This study was undertaken to evaluate the monocyte function of uraemic non-responders to hepatitis B vaccination. Therefore, some parameters concerning antigen processing by monocytes (Mo) as antigen presenting cells (APC) were analysed. It was found that in uraemic non-responders, (1) the internalization of HBsAg by monocytes was significantly decreased—HBsAg complexed with specific IgG or as immune complex isolated from patients is better internalized compared with free HBsAg; (2) during antigen presentation the expression of adhesion (ICAM-1) and accessory (HLA-DR/Ia) molecules was significantly decreased in uraemic patients, especially in non-responders; and (3) impaired internalization of HBsAg as well as a decrease in ICAM-1 and HLA-DR/Ia expression, correlated well with the blunted proliferation of CD4⁺ T cells stimulated by autologous monocytes induced by HBsAg.

Key words: Adhesion molecules, Antigen presentation, HBV vaccination, Monocytes, Uraemia

Defective antigen presentation by monocytes in ESRD patients not responding to hepatitis B vaccination: impaired HBsAg internalization and expression of ICAM-1 and HLA-DR/Ia molecules

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Introduction

After immunization with hepatitis B (HBV) vaccine, 95% of healthy individuals develop antibodies against hepatitis B surface antigen (HBsAg). In contrast, only 60–70% of dialysis patients show a seroconversion even after additional injections of the vaccine.¹ Various attempts have been made to enhance antibody production and to clarify the causes of this lack of response. Recent studies have shown that in uraemic non-responders to HBV vaccination, T lymphocytes do not receive the monocyte-derived signals that are essential for adequate antigen presentation.¹ *In vitro*, this defect can partially be counteracted by addition of monocytes from healthy donors^{2–4} or simultaneous supplementation of IL-2, IL-1, IL-6 and IFN- γ .^{1,5} In the present study we tested the monocyte internalization (phagocytic) capacity for HBsAg, as well as adhesion (ICAM-1/intercellular adhesion molecule-1) and accessory molecule (HLA-DR/Ia) expression on monocytes induced by HBsAg. The parameters examined were correlated with responsiveness to HBV vaccination in patients undergoing intermittent haemodialysis (ESRD patients).

Materials and Methods

Patients: This study was carried out according to the Declaration of Helsinki, and the informed consent of each patient was obtained. Thirty haemodialysis patients (20 males, ten females) who had not produced antibodies after at least four injections of H-B-Vax-D⁶ vaccine (U-NR, uraemic non-responders) and ten uraemic haemodialysis patients who had responded to the vaccine (U-R, uraemic responders; anti-HBs antibody titre higher than 10 U/l) were included in the study. Ten healthy volunteers who had responded well to HBV vaccination (C-R, control responder; anti-HBs antibody higher than 10 U/l) and ten healthy blood donors (C-NR, control non-responders; Institute of Transfusiology, University of Cologne) not responding to vaccination were selected as control groups for the trial. The mean age of patients was 25.6 years (range 8–28 years), the mean duration of haemodialysis was 3.1 years (range 1–6 years). The mean age of controls was 28.5 years (range 21–35 years). None of these subjects had the hepatitis B virus infection before introduction to the vaccination schedule (HBsAg-negative, anti-HBc/IgG and anti-HBc/IgM-negative).

Vaccination: Subjects were vaccinated (Gen-H-B-Vax-D; Merck Sharp & Dohme) in accordance with the standard schedule for hepatitis B vaccination (four doses of 40 µg given intramuscularly, deltoid or quadriceps muscle) at 0, 1, 2 and 6 months. Non-responsiveness was defined if the antibody titre after vaccination reached less than 10 U/l 6–8 weeks following the last booster injection.

Purification of monocytes and T cells: Heparinized blood was drawn before the haemodialysis session, after the longest interdialytic interval in order to minimize a direct influence of the previous haemodialysis procedure. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation using Ficoll-Paque (Lymphoflot, Biotest Diagnostics).

