

H. E. H. Liebermann¹

Research Article

¹Varicula Life Science AG,
Willem-Kolff-Platz 1,
D-17166 Teterow, Germany.

Reaction Pattern in Vertebrate Cells (RiV): May the anti-RiV ELISA Detect a Pathologic Cell Proliferation? (First Results)

RiV is mainly represented by characteristic exosome-like particles with a diameter of 30 to 70 nm. RiV particle preparations (RiV-PP) contain identified proteins. In the present paper, the authors started an evaluation of the hypothesis, that RiV-reactive antibodies, detected in patient sera by an indirect ELISA, may be used for the diagnosis of pathologic cell proliferations. On the basis of the frequency distribution curve of about 500 blood donor sera, cut-off values for RiV-reactive IgG and IgM antibodies could be defined. Additionally it was found that 1.6 % of blood donor sera reacted strongly with an RiV-PP (of calf kidney cells) for IgG antibodies. 29 % of cancer patient sera ($n=48$), 17 % of 53 patients with endoprothetics and with lung disorders, 44.3 % of 115 patient sera of a doctor's practice and 28 % of 97 sera of 10 to 11 years old schoolchildren were positive in the anti-RiV ELISA with IgG antibodies. About half of the selected group of schoolchildren contained different autoantibodies and some of these children displayed already insulin dependent diabetes mellitus (as shown by others). A correlation of our ELISA results with the other autoimmune antibody results was not found. Using RiV-PP as an antigen complex, isolated from calf kidney cell cultures or from human FL cell cultures and HeLa cell cultures, results with small differences between human and animal RiV origin were obtained. Using the human RiV, the number of positive sera was not significantly lower. Generally, the anti-RiV ELISA, using RiV-PP from human cell cultures, detect autoantibodies against RiV-specific proteins. This assay may detect, but not diagnose pathologic cell proliferation. The possibilities of RiV ELISA for diagnostics of defined autoimmune diseases, as well as further studies and the more meaningful RiV immunoblot are discussed.

Keywords: Antibodies, Medical biotechnology, Pathologic cell proliferation, Tumor patients sera

Received: August 14, 2007; *revised:* January 25, 2008; *accepted:* February 6, 2008

DOI: 10.1002/elsc.200720233

1 Introduction

The term “reaction pattern in vertebrate cells” (RiV) was defined after extensive electron microscopic experiments in 1986 [1].

Correspondence: H. E. H. Liebermann (herbert_liebermann@web.de or liebermann@varicula.de), Varicula Life Science AG, Willem-Kolff-Platz 1, D-17166 Teterow, Germany.

RiV particles are characteristic exosome-like particles with a mean diameter of 30–70 nm typically seen in ultrathin sections of stressed cells in culture [1, 2]. The isolated RiV particle preparations (PP) have been characterized both in the EM after negative staining and by biochemical analyses [3–6].

Since the RiV particles were produced from primary, secondary or permanent cell cultures of vertebrates after a stress phase, preferably by hunger [1, 4], and cells without or together with the supernatant were used for the preparation of RiV particles, these particles are mainly special intrinsic (intraluminal) particles [7–9].

RiV-PP have been applied as an experimental vaccine against laryngeal papillomatosis [10, 11] and against foot and

mouth disease of guinea pigs [3], mammary carcinoma of mice [12] and mouse myeloma [13, 14]. On a doctor's practice scale, treatment of AIDS, cancer and hepatitis C was also initiated [2, 5, 15–17].

In combination with the development of a vaccine against larynx papillomatosis by Hahnefeld et al. [10], sera were also tested for reactivity with the vaccine preparation (identical with the RiV sample or RiV-PP). Some sera of animals as well as some sera of humans, who not received the vaccine, reacted with the RiV sample. Investigations of patient sera of a gynaecological hospital, of blood donors and of cancer patients by ELISA with RiV-PP as an antigen resulted in the conclusion that RiV-reacting antibodies (anti-RiV antibodies) could be used for the diagnosis of diseases, the nature of which is an uncontrolled cell proliferation [5, 18]. In the present paper, this hypothesis was tested. For that purpose, sera of cancer patients, of patients with endoprothetics and with lung disorders, of a doctor's practice and sera of 10- to 11-year-old schoolchildren were examined. Furthermore, the authors report on a comparison of ELISA using RiV-PP of bovine and of human origin for the exclusion of false positive results with the bovine RiV samples and on first investigations about a possible correlation of anti-RiV ELISA (anti-RiV antibody assay, ARIVA) with some known laboratory parameters such as C-reactive protein (CRP) and borrelia-specific antibodies.

2 Materials and Methods

2.1 Cells and Preparation Procedure for the RiV-PP as an Antigen

Primary calf kidney cells were cultivated and the RiV sample was prepared as previously described [4, 19]. Human FL cell line (amnion, HeLa markers) and human HeLa cell line (*epitheloid cervix carcinoma*) were obtained from the Friedrich-Loeffler-Institut (ZBV, Bundesforschungsinstitut für Tiergesundheit, Greifswald – Insel Riems, Germany) and the *Human A549 non-small cell lung adenocarcinoma cell line* were kindly placed at our disposal from the Friedrich-Loeffler-Institut für Medizinische Mikrobiologie, Universität Greifswald, Germany. For the preparation of a human RiV-PP as an antigen for ELISA HeLa cells, FL cells and A549 cells were cultivated as monolayers. After confluence (>80%) of cell cultures in T175 – or roller bottles – the medium (MEM, Eagle and additive salts and/or nonessential amino acids) with 10% fetal calf serum (FCS) was replaced by medium without FCS after washing steps using PBS. The further procedure was the same as for calf kidney cells.

2.2 Sera

RiV Specific Antiserum: A polyclonal antiserum pool against an RiV-PP sample was produced by subcutaneous and intramuscular injections into rabbits as previously described [4] and it was used as a standard RiV-specific antiserum (1:500) for RiV antigen titration.

2.3 Protein Quantification

The protein content of the samples was determined by a modified method of Lowry et al. [20], using a micro-variant.

2.4 UV Spectroscopy

For the measurement of the ultraviolet absorption spectrum of RiV-PP, a universal spectral photometer was used. The calculation of protein concentration was performed as described previously [4].

