Clin. Lab. 2020;66:XXX-XXX
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ORIGINAL ARTICLE

Vitamin D - Deglycosylated Vitamin D Binding Protein Dimer: Positive Synergistic Effects on Recognition, Activation, Phagocytosis and Oxidative Stress on Macrophages

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SUMMARY

Background: **We have recently shown positive effects in the quality of life in autism and amyloid lateral sclerosis patients using a newly developed 25-OH vitamin D deglycosylated vitamin D binding protein complex (VitD~dgVDBP) by reducing oxidative stress. The question arises whether this reduction of oxidative stress was due to a synergistic effect of the dimer in the recognition and activation of phagocytosis on macrophages combined with a lower oxidative burst compared to the VitD free proteins, namely vitamin D binding protein (VDBP: Gc Protein) and deglycosylated dgVDBP (GcMAF).**

Methods: **VDBP sandwich ELISA of equal protein concentration of VDBP, dgVDBP, and VitD~dgVDBP (1 µg/mL by BCA protein technique) was used to identify immune affinity to polyclonal antibodies raised against human VDBP. The 25(OH) vitamin D levels of VDBP, dgVDBP and VitD~dgVDBP were estimated by a competitive immune assay using a monoclonal antibody. Macrophage phagocytosis and oxidative burst in absence or presence of 400 pg/mL VDBP, 400 pg/mL dgVDBP, and 400 pg/mL VitD~dgVDBP was measured.**

Results: **The recognition of the antibody against VDBP protein was significantly more than 4-fold higher for VitD~dgVDBP (769.2 +/- 35.1%) compared to dgVDBP (186.5 +/- 16.8 %; $p < 0.01$) and 7-fold higher to VDBP (100 +/- 11.4 %; $p < 0.001$). 25(OH) vitamin D levels of VDBP (20.7 nmol/mg; $p < 0.001$) and dgVDBP (28.8 +/- 3.9 nmol/mL; $p < 0.001$) was significantly lower than of VitD~dgVDBP (324.0 +/- 12.8 nmol/mL). The calculated VitD/protein ratio showed significantly higher results in favor of VitD~dgVDBP (1.01 +/- 0.12) compared to dgVDBP (0.06 +/- 0.03; $p < 0.001$) and VDBP (0.05 +/- 0.01; $p < 0.001$).**

The estimation of macrophage phagocytosis rate of VitD~dgVDBP (5,864.3 +/- 742.2 cps) was significantly higher compared to dgVDBP (2,789.6 +/- 102.7 cps; $p < 0.01$) and VDBP (1,134.3 +/- 135.9 cps) whereas the production of macrophage superoxide anion radicals showed significantly higher levels of dgVDBP (255.3 +/- 14.5 cps) in comparison to VDBP (148.6 +/- 24.7 cps, $p < 0.01$) and VitD~dgVDBP (142.3 +/- 20.0 cps; $p < 0.001$). Linear regression between VDBP antibody affinity and macrophage phagocytosis of VDBP, dgVDBP and VitD~dgVDBP resulted in a correlation coefficient of $r = 0.95$ in favor of VitD~dgVDBP.

Conclusions: **VitD~dgVDBP (Il-42) showed higher macrophage activation and lower oxidative burst than VitD free dgVDBP (GcMaf) and VDBP (Gc) which may result from a synergistic effect by presenting protein bound Vitamin D better to macrophages.**

(Clin. Lab. 2020;66:xx-xx. DOI: 10.7754/Clin.Lab.2019.191121)

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Manuscript accepted December 7, 2019

KEY WORDS

vitamin D (VitD), vitamin D binding protein (VDBP), deglycosylated vitamin D binding protein (dgVDBP), vitamin D bound deglycosylated vitamin D binding pro-

tein complex (VitD~dgVDBP)

INTRODUCTION

Vitamin D and its metabolites are involved not only in the endocrine interaction between kidney, bone, and parathyroid hormone, but also in immunity, hypertension, muscular function, autoimmune disease including autism, T-cell regulation, cancer, and antioxidative defense via glutathione metabolism [1-6].

Vitamin D activates numerous genes, which are involved in the immune and inflammatory response [7]. Interestingly, Shah et al. reported that the inactive vitamin D hormone cholecalciferol bound to VDBP had no effect on the chemotactic factor function of VDBP on neutrophils whereas the bioactive vitamin D form calcitriol completely abolished this VDBP function [8].

Vitamin D binding protein (VDBP) is part of the albumin gene family with three domains, which are able to bind simultaneously without apparent alteration in the binding affinity and/or protein function [9,10].

