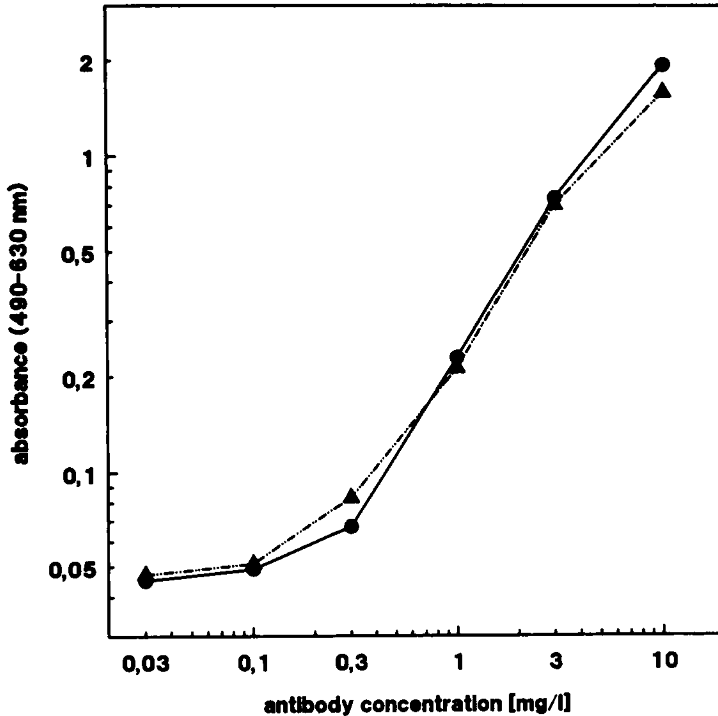
was used. As shown in Fig. 1 the binding capacity of positive sera increased with elevated concentrations of annexins, resulting in saturation at approximately 5 mg/l. Non-specific binding as mainly observed for IgG-anti-annexin AA determination (e.g. anti-annexin V, Fig. 1A) could be reduced by optimization of coating conditions and treatment of the coated plates with a 1% BSA solution.

Sample and conjugate diluents: Sample and conjugate diluents were most important in order to achieve optimum signal/noise ratios. In the sample diluent especially NaCl exerted the major effects (e.g. see Fig. 2 for anti-annexin I/II determination), resulting in the final composition as described under Material and Methods.

Validation of the Assays.

Sensitivity and linearity: Polyclonal antibodies, which had been raised against each annexin in rabbits were used in order to estimate the linearity and sensitivity of the ELISAs. Addition to a human serum pool, which had been depleted from AA by immunoadsorption, yielded sufficient dose/response curves using the standard ELISA procedure except for applying goat-anti-rabbit/HRP conjugate for the detection of bound antibodies. As shown for example in Fig. 3 for the anti-annexin IV determination, linearity was obtained ranging from 0.3 to 10 mg/l, which was equivalent to 3 to 100 mg/l in undiluted sera. The detection limit of the ELISA was 0.1 mg/l as calculated from the mean values plus three-times SD measured in samples without added anti-annexin antibodies. Only in the lower concentration range some minor deviations could be observed between the curves obtained using either serum or sample buffer as diluent (Fig. 3). The same linearity

Downloaded At: 11:35 16 January 2011



Downloaded At: 11:35 16 January 2011

FIGURE 3

Reference curve in the standard anti-annexin AA ELISA. Rabbit anti-annexin IV antibodies and anti-rabbit IgG/HRP conjugate had been used. The influence of sample diluent (—●—) on the determination of the antibody titre in comparison to same sample diluted in human serum (··–▲··–) is shown.

was obtained diluting anti-annexin positive sera in either buffer or negative sera as can be seen e.g. for IgG-anti annexin VI determination in Fig. 4A and for IgM-anti annexin I/II determination in Fig. 4B. Also no significant differences in absorbances were detected applying either