2.5 ELISA

The assay for the RiV antigen titration was carried out as an indirect assay with horseradish-conjugated secondary anti-rabbit antibodies and *O*-phenylenediamine as a substrate as previously described, using a rabbit RiV-specific antiserum as a standard in a dilution of 1:500 [4].

For the examination of human sera by ARIVA (anti-RiV antibody assay), the assay was performed analogously to earlier described ELISA [21]. Polystyrene microplates (MP) with a high adsorption capacity (Greiner, Frickenhausen, Germany) were coated with a defined dilution of RiV antigen in PBS, pH 7.4 (2 h at 37 °C). As a standard antigen (SA), an RiV sample of calf kidney cell cultures was used. Then and after each of the following steps, the plates were washed 4 times with Immuno Wash (Bio-Rad, Hercules, CA, USA). Normally, two wells of the plates were incubated with a 1:50 dilution of a human serum for 2 h at 37 °C. A pool of normal sera (negative sera pool) and a pool of positive sera, the latter also in the dilutions 1:125, 1:312.5 and 1:781.25 were always carried along. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-human-IgG-HRP and anti-human-IgM-HRP, respectively) for 45 min at 37 °C, bound antibodies in the test sera were detected using *o*-phenylenediamine. Optical density (OD) was determined with an ELISA reader (anthos 2020, Anthos Labtec Instruments, Krefeld) at 492 nm. The OD value of the positive sera pool in the dilution 1:50 must be always >2× cut-off value.

The index values of the sera were demonstrated (ratio of determined OD at 492 nm to cut-off). A temporary cut-off for the RiV-reactive IgG and IgM values resulted from the mean value of the normal (negative) sera pool plus 0.1 OD. A gray area for the index was defined with 1.0 ± 0.1 . Sera with index values <0.9 were normal (or “negative”) and sera with index values >1.1 were positive. Sera with index values ≥ 2.5 were valued as strongly positive.

2.6 Electron Microscopy

Negatively stained RiV samples were prepared on Pioloform-carbon-coated copper grids (400 mesh). After glow-discharging the grids, samples were adsorbed for 10 min, followed by washing two times with double-distilled water and negative staining with 1% uranyl acetate for 30 s.

2.7 Immunogold Electron Microscopy (IEM)

Immunogold electron microscopy was performed as previously described [4].

3 Results and Discussion

3.1 Analysis of RiV Sample Antigen by ELISA, SDS-PAGE, Immunoblot, UV Spectrometry and Electron Microscopy

Following the studies of Solisch and Bergmann [22], at first and for the most sera an RiV sample, obtained from calf kidney cells, was used as a standard antigen in ELISA. RiV-PP were analyzed using ELISA, SDS-PAGE, immunoblot and UV spectroscopy as previously shown [4].

After the titration of the “standard antigen” (SA) in dilutions from 1:20 to 1:1280, a dilution of 1:80 was normally used for this SA in ELISA of patient sera. Annexin I and V were the main components in the SA and other RiV-PP samples as antigens, determined by SDS-PAGE and immunoblots (not shown), see also [4]. The protein content of the used SA was $77 \pm 17 \mu\text{g/mL}$, determined by UV measurement and $105 \pm 14 \mu\text{g/mL}$, respectively, determined by the method of Lowry. For the comparison of the antigen concentration of other RiV samples, prepared from bovine or human cell cultures, not only the protein concentration was determined, but also an ELISA (an antigen titration) of the RiV sample and the standard RiV antigen on the same MP was performed. For this test; a “standard” anti-RiV-PP rabbit serum and comparatively also a pool of positive patient sera were used (not shown).

An RiV sample, also prepared from calf kidney cell cultures, was used for comparison in ELISA of patient sera. This RiV sample displayed a protein content of $90 \pm 10 \mu\text{g/mL}$ and in ELISA a factor F of 1.16 ± 0.19 , using the anti-RiV-PP antiserum of rabbits. (The factor F was defined as the relation of an RiV-PP sample dilution at a defined OD to the standard antigen dilution at the same OD.) The factor F of 1.16 means that

this sample should be used in ELISA for sera evaluation e.g. in a dilution of 1:90 (1:93), if the standard antigen is used in a dilution of 1:80.

Electron microscopy was used for a quality control of RiV-PP. Fig. 1 displays RiV-PP from calf kidney cell cultures, HeLa cell cultures and A549 cell cultures. The RiV particles in Fig. 1C seem to be broken up, a little damaged. The diameters of the RiV particles of these preparations did not significantly differ.

3.2 ARIVA: Frequency Distribution Curve

A possibility to obtain a cut-off value is the creation of a frequency distribution curve with normal blood donor sera. The frequency distribution curve of 486 sera (see Fig. 2A) were used to determine $m + 2s \rightarrow 97.725\%$. For the IgG index, a value of 1.0 (at a mean value of extinctions at 492 nm of 0.1) was obtained (m : mean value; s : standard deviation), which means that the temporary cut-off for BC-RiV-reactive IgG values could be used further and sera with index values $<(1.0 \pm 0.1)$ were normal (or negative) as before. Index values of 1.0 ± 0.1 were in the gray zone and values >1.1 were positive. There were different results for BC-RiV-specific IgM values as shown in Fig. 2B. (One serum was omitted because of a very high index). The much broader distribution curve resulted in an upper limit of 1.62 (on the basis of the “old” temporary cut-off). Index values of 1.782 ± 0.178 are in the new gray zone and IgM index values ≥ 1.96 are positive. (Under the norm would be index values <0.027). Per definition, 2.275% of these sera would be positive (without a gray zone). Obvious runaways were omitted in the calculation. It was found that 1.6% of all blood donor sera reacted strongly with the RiV-PP standard sample (of calf kidney cells) for IgG antibodies, see Fig. 2A. These sera and some also strongly positive patient sera were mixed to an intern “positive control serum standard”. Since sera of blood donors were received completely anonymous, blood donors with strongly RiV-reacting sera could not be medically checked.