It was reported that VDBP binds to the surface of several cell types to deliver vitamin D sterols and G-actin beside the chemotactic cofactor and macrophage activating function including B-lymphocytes [11-13], T-lymphocytes [14], monocytes [15] and neutrophils [16]. Furthermore, VDBP is an important protein of the plasma actin-scavenger system. As such, it has been shown to bind free actin and prevent hypercoagulation and shock in patients with massive actin release resulting from severe tissue injuries [17].

Stepwise deglycosylation of VDBP by T- and B-lymphocytes converts the VDBP to a macrophage-activating factor (GcMAF; DBP-MAF; deglycosylated VDBP: dgVDBP) [18,19]. We recently reported a newly developed dimeric compound, namely cholecalciferol-N-acetylgalactosamine-albumin (VitD~dgVDBP) and its positive and non-toxic effects in mice after intravenous injection compared to a sham group [20], effectively increasing the vitamin D level. Furthermore, this dimeric compound showed a positive effect in the behavior on (i) autistic children after oral administration after a 5-week supplementation [21] or (ii) on a patient with amyloid lateral sclerosis (case report). In both studies VitD~dgVDBP effectively reduced the oxidative stress parameters in blood.

Questions arise whether (i) the conversion of VDBP to dgVDBP and (ii) the binding of cholecalciferol to dgVDBP (= VitD~dgVDBP) results in any change of the protein structure, its activation on macrophage phagocytosis, and macrophage free radical formation compared to dgVDBP (GcMAF).

MATERIALS AND METHODS

Gc-protein was purchased from Sigma Aldrich (G8764; Vienna, Austria). Production of dgVDBP by stepwise treatment of purified VDBP protein with immobilized b-galactosidase and sialidase to produce dgVDBP was done according to Yamamoto et al. The immobilized enzymes were removed by centrifugation. VitD~dgVDBP was purchased from HG Pharma, (Vienna, Austria). Goat-anti-human-Gc-IgG (SAB2501100) and rabbit-anti-goat-IgG-HRP (AP106P) were obtained from Sigma Aldrich (Vienna, Austria). Protein determination of VDBP, dgVDBP and VitD dimer was performed with the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Vienna, Austria).

Determination of Vitamin D Binding Protein (VDBP)

This enzyme immunoassay is a sandwich assay for the quantitative determination of VDBP-proteins (Immundiagnostik AG, Bensheim, Germany). The wells of the microtiterplate are coated with polyclonal anti-VDBP antibodies. In a first incubation step, the VDBP in the pre-diluted samples is bound to the wells coated with polyclonal rabbit antibodies. To remove all unbound substances, a washing step is carried out. In a second incubation step, a polyclonal peroxidase-labeled rabbit anti-VDBP antibody is added. After another washing step, to remove all unbound substances, the solid phase is incubated with the substrate, tetramethylbenzidine. An acidic stopping solution is then added. The color converts to yellow. The intensity of the yellow color is directly proportional to the VDBP concentration in the sample. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated, using the values obtained from the standard. VDBP, present in the samples, is determined directly from this curve.

Measurement of 25(OH)-Vitamin D

The 25(OH)-vitamin D direct assay was intended for the quantitative determination of the 25-OH-Vitamin D (Immundiagnostik AG, Bensheim, Germany). The assay utilized a competitive ELISA technique with a selected monoclonal antibody recognizing 25(OH)-vitamin D. For a reliable determination of 25(OH)-vitamin D, it was necessary to release it from the 25(OH)-vitamin D-VDBP-complex. Standards, controls and samples which were assayed for 25(OH)-vitamin D were pre-diluted with the releasing reagent and transferred to the microplate coated with 25(OH)-vitamin D. After an incubation to release the 25(OH) vitamin D, an anti-25(OH)-vitamin D antibody was added. After washing the microtitration plate, a peroxidase-conjugated antibody was added into each microplate well. A complex of 25(OH)-vitamin D-anti-25(OH)-vitamin D antibody-peroxidase conjugate was formed. Tetramethylbenzidine (TMB) was used as a peroxidase substrate. Finally, an acidic stop solution was added to terminate the reac-

tion, whereby the color changes from blue to yellow. The intensity of the yellow color was inversely proportional to the concentration of 25(OH)-vitamin D. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration was generated using the values obtained from the standard. The 25(OH)-vitamin D in the samples was determined from this curve.