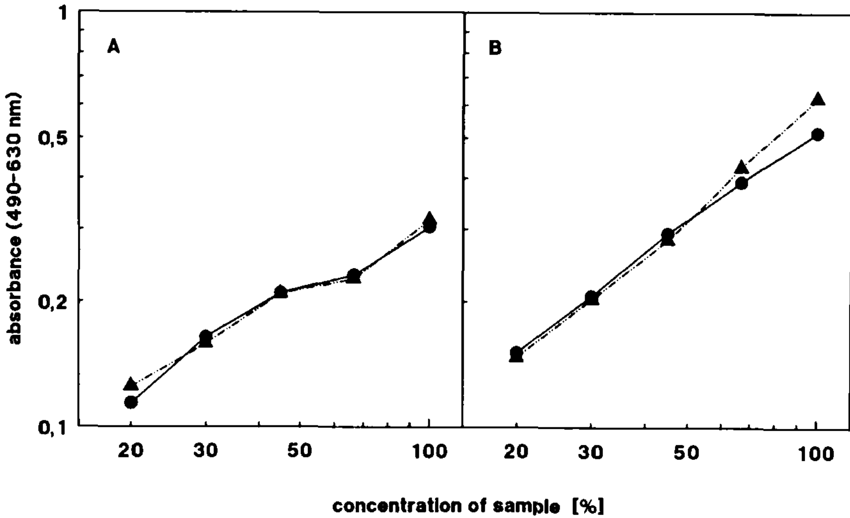


FIGURE 4

Linearity of the anti-annexin AA assay. Human sera with high titres of anti-annexin AA were diluted either with buffer (—●—) or with serum (··–▲··–). The absorbance determined decreases linear with decreasing anti-annexin AA titres independently from the type of diluent used. For IgG-determination a serum with anti-annexin VI AA was used (A), for IgM-determination data are shown for a serum with anti-annexin I/II AA.

Downloaded At: 11:35 16 January 2011

serum or sample buffer as diluent. However, the recovery of the samples exceeded 100% when sera were diluted above 1:20. But throughout these examinations all samples were diluted 1:10 in sample diluent.

Specificity: Only marginal suppression of the signals relative to untreated samples could be observed upon addition of free annexins to sera with high titres of AA

up to concentrations of 1 g/l. However, titration of human anti-annexin AA positive sera with polyclonal antibodies, which had been raised against the same annexin as being used for coating of the microtitration plates, resulted in competition between human and rabbit antibodies, thus corroborating the specificity of the assay. In two positive sera with similar signals (about 0.5 absorbance units) in the ELISA for IgG- or IgM-type anti-human annexin III AA, as shown in Fig. 5, half-maximum suppression of the absorbance was achieved with 12.5 mg/l, respectively, about 33 mg/l of rabbit anti-annexin III. For another sample with an absorbance unit of 0.5 for IgM-anti annexin I and II AA half-maximum suppression was at about 170 mg/l of an equal mixture of rabbit anti-annexin I and II antibodies. Assuming similar affinities of human and rabbit antibodies to the annexins it can be estimated that for these patients about 0.2% of total IgG and 2 to 14% of total IgM were specific for the respective annexin.

Downloaded At: 11:35 16 January 2011

Precision: Intra-assay precision was examined using anti-annexin AA positive sera with either low (< 0.1), medium (0.1 to 0.4) or high (> 0.4 absorbance units) titres applying the respective anti-annexin AA ELISA. The sera with the same specificity were added to 8 wells of the same plate. The mean intra-assay coefficients of