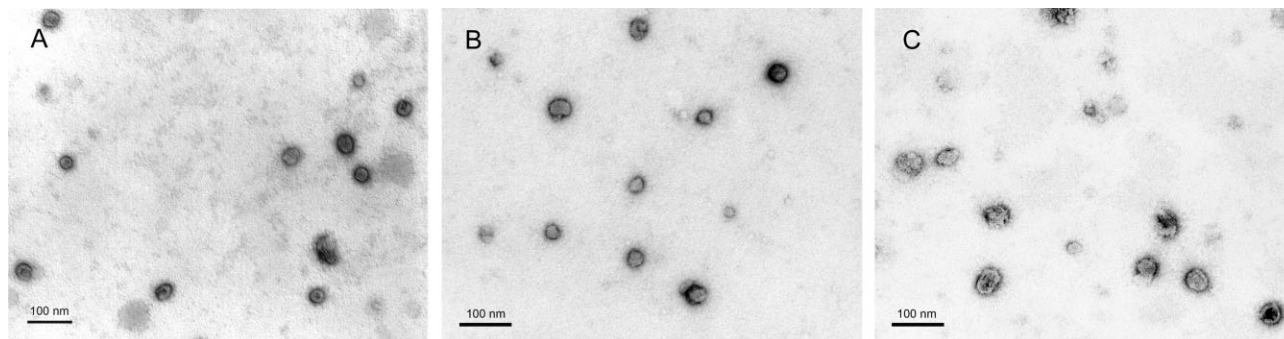


Figure 1. Electron microscopy of RiV samples (RiV particle preparations, RiV-PP). (A) RiV-PP, produced from calf kidney cells. The image was recorded at an instrumental magnification of 60 000. (B) A RiV-PP from HeLa cells. (C) An RiV-PP from A549 cells. The RiV particle diameters in Fig. 1A vary from 32 nm to 41 nm with a mean value of 37 nm, in Fig. 1B from 30 nm to 46 nm with a mean value of 37 nm and in Fig. 1C from 37 to 51 nm with a mean value of 44 nm.

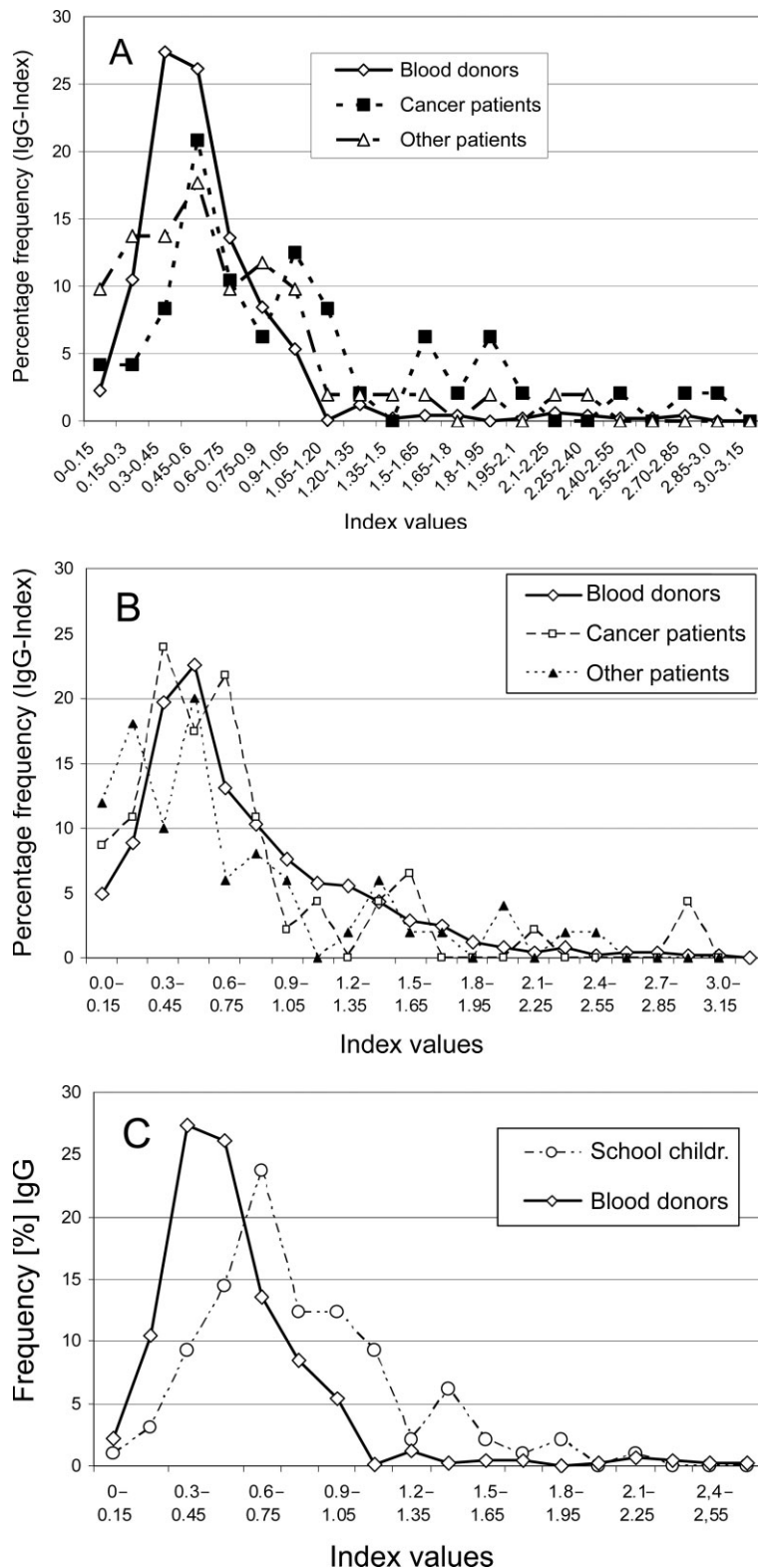


Figure 2. Frequency distribution curves of RiV-PP reactive values, determined by ELISA/ARIVA. (A) IgG values of 486 blood donor sera, of 48 cancer patients and of 51 patients with endoprothetics and with pneumonological diseases. (B) IgM values of 484 blood donor sera. (C) IgG values of blood donor sera and of 97 sera of 10- to 11-year-old pupils, see text.