Macrophage phagocytosis and superoxide anion radical production analysis in the absence or presence of lipopolysaccharide (LPS), dgVDBP, and VitD~dgVDBP

Quantitative determination of the phagocytic activity of monocytes in heparinized human whole blood with 100 ng/mL LPS, 400 pg/mL dgVDBP, and 400 pg/mL VitD~dgVDBP was performed with the PhagotestTM (Glycotope Biotechnology, Heidelberg, Germany). The phagocytosis test kit contains fluorescein-labelled opsonized Escherichia coli bacteria. Heparinized whole blood was incubated with reagent B (FITC-labelled *E. coli* bacteria) at 37°C; a negative control sample remains on ice. The phagocytosis was stopped by placing the samples on ice and adding reagent C (quenching solution). This solution allowed the discrimination between attachment and internalization of bacteria quenching the FITC-fluorescence of surface bound bacteria leaving the fluorescence of internalized particles unaltered. After two washing steps with reagent A (wash solution) erythrocytes were then removed by addition of reagent D (lysing solution). The DNA staining solution (reagent E) was added just prior to flow cytometric analysis and excluded aggregation artifacts of bacteria or cells. The *E. coli* bacteria were opsonized with immunoglobulin and complement of pooled sera. Cells were analyzed by flow cytometry in absence or presence of 1 ng/mL LPS, 400 pg/mL VdgVDBP, and 400 pg/mL VitD~dgVDBP using blue-green excitation light (488 nm argon-ion laser).

Phagoburst™ was used for the determination of leukocyte oxidative burst (Glycotope Biotechnology, Heidelberg, Germany) in presence of 100 ng/mL LPS, 400 pg/mL dgVDBP, and 400 pg/mL VitD~dgVDBP. The Phagoburst™ kit contains unlabeled opsonized *E. coli* bacteria (reagent B) as particulate stimulus, the protein kinase C ligand phorbol 12-myristate 13-acetate (PMA, reagent D) as high stimulus and the chemotactic peptide N-formylMetLeuPhe (fMLP, reagent C) as low physiological stimulus, dihydrorhodamine (DHR) 123 (reagent E) as a fluorogenic substrate and necessary reagents. Heparinized whole blood was incubated with the various stimuli at 37°C. A sample without stimulus serves as negative background control. Upon stimulation, granulocytes and monocytes produce reactive oxygen metabolites (superoxide anion, hydrogen peroxide, hypochlorous acid), which destroy bacteria inside the phagosome. Formation of the reactive oxidants during the oxidative burst was monitored by the addition and oxidation of DHR 123. The reaction was stopped by addition of lysing solution (reagent F), which removes erythrocytes and results in a partial fixation of leukocytes. After one washing step with wash solution (reagent A), DNA staining solution (reagent G) was added to exclude aggregation artifacts of bacteria or cells. The percentage of cells having produced reactive oxygen radicals in absence or presence of 1 ng/mL LPS, 400 pg/mL dgVDBP, and 400 pg/mL VitD~dgVDBP were then analyzed as well as their mean fluorescence intensity (enzymatic activity, 488 nm excitation argon ion laser).

Statistics

Descriptive statistics were calculated using the Excel program (Microsoft, Seattle, WA, USA). The Kolmogorov-Smirnov test was used for the distribution analyses of the data. Group comparisons were made using *t*-tests where appropriate and indicated. Comparisons of several groups were made using the one-way analysis of variance (one-way ANOVA) with post-hoc Dunn test. Different multivariate logistic regression models with the target variable „anti-VDBP activity'' were adapted, taking „macrophage activity" as covariates (SPSS 25, SPSS Inc., Chicago, IL, USA). All values were given as mean value with standard deviation. Statistical significance was considered for $p < 0.05$.

RESULTS

VitD~dgVDBP has the highest binding affinity to anti-VDBP-IgG compared to VDBP and dgVDBP. In Figure 1, the binding affinity of anti-VDBP-IgG against VDBP, dgVDBP, and the dimeric compound VitD~dgVDBP is shown. The affinity to VDBP was about 0.19 +/- 0.02 µg/mL, which was set to 100 +/- 12%. dgVDBP increased significantly to 186.5 +/- 11.8% ($p < 0.01$ in comparison to VDBP), but VitD~dgVDBP increased more than 4-fold to 769.1 +/- 35.1% with a high significance compared to VDBP ($p < 0.001$) and more than 7-fold to dgVDBP ($p < 0.001$).

Vitamin D concentrations of all three vitamin binding proteins were estimated using a competitive ELISA with a specific monoclonal antibody against vitamin D. Figure 2a shows a significantly higher concentration of vitamin D on VitD~dgVDBP 324.1 +/- 12.8 nmol/mg, compared to VDBP (20.7 +/- 2.5 nmol/mg; $p < 0.001$) and dgVDBP (25.8 +/- 3.9 nmol/mg; $p < 0.001$), whereas the same protein content could be verified by the vitamin D binding protein ELISA (Figure 2b): 405.8 +/- 45.8 ng/mL (VDBP), 412.5 +/- 23.4 ng/mL (dgVDBP) and 318.9 +/- 22.8 ng/mL (VitD~dgVDBP). Protein results did not differ significantly from each other.