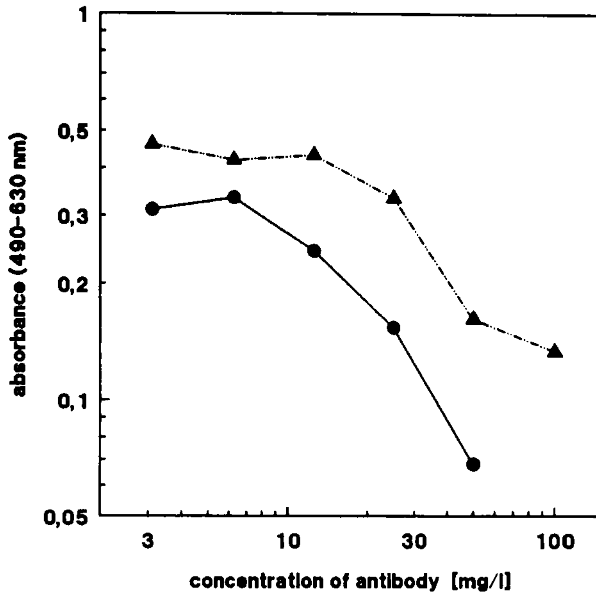


FIGURE 5

Specificity of the anti-annexin AA assay. Polyclonal anti-annexin III antibodies from rabbit were added to human sera which showed about 0.5 absorbance units in the anti-annexin III ELISA for either IgG- (—●—) or IgM- (··▲··) type AA. The polyclonal antibodies competed with human AA for annexin III on the surface of the microtitration plate. Half-maximal suppression was achieved at about 12.5 and 33 mg/l for IgG- and IgM-type AA respectively in the undiluted sera.

Downloaded At: 11:35 16 January 2011

variation (CV) for all IgG-type anti-annexin AA were 26% for the group with low titres, 8% for the group with medium and 3% for the group with high anti-annexin AA titre. For IgM-type AA determination the intra-assay CVs were 9 %, 7 % and 9%.

Influence of rheuma factor: The influence of rheuma factor (human IgM-anti-human IgG) on IgM-AA determination is shown in Table 1 for serum samples positive for either IgG- or IgM-anti-annexin VI AA. Addition of rheuma factor positive serum (125 IU/ml) did not influence IgM-determination in the IgG-anti annexin VI negative serum, while the absorbance values as determined for the IgG-anti-annexin VI positive serum increased with increasing rheuma factor concentrations. The signals of the control (IgG- and IgM-anti annexin VI negative serum) were not influenced. The false positive signal due to the presence of rheuma factor could be suppressed by addition of a γ-globulin fraction from rabbit (Behringwerke, Marburg, FRG) to the sample buffer (final concentration in the assay 1:20) as shown in Table 1.

Downloaded At: 11:35 16 January 2011

Population Studies.

Normal ranges of anti-annexin AA: The sera from 114 apparently healthy, male blood donors were analyzed for IgG- and IgM-AA against each member of the annexin family for which a solid-phase, sandwich-type ELISA had been developed as described in the previous sections: annexins III to VI and annexin I and II (ratio annexin I:II about 2:1). The statistical analysis of the resulting distribution profiles is listed in Table 2. The distribution profiles for all annexins tested are

TABLE 1

Influence of Rheuma Factor on IgM-Determination in the Anti-Annexin Autoantibody ELISA.

Sera with either low (-) or high (+) titre of autoantibodies of either the IgG- or IgM-type against human annexin IV were mixed with various concentration of rheuma factor. The rise in absorbance determined in the IgG+/IgM- sera could be suppressed by addition of a rabbit γ-globulin fraction to sample buffer. Indicated are the absorbance values as obtained with the anti-annexin IV Elisa. (IgG-/IgM- = IgG- and IgM-anti-human annexin IV negative serum; IgG+/IgM- = IgG-anti-human annexin IV positive serum; IgG-/IgM+ = IgM-anti-human annexin IV positive serum.)

Downloaded At: 11:35 16 January 2011

IgM-anti-annexin IV determination after addition of rheuma factor positive serum.

Rheuma Factor (IU/ml)	IgG-/IgM-	IgG+/IgM-	IgG-/IgM+
0	0.037	0.091	0.228
0.8	0.026	0.093	0.200
1.6	0.042	0.105	0.208
3.1	0.032	0.111	0.231
6.3	0.034	0.149	0.250
12.5	0.024	0.176	0.215
25.0	0.018	0.201	0.209

Suppression of false positive IgM-anti-annexin IV determination by addition of a rabbit γ-globulin fraction to sample buffer.

Dilution of γ-Globulin Solution	IgG+/IgM- Serum + Rheuma Factor Serum (6.5 IU/ml)
none	0.157
1:160	0.147
1:80	0.126
1:40	0.140
1:20	0.111
1:10	0.112

TABLE 2

Distribution Analysis of Anti-annexin Autoantibody Titres as Determined in 114 Samples from Healthy Blood Donors.