3.3 ARIVA using Different Patient Sera

Sera of cancer patients ($n=48$) (from G. Schulze, Berlin-Buch) and of patients with endoprothetics and with lung diseases (from O. Missal, Sommerfeld) displayed in the IgGARIVA stronger deviations to the distribution curve of blood donor sera (see Fig. 2A). 29% of cancer patient sera were positive in the IgG ELISA and in comparison 17% of 53 sera of patients with endoprothetics and with pneumonological diseases were also positive in IgGARIVA. (Two sera of the last group were not considered because of very high index values). 44.3% of 115 sera of patients with different diseases of a general practice were positive in IgG ELISA and 40.9% in IgM-ELISA on the basis of the temporary cut-off. Two patients of this group with a prostate hyperplasia (BPH) displayed strongly positive values in IgG ELISA and normal values in IgM ELISA.

Furthermore, 97 sera of 10- to 11-year-old schoolchildren were tested in ARIVA. A part of 52% of these sera contained different autoantibodies, GADA (glutamic acid decarboxylase autoantibody), or/and IAA (insulin autoantibody), IA-2A (protein tyrosine phosphatase autoantibody), ICA (islet cell antibody) and 10 of these schoolchildren with autoantibodies displayed already IDDM (Insulin dependent diabetes mellitus) [23, 24]. It was interesting that on the basis of the temporary cut-off, 65% of these sera were positive in IgM ARIVA. 28% of these sera were positive in IgGARIVA (Fig. 2C). The authors did not find a good correlation of our ARIVA results with the other autoimmune antibody results. Such a correlation would have been coincidentally, because RiV-reactive antibodies are not identical with the other autoimmune antibodies. It seems that schoolchildren have more RiV-reacting antibodies of the IgM class than adult persons, because only 38.8% of a reference group ($n=49$) of 18 to 21 years old persons were positive in IgM ARIVA on the basis of the temporary cut-off and 25% in IgGARIVA. Because the IgG index percentage values are also relatively high, it may be speculated that there is a dependence of age. But much more sera have to be tested for such a statement. The greater part of positive RiV-reactive antibody values (or of "autoantibodies") found in the age of schoolchildren might also be temporary because of virus and other infections and of vaccinations, inducing also the development of cross reactive antibodies.

More positive results were obtained with the ELISA of patient sera, when crude (unpurified) RiV antigen was used – the suspension with destroyed cells together with supernatant after clarification. But the influence of the purity of RiV antigen, especially RiV-PP and other RiV preparations,

on ELISA results with patient and blood donor sera was so far not further investigated.

3.4 ARIVA: Correlation with Some Other Laboratory Parameters

An evaluation of patient sera was started regarding a possible correlation of RiV ELISA/ARIVA values with known laboratory parameters. Different sera with known CRP values and borrelia-specific antibody titers and also GGT, ALAT and ASAT (aspartataminotransferase, EC 2.6.1.1) values were tested in ARIVA. If such a correlation with a high correlation coefficient or eventually a regression would exist, the ARIVA would be unnecessary or not essential. CRP was selected, because this acute phase protein is a parameter for an inflammation. On the other hand, annexin I as a major protein in RiV-PP acts anti-inflammatorically, so that RiV- or annexin I-reactive antibodies could perhaps correlate with CRP values in a reverse manner. Patient sera with *Borrelia*-specific antibodies were selected, because two patients of a doctor's practice with such antibodies displayed also stronger RiV-reactive antibody values and a cross reactivity could be possible. The results are shown in Figs. 3A and B. In Fig. 3A, the correlation coefficient is -0.323 and the degree of certainty $B\%$ (coefficient of determination) is only 10.4%. The correlation coefficient is the measured value for the dependence for the linear correlation of two quantitative features [25]. The linear trend line in Fig. 3B is gentle positive and the potential trend line is gentle negative, the degree of certainty is only 2.76%. Also, sera with known ALAT, GGT and ASAT values and with determined tumor markers were tested and e.g. with GGT, the degree of certainty was found to be also only 6.51%. (If this correlation is coincidentally, then it is non-sensical). At the great mean variation in Fig. 3A it seems to be necessary to test more sera.

Generally, the low correlation coefficients and degrees of certainty found indicate that the ARIVA may not be replaced by one of these other tests and ARIVA should be in principal of interest. It seems to be sensible that further experiments should be performed to obtain a correlation (or possibly a regression) of ARIVA with some selected tumor markers. In this case, only patients without an immunosuppressive acting treatment can be considered. Sera and the laboratory parameters were obtained by G. Menzel, F. Wegner and K. Meinck, (*Ärztliche Laborgemeinschaft der Hansestadt Greifswald, D-17489 Greifswald*).

3.5 ARIVA, using RiV Antigen of Human Origin

A disadvantage of the previous ELISA results of patient sera is the fact that they were obtained using an RiV antigen of calf kidney cells and not of human cells. That means, a patient ser-

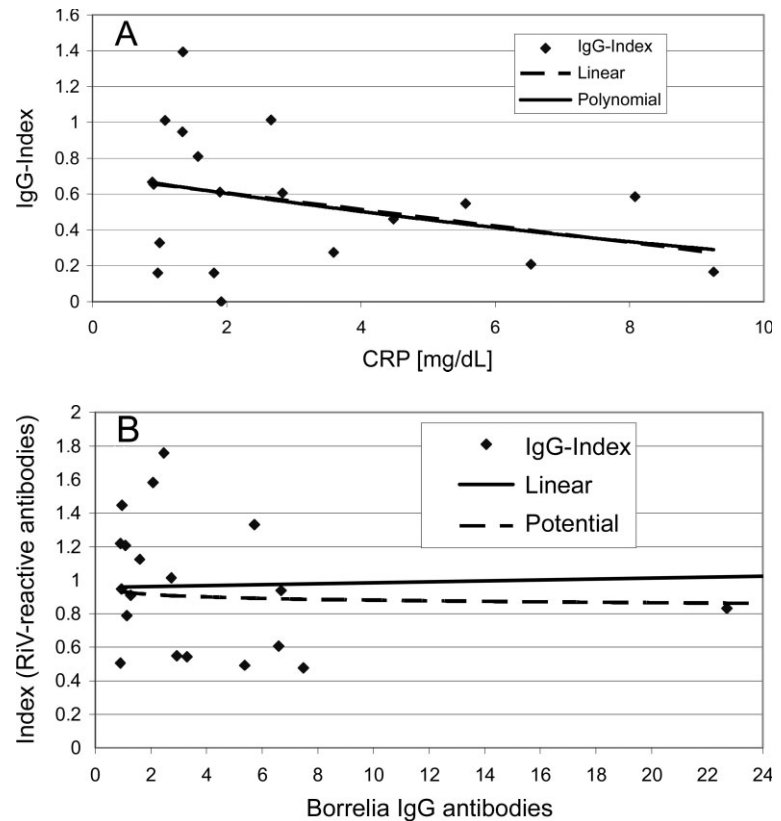


Figure 3. Some laboratory parameters and RiV-PP reactive antibody values (IgG ELISA). RiV-PP reactive IgG antibody values in dependence of (A) C reactive protein (CRP) and (B) *Borrelia*-specific IgG antibody titers, see text. (Linear: linear trend line; polynomial: polynomial trend line; potential: potential trend line).