Monocytes were prepared from PBMC by adherence to glass Petri dishes. Cells at 2×10^6 /ml were incubated in a 1 ml volume for 2 h at 37°C in flat-bottom 24-well culture plates (Nunc, InterMed Nunc, Denmark). Adherent cells consisting of about 95% pure monocytes as assessed by measurement of CD14 molecule expression (flow cytometry), were used as APC.⁴

Non-adherent cells were removed by washing. CD4⁺ T cells were prepared from non-adherent cells by using the E-rosetting technique with subsequent passage over the nylon wool column (to deplete residual B cells and monocytes). Then the CD4⁺ T cells were purified by negative selection by using a panning technique to deplete contaminating Ia positive cells and CD8 cells. Flow cytometry analysis of the resultant CD4⁺ population showed that contamination with residual B cells (CD19), monocytes (CD14) and CD8⁺ cells was less than 1%.

In order to exclude an influence of nonspecific monocyte activation by adherence, all experiments were performed simultaneously with monocytes originating from uraemic patients and controls (non-responders, responders).

Internalization activity of monocytes: Internalization analysis of HBsAg by monocytes was based on the technique used in the Phagotest (Orpegen, Med-Molekularbiologische Forschungsanstalt GmbH, D-6900 Heidelberg), where either free HBsAg or immune complexes containing HBsAg were used instead of opsonized FITC-conjugated *E. coli*. Labelling of protein molecules with FITC is based on the phenomenon that some amino acids, mainly lysine, can bind fluorochrome molecules (FITC).^{7–11} The following HBsAg preparation were used in our experiments: (1) free HBsAg (ad or ay subtypes, Biodesign-International); (2) HBsAg conjugated *in vitro* with specific IgG (anti-HBs, goat IgG, B124046, Biodesign-International); affinity of anti-HBs to HBsAg ranged between 2×10^6 and 9×10^7 mol/l;¹² and (3) specific immune complexes containing

HBsAg (Ic-HBsAg-IgG) isolated from sera of children suffering from glomerulonephritis associated with HBV infection.¹³ Sera were fractionated by means of a two-stage chromatography system with Sephadex G-200 filtration followed by Sepharose 6B, using calibrated columns.¹⁴

A solution of fluorescein 5-isothiocyanate (FITC, 3 mg, isomer I F7250, Sigma, C₂₁H₁₁NO₂S) in borate buffer (pH 9.25, 100 ml) was used for labelling HBsAg-containing proteins.^{7,8} The ratio of the fluorochrome molecule (F) conjugated to the protein molecule (P, HBsAg preparation) (F/P ratio) was estimated photometrically in the FITC-HBsAg solution. The absorbance at 495 nm and at 280 nm was determined using double-distilled water as a blank. The results were calculated according to Equation 1.

$$\frac{F}{P} = \frac{6.85 \times E_{495}}{E_{280} - 0.35 \times E_{495}} \quad (1)$$

The internalization of the HBsAg-preparation conjugated with FITC was assayed using a flow cytometer (FACScan; Becton-Dickinson) using the live gate, similar to that in the Phagotest procedure. FITC positive cells gated as monocyte clusters were proportional to the monocyte number that had phagocytosed HBsAg. Fluorescence intensity, however, was proportional to the number of internalized HBsAg molecules.

Antigen presenting function of monocytes: The antigen presenting function of monocytes (Mo) was tested by preparing HBsAg-pulsed monocytes (P-Mo, 2×10^4 cells/ml), which were subsequently cultured with autologous CD4⁺ T cells (1×10^5 /ml) for 6 days. P-Mo were prepared by placing 1×10^6 cells/ml in polysulphon tubes, irradiating (2 500 rad) the cells and then incubating them with or without HBsAg, at a concentration of 100 ng/ml, in complete medium for 18 h. Irradiated monocytes incubated with HBsAg internalize and digest this antigen which is re-expressed on the surface of the cell in association with HLA class II (MHC) and is presented onto T cells during 6-day culture.^{3,15,16} These irradiated monocytes are not able to transform by cell division during the culture, but they are able to perform the function of surface receptor expression and monokine synthesis; T lymphocytes then respond to the altered surface of monocytes by proliferating. Only free HBsAg (Biodesign-International) was used for the *in vitro* stimulation in order to avoid the nonspecific monocyte activation of IgG contained in immune complexes via its Fc receptor.