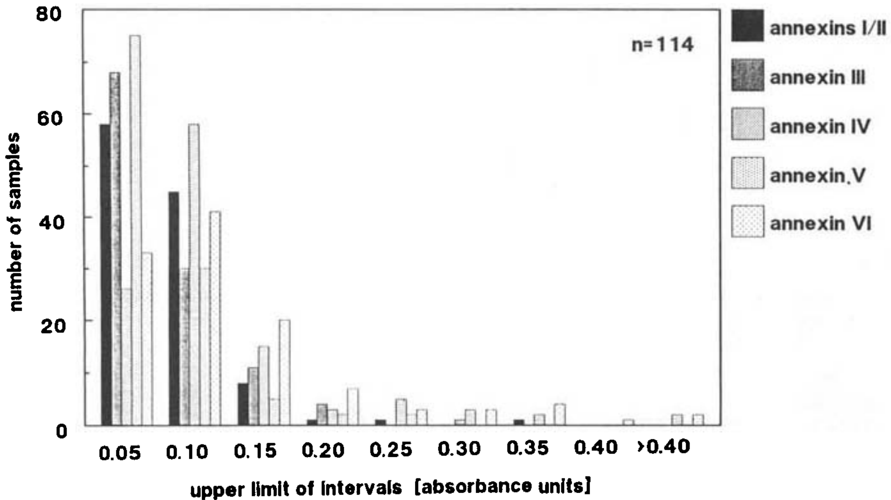
The values are expressed in absorbance units separately for each annexin and type of antibody.

Annexin	I/II	III	IV	V	VI
IgG-type AA					
Mean	0.057	0.058	0.099	0.051	0.104
Median	0.049	0.041	0.067	0.039	0.078
90%-Percentile	0.109	0.105	0.177	0.090	0.193
95%-Percentile	0.140	0.131	0.224	0.112	0.248
IgM-type AA					
Mean	0.078	0.054	0.085	0.050	0.072
Median	0.063	0.043	0.069	0.040	0.061
90%-Percentile	0.136	0.098	0.154	0.093	0.141
95%-Percentile	0.170	0.123	0.193	0.117	0.181

Downloaded At: 11:35 16 January 2011

presented in Fig. 6 for IgG-type AA and Fig. 7 for IgM-type AA. They are skewed and follow a logarithmic normal distribution (the median equals the resulting mean after logarithmic transformation of the distribution classes). These logarithmic normal distributions were used to estimate the true 90%- and 95%-percentiles of the underlying distribution.

The data also demonstrate that the signals obtained for anti-annexin IV and VI AA determinations are generally



Normal distribution of human IgG-AA directed against several members of the annexin family. The absorbances for anti-annexin AA of the IgG-type were determined in normal, healthy blood donors (n=114) for annexins III to VI and a mixture of annexins I and II. The bars indicate the numbers of samples which fell into the appropriate distribution class of absorbance units.

Downloaded At: 11:35 16 January 2011

higher than those for the other annexin AA as expressed in higher mean and median values (Table 2). This is due to the fact that for conveniance a general assay scheme had been applied to all anti-annexin AA ELISAs, although optimum conditions are somehow different especially as it concerns coating solutions and diluents.

Occurrence of anti-annexin AA in patients with malignant melanoma: The distribution profiles obtained from

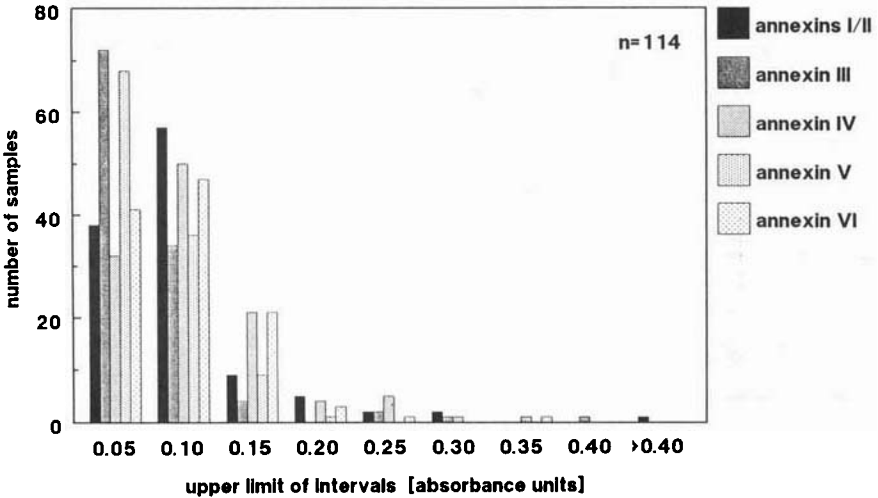


FIGURE 7

Distribution profile of human IgM-AA directed against several members of the annexin family. For explanation see legend to Fig. 6.