um with reacting antibodies (a positive serum) could contain antibodies only against proteins of bovine origin and had not to have autoantibodies. For the exclusion of false positive sera it was necessary to use an RiV-PP sample of human cells. But the yield of RiV-particles of HeLa cell cultures and FL cell cultures was low in the first experiments so that a mixture of RiV antigen from HeLa cells and FL cells was used for the ELISA. Fig. 4A–C confirm that there are some differences in the results using the bovine or human RiV antigen. Some index values of sera with the bovine RiV antigen display runaways in comparison to ELISA results with human RiV antigen (Fig. 4A). But also some sera display much higher values with human RiV antigen than with bovine RiV antigen. A reason for these differences in the corresponding values may be the existence of anti-bovine antibodies. Other reasons may be different concentration ratios of the proteins in the RiV antigens and/or a deviating protein composition of the human and bovine RiV-PP and different antibody spectra of the patients sera. The SDS-PAGE pattern of the two RiV-antigen complexes (RiV-PP) was similar (not shown). Typical SDS-PAGE pattern of bovine RiV-PP samples were shown in [4].

Figs. 4B and 4C demonstrate that the courses of the antibody development of patients, reacting with the human and bovine RiV antigens, are similar. But in Fig. 4B, all index values, using human RiV antigen, are lower than the index values,

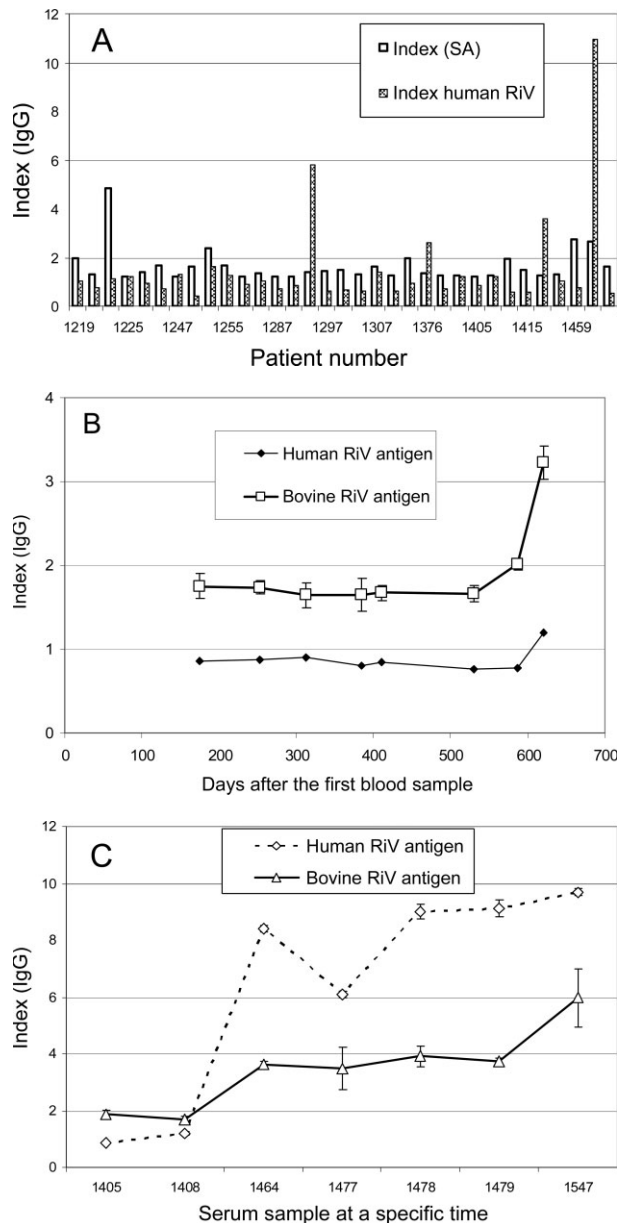


Figure 4. ELISA: Comparison of the RiV-reactive antibody determination using human and bovine RiV-PP, respectively. (A) RiV-PP from FL cell cultures and Hela cell cultures (Index human RiV) as antigen and bovine RiV-PP from calf kidney cell culture (Index SA) as standard antigen (SA) and 33 sera of 30 patients were used. (B) A section of the development (course) of antibodies of sera of patient J. S. with a gliosarcoma. The patient was treated with bovine RiV-PP. The standard deviations of two to four independent experiments for bovine RiV as an antigen and in one experiment for human RiV as an antigen are shown. (C) A section of the course of antibodies in sera of patient M. H. with a chronic hepatitis C and treated at first with peginterferon alpha-2a and Ribavirin, then with the bovine RiV-PP and after a rest the treatment was started once more with a different “immunomodulating” vaccine some weeks before the serum sample number 1464 and the following samples were obtained, see text. The standard deviations are shown.

using bovine RiV antigen. In Fig. 4C, they even arise stronger, starting with serum sample number 1464. Fig. 4C displays a section of the course of antibodies in the sera of a patient with a chronic hepatitis C and treated at first with a pegylated α -interferon (PEGASYS) and Ribavirin [26]) (and stopped because of intolerance), later treated with an RiV-PP sample for months without side effects and with disappearance of liver enlargement and disappearance of ascites (and stopped because of a non-medical reason). After a rest, the treatment was started again with a different “immunomodulating” vaccine some weeks before the serum sample number 1464 was obtained. The last treatment had to be stopped because of the development of an intolerance.