Figure 2c presents the calculated ratio of bound vitamin D to protein (vitamin D/protein ratio): 1.01 +/- 0.12 ratio of the VitD~dgVDBP compound was significantly higher compared to VDBP (0.05 +/- 0.01; $p < 0.001$) and dgVDBP (0.06 +/- 0.03; $p < 0.001$). Obviously, VDBP and dgVDBP were not significantly different because of the missing vitamin D on these proteins.

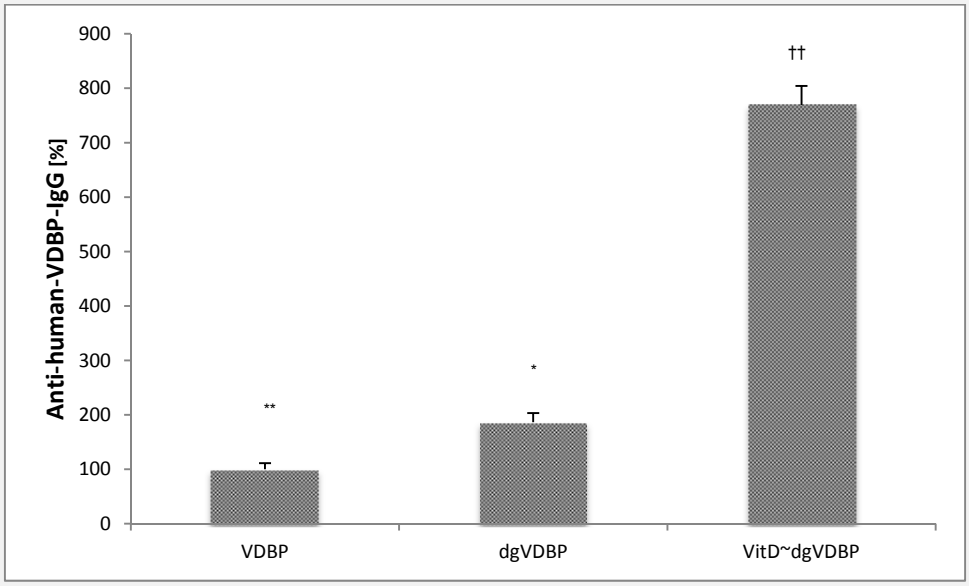

Figure 1. Binding affinity measurements of anti-VDBP-IgG against VDBP, dgVDBP, and VitD~dgVDBP expressed in percentage (100 +/- 12% = signal of VDBP).

*** Significance between VDBP and dgVDBP: p < 0.01. ** Significance between VDBP and VitD~dgVDBP: p < 0.001; †† Significance between dgVDBP and VitD~dgVDBP: p < 0.001.**

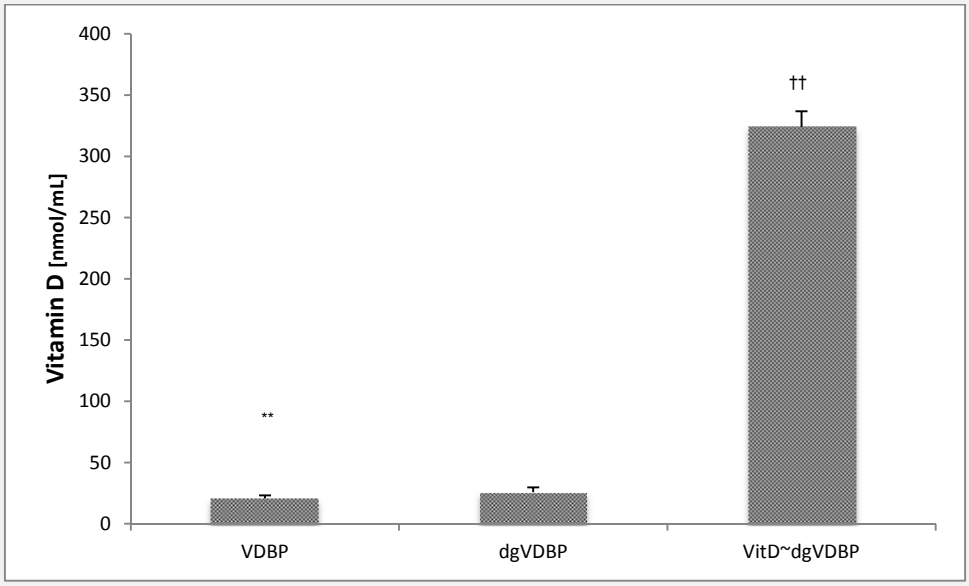

Figure 2a. Estimation of vitamin D (nmol/mL) of VDBP, dgVDBP, and VitD~dgVDBP.