Downloaded At: 11:35 16 January 2011

measurements in apparently healthy blood donors were used for the definition of 'positive' anti-annexin AA samples. In an examination of 46 patients with malignant melanoma serum samples having absorbance values above the 95%-percentile of the corresponding annexin and type of antibody tested for, were regarded upon as 'positive' for the respective annexin and type of AA.

With respect to this definition 14 out of 46 patients (30%) were positive. Out of this pool five positive sera contained AA of the IgG-type, 8 only IgM-type AA and 1

TABLE 3

Distribution of Specificities of Autoantibodies against Annexins in 13 Anti-Annexin AA Positive Patients with Malignant Melanoma.

Annexin	I/II	III	IV	V	VI
IgG-type AA	0	2	3	1	0
IgM-type AA	5	2	1	2	2

Downloaded At: 11:35 16 January 2011

exhibited both types. The individual patient exerted a high sensitivity: all IgG-anti annexin AA were directed against only one annexin, while in 3 patients the IgM-anti annexin AA had two specificities. The specificity was evenly distributed against all annexins tested for as listed in Table 3. Due to the multiple specificities observed in some patients as outlined above, the sum of positive observations in Table 3 is higher than the number of positive patients.

To allow a comparison of the data of the LAA-titres against all annexins the absorbances were related to a neutral measure, e.g. the 95%-percentile of the distribution of the respective anti-annexin and type of antibody tested for, yielding relative absorbances (rel. abs.):

$$\text{rel. abs.} = \frac{\text{absorbance in Elisa}}{\text{absorbance at 95\%-percent interval of normal distribution}}$$

The relative absorbances may be added, revealing the abundance of sera with high AA-titres, as is presented in Fig. 8 for the data obtained with sera from apparently healthy blood donors and from patients with malignant melanoma, for IgG- and IgM-type AA separately. According to this figure there were no differences in the abundance of IgG-anti annexin AA to those observed in healthy persons, which even tended to have higher titres of IgM-type anti-annexin AA.

Downloaded At: 11:35 16 January 2011

DISCUSSION

Despite the possible role of anti-annexin autoantibodies (AA) in the development of uncontrolled, chronic inflammation, it took 10 years since the first examinations of Hirata et al. (15) that the occurrence of anti-annexin AA in chronic, inflammatory diseases was again investigated by other research groups (13,16-18). On the basis of solid-phase ELISA technique using annexin I a correlation between anti-annexin I AA titres and chronic, inflammatory diseases or corticosteroid requirement could not convincingly be demonstrated.