(Sera of both patients, see Figs. 4B and 4C, were tested against a human RiV sample as an antigen on the same microplate). It is assumed that besides another reactivity of the patients (another antibody spectrum and different diseases, see legends) the treatment with the other different vaccine, see Fig. 4C, resulted generally in a strong antibody production. If the author used a second bovine RiV-PP sample in comparison with the standard RiV-PP as an antigen, no significant differences e.g. in the respective IgM index values of 6 sera of a cancer patient were obtained (not shown).

3.6 Sensitivity and Specificity of ARIVA

The sensitivity is the ratio of test positives to the whole number of the diseased individuals, the actual positive individuals and it explains, with which probability the assay really responds at the existence of a disease.

At a cancer disease there are uncontrolled cell proliferations. If the hypothesis can be corroborated that the ARIVA displays this cell proliferation then the sensitivity must be very good. But e.g. only 14 of 48 sera of cancer patients (a gift of G. Schulze, Berlin-Buch) were positive in ARIVA for IgG values, corresponding to a sensitivity of 29%. Using sera of patients before chemo- and radiotherapy, i.e. before the immunosuppression, 8 of 19 sera, i.e. 42%, reacted positive.

The specificity is the ratio of test negative ones to the whole number of the clinically healthy individuals, and it explains, how well the assay displays the real existence of the disease. For additional blood donor sera, 44 positive sera of 432 sera were found in IgG ARIVA, that means, 388 normal/negative sera. This would result in a specificity of the ARIVA of $388/432 = 0.898$ subjected to the condition that really all donors are healthy. As mentioned above, blood donor sera were obtained without identification number and it was not possible to check the corresponding blood donors (positive sera) with regard to a preclinical illness with a pathologic cell proliferation.

For “further” evaluations of the hypothesis that a positive result in ARIVA displays a pathological cell proliferation, transplantations of different tumor cells in mice should be carried out and the development of RiV-reactive antibodies should be studied. But syngene mouse models were not offered at that time by our partner epo (Fa. Experimentelle Pharmakol. & Onkol. (epo) GmbH, R.-Roessle-Str. 10, 13122 Berlin, Germany). Corresponding experiments with a mouse myeloma and Balb/c-mice (not T cell deficient) are planned.

3.7 Immunogold Electron Microscopy (IEM)

Some in ARIVA strongly positive blood donor sera were tested also in IEM against two bovine RiV-PP 010104 and 030604. As shown in Figs. 5A and B, RiV particles are weakly gold labeled, which means, these blood donor sera reacted with RiV particles of these samples. In comparison, Fig. 5C displays a stronger reaction of an RiV-PP specific antiserum of rabbits with an RiV-PP sample. A gold-labeled antibody against annexin I in a dilution of 1:20 did not label the RiV particles, although annexin I is an RiV-specific protein, displayed by immunoblot [4].

The experiments should be continued with higher RiV particle concentrations and also with in ARIVA strongly positive sera of patients with known diseases.

4 Conclusions

First ARIVA results display that 29 % of cancer patient sera ($n=48$), 17 % of patients with endoprothetics and with lung diseases ($n=53$), 44.3 % ($n=115$) of patient sera of a doctor's practice and 28 % of sera ($n=97$) of 10- to 11-year-old schoolchildren were positive in the IgG-RiV ELISA. But 42 % of the sera of the cancer patients before chemo- and/or radiotherapy were positive in this RiV ELISA (IgG antibodies) and only 21 % of patients with a chemo- or radiotherapy. Despite its low numbers, the experiments gave hints that the values obtained by ARIVA are falsified (too low) for patients with immunosuppressive acting treatment. Also, tumor therapy course studies with ARIVA confirmed that this assay is unsuitable for patients, who obtain a suppressive acting medical treatment and an ELISA for the detection of RiV (particles as) antigen may be more informative than one designed to detect anti-RiV antibodies [27].

The results led to the conclusion that positive ARIVA values (IgG) may correspond to different symptomatic and/or pre-symptomatic diseases, and sometimes there might also be a pathologic cell proliferation, especially cancer. Since the RiV-

PP contain only some tumor associated proteins, it could be that only some certain cancer diseases will be detected, see below. In the case of a positive ARIVA result it should additionally be checked, if the values exist only momentarily/temporarily. RiV reactive autoantibodies of class IgG or IgM may exist only temporarily, i.e. after an infection (cross reactivity) or after vaccination of schoolchildren. Is the result negative in ARIVA, i.e., normal values were obtained with the human RiV-PP antigen, may be this result is wrong regarding to a disease. This may be because of a immunosuppressive treatment or because of a specific weakness of immune defense or because of the small spectrum of RiV-reactive autoantibodies.

The ARIVA is useable only for a screening of sera for (pre-symptomatic) diagnosis of a defined spectrum of diseases, especially autoimmune diseases, but in some cases also for some tumors. After the screening of the sera by ARIVA, using RiV-PP of human origin, positive sera have to be analyzed then by sensitive immunoblot against a human RiV-PP sample as an antigen complex for a more detailed diagnosis. The production of RiV particles, e.g. in HeLa cell cultures and/or FL cell cultures, and the preparation procedure for RiV particles as an RiV antigen in ARIVA needs to be optimized in our laboratory.

The detection of autoantibodies against a complex of proteins as the biocomplex RiV may be very useful for the diagnosis of a defined spectrum of autoimmune diseases, compare [28]. The analysis of RiV-PP, produced from cell cultures of bovine origin, resulted in the identification of the following proteins: annexin I and annexin V, S-100A2 and S100A4, actin, α -actinin, ezrin and HSP70; enolase; vimentin, aldolase and gCAP39, 14-3-3 ϵ and galectin-3, 14-3-3 ζ , natural killer (NK) cell enhancing factor A, Nm23, glutathion-S-transferase, myosin (light chain), histon 2B, histon 2A, ribosomal protein L7, nucleolin, HSP27, proteasom C6, proteasom (δ), proteasom (I), ER60, PDI, and a stratherin homologous protein (approx. 50 kD) and an annexin-I-isoform (H156Y) [5,6]. The same or a very similar protein composition in human RiV-PP provided, autoantibodies were found inter alia against annexin V, annexin I and galectin. The proteins S100, glutathion-S-transferase, aldolase, vimentin, enolase are cited also as tumor