Proliferation assays: The proliferative response of CD4⁺ T cells stimulated by P-Mo was determined by ³H-thymidine incorporation (0.5 µCi/well; Sp. Ac. 5 Ci/mmol; Radiochemical Centre, Amersham Co., Oakville, Ontario, Canada). Assays were performed

in triplicate and the radioactivity was expressed as the mean count per minute (cpm) with standard deviation (S.D.). The results are given as a stimulation index (SI), calculated as in Equation 2.

$$SI = \frac{\text{cpm of CD4}^+ \text{ T cells + P-Mo(HBsAg)}}{\text{cpm of CD4}^+ \text{ T cells + Mo(w/oHBsAg)}} \quad (2)$$

Flow cytometry: PBMC were fluorescence labelled using, simultaneously, fluorescein (FITC)-conjugated anti-ICAM-1 (CD54, Dianova), or anti-HLA-DR/1A mAb and phycoerythrin (PE)-labelled anti-CD14 mAb (Becton–Dickinson). Analysis was performed by flow cytometry (FACScan, Becton–Dickinson) in double colour, direct fluorescence. The instrument was calibrated with inert beads to which a known number of fluorescein molecules were covalently bound (Flow Cytometry Standards Corp., Research Triangle Park, NC).¹⁷ Data were analysed using Consort-30 software (Becton–Dickinson). Receptor expression was presented as receptor density (RD) on the cell surface, shown as the mean fluorescence intensity (MFI) in arbitrary units. MFI was proportional to the number of receptor binding sites on the cell surface.

Statistical analysis: All data were analysed by using Student's paired *t*-test (Statgraphics software package). Non-parametric equivalents of this test (Kruskal–Wallis and Wilcoxon matched-pair rank sum test) were also performed to confirm the results. The significance level was $p < 0.05$.

Results

Internalization activity of monocytes: Internalization of the HBsAg preparation by monocytes was measured by assessing phagocytic activity, using flow cytometry (Fig. 1). In ESRD patients the mean number of particles containing HBsAg phagocytes by monocytes was significantly decreased compared with that in controls ($p < 0.001$). Moreover, the mean value of internalized molecules containing HBsAg was especially low in uraemic non-responders compared with the value in uraemic responders ($p < 0.05$) and in controls ($p < 0.001$). There were no differences in phagocytic (internalization) activity between ad- and ay-subtypes of HBsAg. However, the pure HBsAg preparation was much less internalized in all groups of examined patients and controls than was the HBsAg complexed with IgG; the best internalization was observed by using specific immune complexes (IC–HBsAg–IgG) isolated from patients suffering from HBV related glomerulonephritis. The monocytes are additionally able to bind IC–HBsAg–IgG by their receptors to the Fc fragment of IgG, as well as to the C_{1q} receptors. Hence, this

facilitation of internalization processes has to be taken into account.

Expression of adhesion and accessory molecules (ICAM-1; HLA–DR/Ia) on monocytes: The percentage of positive cells with regard to the defined receptors (ICAM-1; HLA–DR/Ia) revealed no significant differences compared with controls. The expression of accessory and adhesion molecules was interpreted as RD. On freshly isolated uraemic monocytes the density of ICAM-1 (CD54) and HLA–DR/Ia molecules was not significantly decreased ($p > 0.05$) (Table 1).

Induction of monocytes for 18 h with HBsAg as a soluble protein (Table 1) resulted in an increased HLA–DR/Ia expression both on uraemic and control monocytes. Simultaneously, the differences between non-responders and responders in the ESRD patient group were enhanced. ICAM-1 density was also higher compared with that on freshly isolated monocytes, but no differences between uraemic patients and controls could be detected.

When monocytes were cultured with CD4⁺ T lymphocytes these cell-to-cell interactions resulted in a further increase of HLA–DR/Ia and ICAM-1 expression on monocytes but their expression was lower in uraemic non-responders (Table 1).

Proliferative response of helper-inducer (CD4⁺) T cells induced by autologous monocytes presenting HBsAg: The proliferative response of CD4⁺ T cells induced by monocytes presenting HBsAg was significantly decreased in ESRD patients ($p < 0.05$), especially in non-responders ($p < 0.001$) (Fig. 2).