**** Significance between VDBP and VitD~dgVDBP: p < 0.001; †† Significance between dgVDBP and VitD~dgVDBP: p < 0.001.**

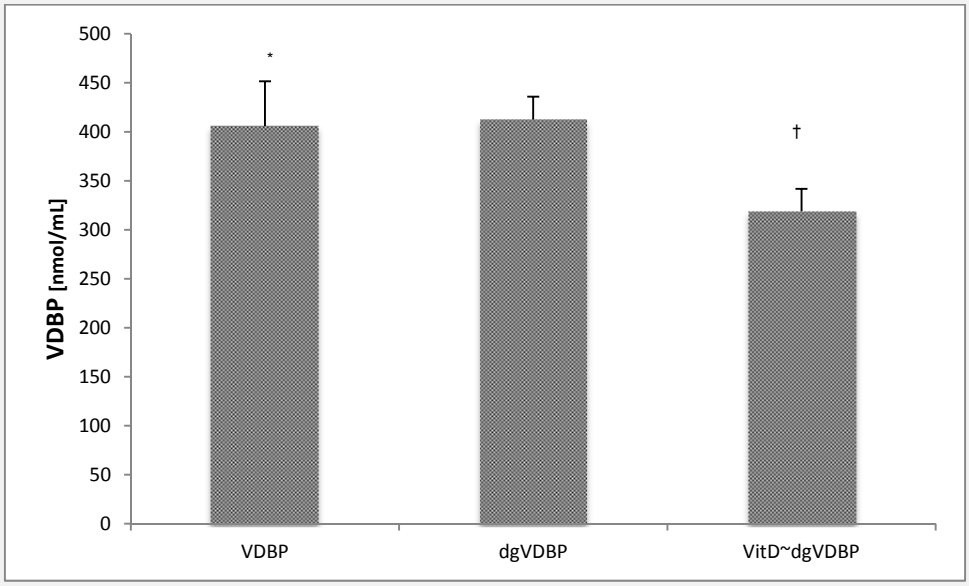

Figure 2b. Estimation of VDBP concentrations of VDBP, dgVDBP and VitD~dgVDBP before VitD measurements.

[*] Significance between VDBP and VitD~dgVDBP: p < 0.01; [†] Significance between dgVDBP and VitD~dgVDBP: p < 0.01.

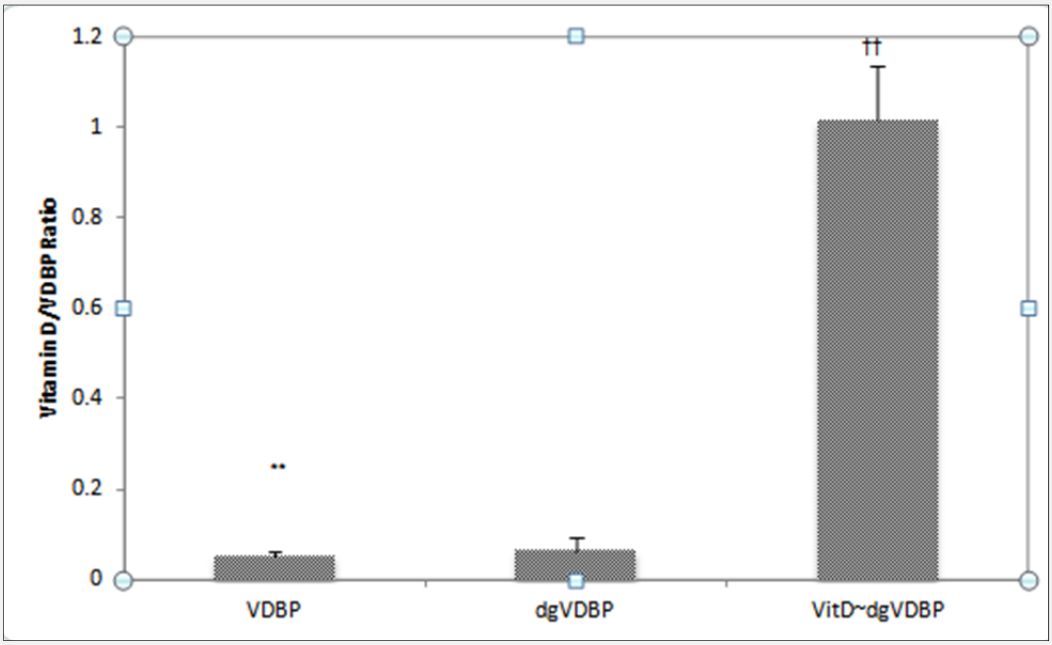

Figure 2c. Calculated vitamin D/VDBP ratio of VDBP, dgVDBP, and VitD~dgVDBP.

[**] Significance between VDBP and VitD~dgVDBP: p < 0.001; [††] Significance between dgVDBP and VitD~dgVDBP: p < 0.001.