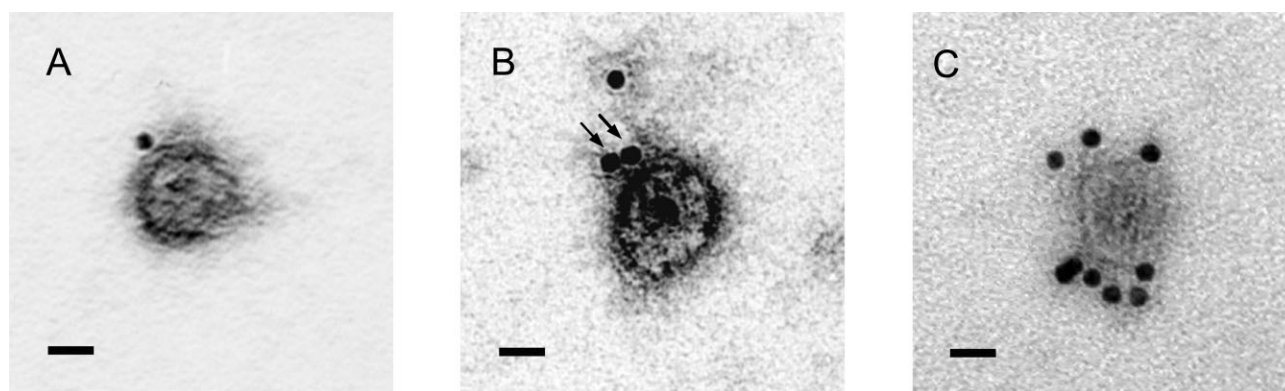


Figure 5. Immunogold electron microscopy of an RiV-PP sample, produced from calf kidney cells. (A) Reaction with a blood donor serum. (B) Reaction with a second blood donor serum (see also arrow) and see text. The sera were used in a dilution of 1:20. These blood donor sera were tested in ARIVA as strongly positive. (C) Reaction of the RiV sample with rabbit anti-RiV antiserum, diluted 1:400. Scale bar, 20 nm. Gold-tagged protein A was used in a dilution of 1:20.

markers and NDPK (Nm23) as “metastasis suppressor gene” protein and a member of the ezrin family as a tumor suppressor (original literature see review of Kalies [29]).

Generally, antibodies against up to 29 different RiV proteins may be detected. In the present ELISA, RiV-PP with complete RiV particles are used. By the binding of RiV particles to the MTP there are only conformation changes and antibodies can only bind to the not hidden proteins. The detection possibility of the ELISA with the native RiV antigen complex is more or less weaker than the ELISA with a disrupted antigen complex, since by the second assay much more RiV-specific proteins can be detected. Such experiments for the comparison are outstanding.

Many efforts are made all over the world to develop methods of pre-symptomatic tumor diagnostic. About the importance especially of autoantibodies against tumor associated antigens (TAA) for the early tumor diagnosis was reported [30, 31].

Since a part of RiV proteins are known as tumor markers or as tumor suppressor proteins, a detection of some tumor-specific antibodies or tumor-specific antigens by ARIVA or by RiV-antigen detection is possible. An immune response manifested by the common occurrence of annexins I and II autoantibodies in lung cancer has been described [32] and annexin I is a main protein in RiV-PP samples as shown earlier [4, 5]. As mentioned, it is possible that a main part of the different RiV proteins was hidden in the RiV particles. In this case, greater differences in the results of ELISA and immunogold electron microscopy, on the one hand, and immunoblot on the other, are possible. Such experiments for the comparison are still outstanding. Mouse experiments with syngene tumor models shall demonstrate if an early detection of RiV-reactive antibodies is possible. A further publication will inform about studies of the course of tumor therapy using ARIVA [27].

Acknowledgements

The author is grateful to O. Missal, Ulrici-Kliniken Sommerfeld, now Sana Kliniken Sommerfeld/Hellmuth-Ulrici-Kliniken, G. Schulze, Rössle-Klinik, Berlin-Buch, G. Menzel, F. Wegner und K. Meinck (Ärztliche Laborgemeinschaft der Hansestadt Greifswald), S. Solisch, Greifswald-Riemserort, G. Lang, Greifswald), A. Greinacher, Institut für Immunologie und Transfusionsmedizin, Universität Greifswald, M. Ziegler, Institut für Pathophysiologie Karlsburg, Universität Greifswald, Germany, for the supply of sera and partially laboratory values. The author would also like to thank R. Riebe, Greifswald-Insel Riems and R. Mentel, Greifswald, for the supply of HeLa and FL cells, R. and Ch. Lenz for technical assistance, W. Wazel and R. Lenz for the preparation of RiV antigen, A. Meuche and R. Sietmann, Institut für Mikrobiologie und Molekularbiologie, Universität Greifswald, for excellent electron microscopic preparations and electron microscopic images (see Figs. 1 and 5). Special thanks are due to P. Solisch, Isle of Riems, and R. Sietmann for helpful advices. The author is also grateful to H. Bergmann for help with premises and equipment in the restart of RiV ELISA and D. Griffel, Neu-

brandenburg, for help and advices. This work was supported by a grant from the Wirtschaftsministerium Mecklenburg-Vorpommern, Schwerin (V230-630.08-TIFA-076).