Discussion

Impaired response to HBV vaccination in ESRD patients still remains a pivotal problem in the prevention of HBV infection among the patients and hospital staff in dialysis units.^{18–20} Therefore, the present study was focused on some events which are associated with antigen processing by monocytes as APC. We found that in uraemic non-responders to HBV vaccination the internalization of HBsAg by monocytes was significantly decreased; HBsAg was better internalized as the immune complex HBsAg–IgG. In the presentation processes the expression of adhesion molecules (ICAM-1) and accessory molecules (HLA–DR/Ia) was significantly decreased, especially in uraemic non-responders. Impaired internalization of HBsAg, as well as a decrease in ICAM-1 and HLA–DR/Ia expression, correlated well with the blunted proliferation of CD4⁺ T cells stimulated by autologous monocytes induced by HBsAg.

The lack of response to HBV vaccination in ESRD patients may not only be related to uraemic toxicity or intermittent exposure to the haemodialysis environment^{2,21} but also seems to depend on individual predisposition, associated with HLA-configura-

FIG. 1. Internalization capacity of monocytes according to different HBsAg preparations in ESRD patients and controls, in groups of responders and non-responders to hepatitis B vaccination. Experiments were performed with free HBsAg, HBsAg prepared *in vitro* as an immune complex with specific IgG (HBsAg-IgG) and with immune complexes (IC) isolated from serum of patients suffering from glomerulonephritis associated with HBV infection (IC-HBsAg-IgG). Internalization of HBsAg preparations conjugated with FITC was assessed by flow cytometry (FACScan, Becton-Dickinson). The live gate (Consort-30 Software) was estimated on the monocyte cluster by simultaneously defined forward scatter (FSC) and side scatter (SSC) parameters. Internal mean green fluorescence intensity (FL1-axis X) represents the mean number of internalized HBsAg-FITC molecules per cell. Relative cell number (%; axis-Y) indicates the number of monocytes internalizing HBsAg-FITC. U, ESRD patient; C, control; NR, non-responder; R, responder.

tion.^{5,6,22,23} Available data concerning the impaired function of monocytes in ESRD patients are based mainly on the fact that addition of monocytes isolated from healthy donors or, alternatively IL-2, restores the inhibited proliferation of T cells in uraemic non-responders.²⁴⁻²⁷ Unfortunately, the exact mechanism involved in the process of antigen presentation and recognition has not yet been resolved. Studies performed *in vitro* have revealed a defect of IL-2 secreted by activated T cells,^{28,29} while a higher IL-2 receptor expression has been observed in patients not responding to HBV vaccination.^{21,30,31}

The dysregulation at the level of the TCR/CD3 receptor complex in T cell-monocytes cooperation might result in an inadequate expression of adhesion molecules (e.g. ICAM-1) as well as accessory or co-

stimulatory molecules (e.g. HLA-DR/Ia; CD4).¹⁷ A co-stimulatory signal is essential for the induction of IL-2 synthesis and T cell proliferation. This co-stimulatory activity delivered by monocyte-T cell interactions involves the expression of adhesion molecule ligands (e.g. ICAM-1/LFA-1) as well as the synthesis and release of IL-1 and IL-6.^{32,33} Moreover, the preactivation state in uraemia characterized by a high IL-2 receptor (IL-2R) expression and a high density of LFA-1 β and CD4 molecules on helper-inducer T cells may be a consequence of impaired monocyte co-stimulatory function.^{17,21,30,32-35} In our experimental system, in which CD4⁺ T cells stimulated by autologous activated monocytes served as APC, the decrease in CD4⁺ T lymphocyte proliferation in uraemic non-responders not only resulted

Table 1. ICAM-1 adhesion molecule expression and expression of HLA-DR/la accessory molecule expression on monocytes^a

	Before culture		After 18 h induction with HBsAg		After 6 days culture with autologous CD4 T cells	
	NR	R	NR	R	NR	R
ICAM-1	<div style="display: flex; justify-content: space-around; align-items: center;"> ③ ② ① </div>					
ESRD patients	180 ± 35 ^b	192 ± 43	600 ± 110	640 ± 125	305 ± 72	450 ± 68
Controls	250 ± 53	305 ± 49	490 ± 95	510 ± 78	682 ± 145	835 ± 121
HLA-DR/la	<div style="display: flex; justify-content: space-around; align-items: center;"> ① ① ③ </div>					
ESRD patients	410 ± 120	420 ± 110	550 ± 121	693 ± 193	301 ± 135	410 ± 92
Controls	432 ± 93	441 ± 105	833 ± 190	945 ± 114	995 ± 232	1115 ± 167

^aTwo-colour flow cytometry (Becton–Dickinson, FACScan) was performed by using monoclonal antibodies anti-ICAM-1 and anti-HLA-DR conjugated with FITC and anti-CD14 labelled with PE. Analysis was undertaken before and after the 6 day culture of helper-inducer T cells with autologous monocytes presenting HBsAg. ICAM-1 and HLA-DR/la were also estimated on monocytes following induction with HBsAg and directly before institution to the culture.