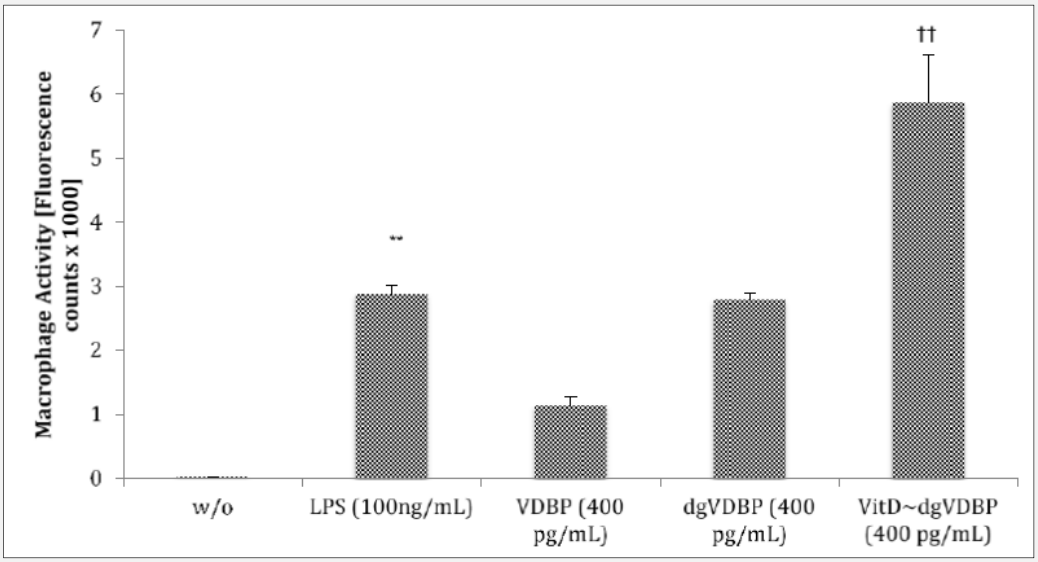

Figure 3a. Macrophage activation by LPS, VDBP, dgVDBP, and VitD~dgVDBP compared to negative control (w/o).

** **Significance between VDBP and dgVDBP: $p < 0.001$;** †† **Significance between dgVDBP and VitD~dgVDBP: $p < 0.001$.** ## **Significance between VDBP and VitD~dgVDBP: $p < 0.001$.**

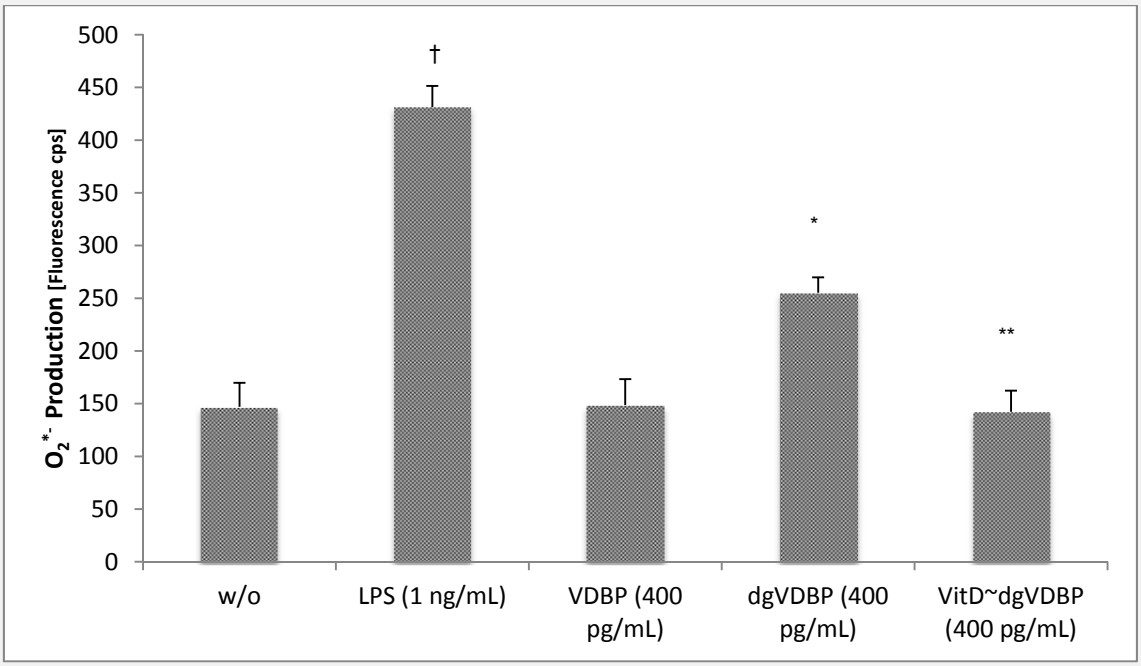

Figure 3b. O_2^{*-} generation of macrophages by LPS, VDBP, dgVDBP, and VitD~dgVDBP compared to negative control (w/o).