References

- [1] P. Solisch, A hitherto unrecognized pattern of reaction in vertebrate cells (RiV)–ultrastructural studies of virus-host cell relationship and cell cultures, *Zentralbl. allg. Pathol. pathol. Anat.* **1986**, *131*, 49–57. (in German)
- [2] P. Solisch, H. Bergmann, Reaction pattern in vertebrate cells (RiV), biological effect: cell culture, laboratory animal, *J. KVMV* **1994**, *3*, 13–16, 21–24. (in German)
- [3] P. Solisch, A. Nöckler, D. Schäfer, R. Riebe, U. Holl, H. Schirrmeyer et al, A hitherto unrecognized pattern of reaction in vertebrate cells (RiV). Second report. The protective effect of RiV-particle preparation against foot-and-mouth disease of guinea pigs, *Zentralbl. allg. Pathol. pathol. Anat.* **1986**, *131*, 563–568. (in German)
- [4] H. E. H. Liebermann, R. Sietmann, R. Bange, W. Wazel, R. Riebe, RiV particles are heat stable, *Eng. Life Sci.* **2005**, *5* (3), 240–246.
- [5] P. Solisch, K.-U. Kalies, S. Bergmann, D. Euhus, H. Bergmann, Biocomplex RiV: a report to treatment. Immunology, statistics and biochemistry, *Bericht an das Wirtschaftsministerium von Mecklenburg-Vorpommern*, **1997**. (in German)
- [6] P. Solisch, K.-U. Kalies, S. Bergmann, Use of BC-RiV preparations, method of production and containing proteins, *Patent EP 0889 053 A2*, **1998**. (in German)
- [7] K. Denzer, M. J. Kleijmeer, H. F. G. Heijnen, W. Stoorvogel, H. J. Geuze, Exosome: from internal vesicle of the multivesicular body to intercellular signaling device, *J. Cell Sci.* **2000**, *113*, 3365–3374.
- [8] C. Thery, M. Boussac, P. Veron, P. Ricciardi-Castaguoli, G. Raposo, J. Garin et al., Proteomic analysis of dendritic cell-derived exosomes: A secreted subcellular compartment distinct from apoptotic vesicles, *J. Immunol.* **2001**, *166*, 7309–7318.
- [9] B. Fevrier, G. Raposo, Exosomes: endosomal-derived vesicles shipping extracellular messages, *Current Opin. Cell Biol.* **2004**, *16*, 415–421.
- [10] H. Hahnefeld, P. Solisch, R.-H. Brandt, H. E. H. Liebermann, Procedure for production of a vaccine against laryngeal papillomatosis of man, *Patent DD137785 A4*, **1975**.
- [11] R. H. Brandt, B. Christoph, H. Hahnefeld, P. Solisch, H. E. H. Liebermann, Vaccination therapy of laryngeal papillomatosis, *Erkrank. Atm.-Org.* **1980**, *155*, 254–261. (in German)
- [12] P. Solisch, W. Wazel, W. Steinmann, R. Riebe, U. Meyer, A hitherto unknown reaction pattern in vertebrate cells (RiV), 3th Communication: Therapeutic experiments with RiV particle preparations at mice with mammary carcinoma, *Zentralbl. allg. Pathol. pathol. Anat.* **1987**, *133*, 293–298. (in German)
- [13] I. Klötting et al., Personal Communication, **1994** and **1995**.
- [14] I. Klötting, H. E. H. Liebermann, R. Bange, F. Helbig, *Patent DE 102004036079 A1*, **2004**.
- [15] P. Solisch, H. Bergmann, A drug for therapy of aids, *Patent EP 0702 955 1*, **1994**.

- [16] P. Solisch, H. E. H. Liebermann, W. Wazel, The biocomplex (BC) RiV – a new concept for diagnosis and therapy, in Proceedings of the *Pre-Symptomatic Tumour Diagnosis Conference*, Senftenberg, Germany, October 4–5, 2001, 20–21 (in German). (www.tumornetzwerk.de)
- [17] P. Solisch, H. E. H. Liebermann, W. Wazel, AIDS therapy with Biocomplex (BC) RiV: first results, in *Conference of Society of Virology*, Berlin, 2003, 171. Poster CLI 09.
- [18] P. Solisch, H. Bergmann, *Patent P 42 38766.3*, 1992.
- [19] P. Solisch, H. Bergmann, *Registered Design G 9218127.9*, 1992.
- [20] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 1951, 193–265.
- [21] H. E. H. Liebermann, R. Mentel, L. Döhner, S. Modrow, W. Seidel, Inhibition of cell adhesion to the virus by synthetic peptides of fiber knob of human adenovirus serotypes 2 and 3 and virus neutralization by anti-peptide antibodies, *Virus Res.* 1996, 45, 111–121.
- [22] P. Solisch, H. Bergmann, Reaction pattern in vertebrate cells (RiV), Immunology: results and theses, *J. KVMV* 1993, 10, 15–18. (in German)
- [23] M. Ziegler, Personal Communication, 2001.
- [24] M. Schlosser, M. Strebelow, R. Wassmuth, M.-L. Arnold, I. Breunig, I. Rjasanowski et al., The Karlsburg type 1 diabetes risk study of a normal schoolchild population: Association of β -cell autoantibodies and human leukocyte antigen-DQB1 alleles in antibody-positive individuals, *J. Clin. Endocrinol. Metab.* 2002, 87 (5), 2254–2261.
- [25] *Medizinische Biometrie* (Eds: H. Exner, D. Renner), Thieme Verlag, Stuttgart, New York, 1999, 11–15.
- [26] K. R. Reddy, M. W. Modi, S. Pedder, Use of peginterferon alfa-2a (40 kD) (Pegasys) for the treatment of hepatitis C, *Adv. Drug Delivery Rev.* 2002, 54, 571–586.
- [27] H. E. H. Liebermann, H. Müller, Reaction Pattern in Vertebrate Cells (RiV): Studies of the course of tumor therapy using an anti RiV ELISA: initial results, *Eng. Life Sci.* 2008, 8 (2), 148.
- [28] *Immunologie* (Eds: C. A. Janeway, P. Travers, M. Walport, M. Shlomchik), Spektrum Akademischer Verlag GmbH, Heidelberg, Berlin, 5. Auflage, 2002, 537–560.
- [29] K.-U. Kalies, Reaction pattern in cells of vertebrates (RiV): a hypothesis about the molecular effects, 2003. (www.varicula.de)
- [30] J.-Y. Zhang, E. K. L. Chan, in *Methods, Possibilities and Perspectives of Pre-symptomatic Tumor Diagnostics* (Eds: K. Conrad, M. Bachmann, W. Lehmann, U. Sack), Pabst Science Publishers, Lengerich, Vol. 1, 2005, 47–54.
- [31] K. Conrad, D. Roggenbuck, M. Bachmann, in *Methods, Possibilities and Perspectives of Pre-symptomatic Tumor Diagnostics* (Eds: K. Conrad, M. Bachmann, W. Lehmann, U. Sack), Pabst Science Publishers, Lengerich, Vol. 1, 2005, 55–77.
- [32] F. M. Brichory, D. E. Misek, A.-M. Yim, M. C. Krause, T. J. Giordano, D. G. Beer et al., An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer, *Proc. Natl. Acad. Sci. USA*, 2001, 98 (17), 9824–9829.