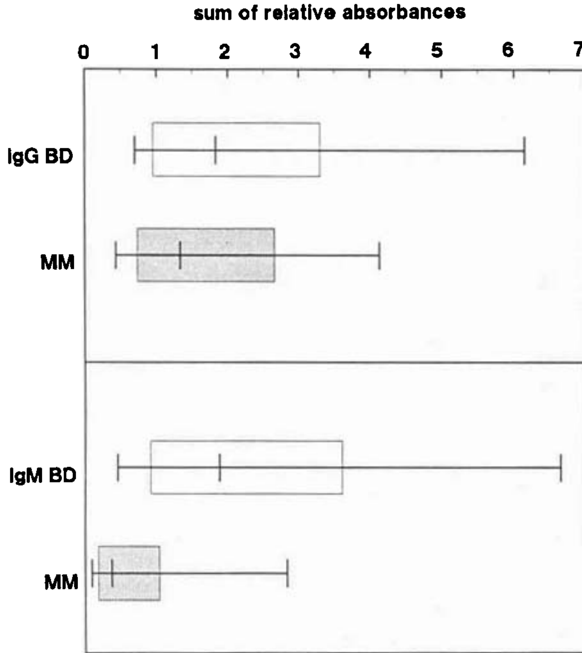


FIGURE 8

Distributions of absorbance values for IgG- and IgM-anti-annexin AA in normal blood donors (n=114) and patients with malignant melanoma (n=46). Presented are the sums of the relative absorbances (absorbance divided by the 95%-percent interval of the respective normal distribution) determined for each annexin and type of antibody. BD, normal distribution as obtained with sera from blood donors; MM, distribution in patients with malignant melanoma. The box delineates the 10% and 90%-percentiles, the bars show the upper and lower ranges and the median of the distributions.

This may partly be due to the fact, that only one member of the annexin family had been taken into consideration. There is, however, too less information about the physiological role of these proteins, which would already allow to omit the other annexins.

Downloaded At: 11:35 16 January 2011

For this reason we developed solid phase enzyme immunoassays for the determination of IgG- as well as IgM-type AA against six members of the annexin family, in particular for anti-annexin I to VI. Employing these assays the distribution of anti-annexin AA titres in sera of normal blood donors allowed the detection of "positive" sera with respect to each annexin and type of antibody.

During development of the assay systems special care had been taken with regard to low background values, but achieving optimum signal/noise ratios. The specificity of the ELISA system employed was investigated with competition experiments using either free annexins or polyclonal antibodies against annexins added to positive human sera. However, we failed to demonstrate a competitive effect with free annexins, while half maximal suppression of absorbance signals in human positive sera was achieved by addition of specific rabbit antibodies. This finding coincides with the observations of Goulding et al. (17), who showed that annexin I could only compete with surface bound antigen when itself absorbed onto E. coli membranes or phospolipid micelles. For some samples with about 0.5 absorbance units in the ELISA assay it may be deduced from competition experiments with specific polyclonal antibodies that about 0.1% of total IgG and between 2 and 14% of total IgM in the patients were specific for the respective annexin.

Downloaded At: 11:35 16 January 2011

Care was taken for elimination of false positive results for IgM-type anti-annexin AA determination in patients with high IgG-type titres due to influence of rheuma factor. This problem, however, seems not to be of particular relevance, since only 1 of 14 anti-annexin AA positive patients revealed high titres of IgG- and IgM-type AA as well, but in this special case they differed in specificity (IgG: anti-annexin IV; IgM: anti-annexin I/II).

The abundance of anti-annexin AA and their specificities were investigated in sera of 46 patients suffering from melanoma, 14 (30%) of which were positive, where "positive" had been defined having an AA titre above the 95%-interval of the distribution of the respective anti-annexin as observed in apparently healthy blood donors. Astonishigly, the sera containing especially IgG-type AA were monospecific and only three out of eight IgM-type AA were bispecific. Furthermore, the spectrum of anti-annexin AA covered all annexins examined. Interestingly, despite the sequence homologies between the members of the annexin family cross-reactivity obviously was low. Thus determination of the six anti-annexin AA yields nearly independent parameters. Therefore, when applying the criteria for a "positive" reaction in retroperspective to the 114 sera of healthy blood donors, it is not

Downloaded At: 11:35 16 January 2011

astonishing that approximately 30% of them (six-times 5% above the exclusion interval) also live with anti-annexin AA without significant pathological symptoms.

The pathophysiological meaning of such AA is yet unknown. It is questionable, whether they are causally related to the development of a disease or if they are generated during its course. Do people generating these AA bear an enhanced risk in developing disease, because their anti-inflammatory (in the term of annexin) potential is partially neutralized by AA? Further studies including other indications are necessary to answer these questions.

Downloaded At: 11:35 16 January 2011

ACKNOWLEDGMENTS

We thank Beate Mathews for excellent technical assistance.
Requests for reprints should be made to Dr. Jürgen Römisch, Research Laboratories, Behringwerke AG, P.O. Box 1140, W-3550 Marburg/L, Germany.

REFERENCES

1. **Klee, C.B.** Ca^{2+}-dependent phospholipic- (and membrane-) binding proteins. Biochem. 1988; 27:6646-6653.