^bMolecule expression is presented as their density (RD) on the cell surface and is given in arbitrary units of mean fluorescence intensity (MFI). The MFI of background staining (Simultest control; IgG1–FITC and IgG2–PE) ranged from 10 to 13 arbitrary units. Mean values ± S.D. of 30 experiments are presented. Differences between responders (R) and non-responders (NR) within the groups of ESRD patients or controls.

① $p < 0.05$; ② $p < 0.01$; ③ $p < 0.001$.

FIG. 2. Proliferative response of helper-inducer (CD4) T cells induced by monocytes presenting HBsAg. Data are expressed as stimulation index (SI). U, uraemic patients; C, controls; NR, non-responders; R, responders. Mean values ± S.D. of 30 experiments are presented.

from impaired IL-2 production, but also from dysregulation in the synthesis of IL-1, IL-6, IL-4 and IFN- γ (data not shown).⁵ These findings, associated with a decreased number of IL-1 receptors and IL-6 receptors and a simultaneous increase in IL-2R expression on freshly isolated CD4⁺ T cells, indicate also that co-stimulation of IL-2-dependent T-cell activation concerning IL-1 and IL-6 activity is impaired.^{5,16,32} It is also possible that other factors produced by activated monocytes (e.g. transforming growth factor- β , leukotrienes, prostaglandins-E, TNF- α) may suppress antigen-induced T lymphocyte proliferation.^{31,32,36}

Using flow cytometry methods, quantitative determination of monocyte phagocytosis was possible.

Monocyte internalization of HBsAg–FITC, measured as phagocytic activity, was significantly decreased in ESRD patients, especially in non-responders. This possibly leads to impaired transformation of this antigen and inadequate presentation to T lymphocytes. However, the use of HBsAg as an immune complex with IgG, and especially as an isolated IC–HBsAg–IgG, improved the blunted monocyte function of antigen internalization, compared with the free HBsAg. This tendency was observed in all groups of patients examined. These immune complexes also resulted in an improvement of ICAM-1 and HLA-DR/la expression (data not shown).

The mechanism of impaired phagocytosis and/or internalization observed in uraemia is not well known. However, some authors suggest disorders in ATP content and resting levels of cytosolic calcium (Ca_i) in leukocytes^{37–39} as well as the negative influence of parathormone on these events. The present study was performed only with leukocytes without separation of monocytes, as APC with regards to HBsAg. Hence, similar disorders in the internalization processes of uraemic monocytes affecting the antigen signalling pathway should be considered.

Adequate antigen presentation and co-stimulatory events are regulated by the TCR/CD3 receptor complex and various cytokines (e.g. TNF- α , IFN- γ).^{4,16,33,40,41} For instance, IFN- γ induces increased expression of ICAM-1, which correlates well with

enhanced antigen presentation by monocytes. Based on our results in uraemic non-responders the impaired signalling could depend on low ICAM-1 and HLA-DR expression. The decreased production of IFN- γ in ESRD patients, in particular in non-responders (data not shown),⁵ may result in blunted antigen presentation of monocytes. It would also be important to know whether the concentration of soluble forms of ICAM-1 in serum and in culture supernatants is changed in the examined group of uraemic patients and controls. Soluble ICAM-1 might regulate the expression of its ligand (LFA-1) on CD4⁺ T cells as well as serving as a prognostic and diagnostic marker of the inflammatory status of these patients.^{42,43}

In conclusion, the parameters examined only partially involve the phenomena concerning monocyte antigen processing and presentation. Additional events should be taken into account for further investigations, e.g. intracellular processing of HBsAg following its internalization into monocytes, and a wider variety of adhesion and accessory molecules. Thus, further analysis of the precise mechanisms could lead to simultaneous administration of different cytokines to improve the impaired response to HBV vaccination in dialysis patients, by providing the co-stimulatory signal.

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