** **Significance between LPS and VitD~dgVDBP: $p < 0.001$;** * **Significance between LPS and dgVDBP: $p < 0.001$;** † **Significance between dgVDBP and VitD~dgVDBP: $p = 0.001$.**

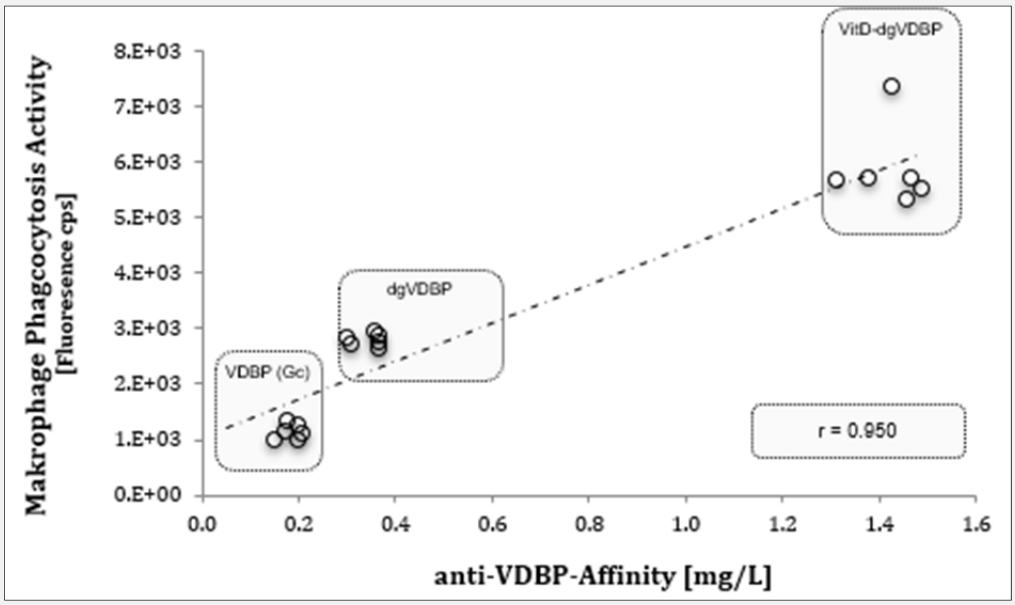

Figure 4: Linear regression of estimated macrophage phagocytosis activity against anti-VDBP antibody affinity of VDBP (Gc), dgVDBP, and VitD~dgVDBP.

Activation of macrophage phagocytosis was measured in the presence of 400 pg/mL VDBP, dgVDBP, the dimeric compound VitD~dgVDBP compared to lipopolysaccharides (LPS) (Figure 3a). The fluorescence of the negative control (w/o) was 22.4 +/- 4.3 cps and of the LPS control 2,869.3 +/- 153.6 cps (LPS). The fluorescence counts demonstrated an equal macrophage activation of dgVDBP compared to LPS. VitD~dgVDBP macrophage activation was significantly higher compared to all other tested components, especially for dgVDBP (2,789.6 +/- 102.7 cps vs. 5,864.3 +/- 742.2 cps; $p < 0.001$) and LPS ($p < 0.001$). It should be pointed out that, LPS was 2,500-fold more concentrated than VDBP and VitD~dgVDBP. Therefore, the comparable stimulation of macrophages should be assumed to be 2,500 higher in VitD~dgVDBP compared to LPS.

Superoxide anion radical (O_2^{*-}) production during macrophage phagocytosis was estimated (Figure 3b) with the same concentrations of LPS, dgVDBP and VDBP and VitD~dgVDBP as used for the macrophage phagocytosis analysis. Whereas the VDBP and VitD~dgVDBP compounds show the same fluorescence intensity like the negative control (142.3 +/- 20.0 cps vs. 147.0 +/- 22.7 cps), dgVDBP produced significantly more O_2^{*-} radicals compared to the dimeric compound (255.3 +/- 14.5 counts; $p < 0.001$) as presented in Figure 3a. The positive control LPS shows the highest O_2^{*-} signal (431.3 +/- 20.3 cps) and was significantly higher than dgVDBP ($p < 0.001$).

Linear regression of macrophage activity against anti-VDBP-IgG binding affinity of VDBP, dgVDBP and VitD~dgVDBP is presented in Figure 4 with a calculated regression of r = 0.950.