2. **Crompton, M.R., S.E. Moss, and M.J. Crumpton.** Diversity in the lipocortin/calpactin family. Cell 1988; 55:1-3.

3. **DiRosa, M., R.J. Flower, F. Hirata, L. Parente, and F. Russo-Marie.** Nomenclature announcement: anti-phospholipase proteins. Prostaglandins 1984; 28:441-442.

4. **Crumpton J.J., and J.R. Dedman.** Protein terminology tangle. Nature 1990; 345:212.

5. **Zaks, W.J., and C.E. Creutz.** Evaluation of the annexins as potential mediators of membrane fusion in exocytosis. J. Bioenerg. Biomembr. 1990; 22:97-120.

6. **Schlaepfer, D.D., and H.T. Haigler.** Expression of annexins as a function of cellular growth state. J. Cell Biol. 1990; 111:229-238.

7. **Reutelingsperger, C.P.M., G. Hornstra, and H.C. Hemker.** Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord. Eur. J. Biochem. 1985; 151:625-629.

8. **Tait, J.F., M. Sakata, B.A. McMullen, C.H. Miao, T. Funakoshi, L.E. Hendrickson, and K. Fujikawa.** Placental anticoagulant proteins: isolation and comparative characterization of four members of the lipocortin family. Biochemistry 1988; 27:6268-6276.

9. **Römisch, J., and N. Heimburger.** Purification and characterization of six annexins from human placenta. Biol. Chem. Hoppe-Seyler 1990; 5:383-388.

10. **Parente, L. C. Becherucci, M. Perretti, E. Solito, C.L. Mudridge, G. Galeotti, M. Raugei, and M. Sanso.** Are the lipcortins the second messengers of the antiinflammatory action of glucocorticoies? Prog. Clin. Biol. Res. 1990; 349:55-68.

11. **Davidson, F.F., and E.A. Dennis.** Biological relevance of lipocortins and related proteins as inhibitors of phospholipase A_2. Biochem. Pharmacol. 1989; 38:3645-3652.

12. **Hirata, F., E. Schiffmann,K. Venkatasuburamanian, D. Salomon and J. Axelrod.** A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticosteroids. Proc. Natl. Acad. Sci. USA 1980; 77:2533-2536.

13. **Goulding, N.J., J.L. Godolphin, P.R. Sharland, S.H. Peers, M.Sampson, P.J. Maddison, and R.J. Flower.** Anti-inflammatory lipocortin-1 production by peripheral blood leucocytes in response to hydrocortisone. Lancet 1990; 335:1416-1418.

Downloaded At: 11:35 16 January 2011

14. **Müller W., and F. Schilling.** Differentialdiagnose rheumatischer Erkrankungen. Basel-Wiesbaden: Basel-Wiesbaden, 1982.

15. **Hirata F., R. Del Carmine, C.A. Nelson, J. Axelrod, E. Schiffmann, A. Warabi, A.L. De Blas, M. Nirenberg, V. Manganiello, M. Vaughan, S. Kumagai, I. Green, J.L. Decker, and A.D. Steinberg.** Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases. Proc. Natl. Acad. Sci. USA 1981; 78:3190-3194.

16. **Podgorski, M.K, N.J. Goulding, N,D. Hall, R.J. Flower, P.J. Maddison, and R.B. Pepinsky.** Autoantibodies to recombinant lipocortin in RA and SLE. Br. J. Rheumatol. 1987; 26:54-55. (Abstr.)

17. **Goulding, N.J., M.R. Podgorski, N.D. Hall, R.J. Flower, J.L. Browning, R.B. Pepinsky, and P.J. Maddsion.** Autoantibodies to recombinant lipocortin-1 in rheumatoid arthritis and systemic lupus erythematosus. Ann. Rheum. Dis. 1989; 48:843-850.

18. **Wilkinson, W. Jr., M.R. Podgorski, N.J. Goulding, J.L. Godolphin, and T.H. Lee.** Circulating autoantibodies to lipocortin in corticosteroid resistant bronchial asthma. Clin. Exp. Allergy 1990; 20:108. (Abstr.)

Downloaded At: 11:35 16 January 2011