DISCUSSION

Usually, the purification of human VDBP was done on a $25(OH)D_3$ affinity column chromatography binding serum VDBP at low salt concentrations. Using high salt concentrations release the binding of VDBP from the affinity column to elute "VitD free" VDBP [22]. A possible *in vitro* dimer VitD~dgVDBP might not bind to a $25(OH)D_3$ affinity column and therefore passes through the column. Following deglycosylation steps using β-galactosidase and sialidase results in "VitD free" dgVDBP (GcMAF), which is able to potentially increase activation of macrophages [23]. We have used the ELISA technique using polyclonal antibodies against VDBP showing that deglycosylation increased the antibody recognition of dgVDBP, but much more of the dimer VitD~dgVDBP. Mohamad SB et al. confirmed higher western blot signal of enzymatically treated VDBP (dgVDBP) compared to VDBP but not of VitD~dgVDBP.

Vitamin D binding protein antibody associates with membrane-bound immunoglobulin on the surface of B-lymphocytes and with IgG Fc receptor on the mem-

branes of T-lymphocytes and neutrophils [24-26]. Our results of the VitD dimeric compound demonstrate a highly significant change in antibody binding capacity in favor of VitD~dgVDBP compared to VDBP and dgVDBP. Therefore, we assume that deglycosylation of VDBP to dgVDBP and formation of VitD~dgVDBP either changes the steric protein conformation on the surface of the protein and/or unmasks the binding area of the antibody used. Binding of vitamin D to dgVDBP forming VitD-dgVDBP seems to increase this effect. Therefore, a significant immune-modulation of VitD~-dgVDBP can be suspected.

The usage of low salt concentration combined with carbohydrates showed a higher binding affinity of VitD on dgVDBP, which results in a 1:1 molar binding of VitD to dgVDBP as calculated in an earlier study [27], whereas Bouillon et al. [28] calculated a number of binding around 0.8. VDBP and dgVDBP in high salt concentration only showed no binding to any VitD, which was used by several studies following the purification steps over a VitD chromatographic column extraction. This may depend also on a slightly different binding capacity of VitD to dgVDBP compared to VDBP.

Vitamin D_3, the main circulating form of VitD, itself can be utilized by monocytes/macrophages and dendritic cells via the CYP27B1 and VitD receptor pathway for intracrine conversion to 1,25-VitD, which is able to promote the antibacterial response to infections [29]. VDBP protein (Gc proteins) has been reported to be a precursor of VDBP protein-derived macrophage activation factor (dgVDBP) in the inflammation-primed macrophage activation cascade. Inducible b-galactosidase of B cells and neuraminidase of T cells convert VDBP protein to dgVDBP (GcMAF). dgVDBP possessed a higher ability in the activation of macrophages compared to VDBP or LPS as shown by us and other researchers, but the VitD~dgVDBP compound to a more than two-fold higher phagocytosis level compared to dgVDBP. Furthermore, we have found out that VitD~dgVDBP was able to decrease the production of free radicals during macrophage activation compared to dgVDBP or LPS. We suggest that the dimeric combination of VitD and the deglycosylation of VDBP to dgVDBP forming VitD~dgVDBP showed, besides the synergistic effect on macrophage activation and phagocytosis, an effective reduction of oxidative burst in the circumstance. It was shown that oxidative stress can have both direct and indirect negative effects on macrophage function like carbonyl stress [30]. VitD is also able to reduce oxidative stress in high dose supplemented patients to decrease oxidized glutathione levels in critically ill ventilated adults via the glutathione pathway [31]. We have seen the reduction of free carbonyls, like malondialdehyde (MDA) and 4-hydroxynonenal (HNE), in a small cohort of autistic children following oral supplementation VitD~dgVDBP for 5 weeks combined with an increased effect on the quality of life [21]. The same situation was seen in a patient with amyloid lateral sclerosis [32].

At least we demonstrated for the first time a positive correlation between macrophage activation and anti-IgG-VDBP affinity in favor of VitD~dgVDBP, which allows the speculation regarding a dramatically higher binding affinity of VitD~dgVDBP compared to VDBP, dgVDBP, and VitD alone.

Beside all these aspects, the question arises whether all VitD mechanisms during inflammation and immune processes, which are attributed mostly to the hormone VitD alone, are coming from interleukin like actin VitD bound VDBP metabolites, especially VitD~dgVDBP.

CONCLUSION

Our data suggest a possible interleukin effect of the dimeric VitD~dgVDBP (Il-42) complex on macrophages, with a highly effective activation of phagocytosis capacity and lower production of radical oxidative species, compared to the VitD free components VDBP or dgVDBP.

Acknowledgment:
We gratefully thank Dr. B. Schuetz at Biovis Diagnostik (Limburg, Germany) for the analysis of macrophage phagocytosis and superoxide anion radical production.

Declaration of Interest:
R. H. is CEO of HG Pharma, which supported this study, and J. G. is shareholder of HG Pharma.

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