

# Hepatitis B virus surface antigen suppresses the activation of monocytes through interaction with a serum protein and a monocyte-specific receptor

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During hepatitis B virus (HBV) infection, high numbers of non-infectious HBV surface antigen (HBsAg) particles are present in circulation. It is shown here that recombinant HBsAg (rHBsAg) particles, which contain the S protein only, bind almost exclusively to monocytes. Attachment of rHBsAg to the THP-1 pre-monocytic cell line occurs upon 1,25-dihydroxyvitamin D<sub>3</sub>-induced differentiation. Binding to monocytes is enhanced by a heat-labile serum protein and is inhibited by Ca<sup>2+</sup>/Mg<sup>2+</sup>, low pH and an HBsAg-specific monoclonal antibody. Furthermore, it is shown that rHBsAg suppresses lipopolysaccharide- and IL-2-induced production of cytokines. These results suggest the existence of a monocyte-specific receptor, the engagement of which by HBsAg suppresses the activity of these cells.

## Introduction

Worldwide, hepatitis B virus (HBV) causes more than 1 million deaths per year and about 350–400 million people are persistently infected with this agent. Most adult individuals will clear a primary infection, which can be asymptomatic or can result in an acute, generally self-limited, liver inflammation causing varying degrees of hepatocellular damage. However, approximately 5–10% of infected subjects will not resolve the primary infection and go on to develop a persistent infection. Vaccination is an effective way to prevent infection, but the giant virus reservoir in persistent carriers is a major obstacle to rapid eradication of the virus.

Embedded in the viral membrane are three related viral membrane proteins, L, M and S, which share 226 carboxy-terminal amino acids. During HBV infection, non-infectious subviral lipoprotein HBV surface antigen (HBsAg) particles are produced in large quantities by the infected hepatocytes and are secreted into circulation, where concentrations of 50–300 µg/ml are attained (Ganem, 1996). Like virions, these lipoprotein particles contain predominantly the S protein, smaller amounts of M protein and hardly any L protein. Recombinant expression of only the S protein in yeast,

mammalian and insect cells demonstrated that this protein has the unique property to form these HBsAg particles, which contain up to 30% of cell-derived lipids. The reason for the existence of, or the possible advantage of, the production of HBsAg remains elusive, until today. It is, however, remarkable that, in both acutely and chronically infected persons, a cellular and humoral anti-HBsAg response is lacking, despite the presence of HBsAg (Milich, 1997). Because anti-envelope antibodies are clearly detectable only in patients who recover from acute hepatitis and not in chronically infected subjects, these are thought to play a critical role in virus clearance.

The mechanism by which HBV establishes a persistent infection is at present still unclear. Studies with HBV transgenic mice led to the generally accepted idea that tolerance at the T cell level is an important underlying mechanism for the establishment of the persistent state, especially in neonates (Milich, 1997; Chisari & Ferrari, 1995; Chisari, 1995). However, defects in the antigen-presenting activity of dendritic cells, rather than functional defects in T or B cells are claimed to be responsible for the induction of HBV persistence (Akbar *et al.*, 1993; Kurose *et al.*, 1997). *In vitro* studies demonstrate a reduced capacity of PBMCs from chronically infected persons to produce cytokines upon stimulation with lipopolysaccharide (LPS) (Muller & Zielinski, 1990, 1992), while HBsAg inhibits the release of LPS-induced cytokines by human macrophages (Jochum *et al.*, 1990). Taken together, these results suggest that

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HBV infection or virus products may interfere with the normal function of antigen-presenting cells, like monocytes, macrophages and dendritic cells, which may add to the development of HBV persistence. To examine if and how HBsAg can influence the activity of monocytes, the physical interaction of HBsAg with PBMCs was studied by FACS, using biotinylated yeast-derived S particles. It is reported here that such particles bind almost solely to monocytes and not to T cells, while some interaction with B cells is observed. It is further shown that recombinant HBsAg (rHBsAg) particles not only inhibit LPS-induced secretion of IL-1 $\beta$  and TNF $\alpha$ , but also inhibit IL-2-induced secretion of IL-8.

## Methods

■ **rHBsAg.** Purified rHBsAg (subtype adw<sub>2</sub>) produced in *Saccharomyces cerevisiae* [lots DVP23 (752  $\mu$ g/ml in PBS) and DVP93/1 (1 mg/ml)] was kindly provided by GlaxoSmithKline Biologicals. The purity of these rHBsAg preparations, as judged by HPLC analysis as well as SDS-PAGE with Coomassie staining, was > 98%. rHBsAg is composed of well-defined subvirus particles, which contain the non-glycosylated S protein only. Similar preparations are used worldwide as human HBV vaccines after adsorption onto aluminium hydroxide.

■ **Biotinylation of rHBsAg.** rHBsAg was biotinylated using an enhanced chemiluminescent protein biotinylation module (RPN 2202, Amersham Pharmacia). 300  $\mu$ l rHBsAg was mixed with 270  $\mu$ l H<sub>2</sub>O, 30  $\mu$ l 0.8 M bicarbonate buffer, pH 8.6, and 15  $\mu$ l biotinylation reagent. The mixture was incubated at room temperature for 1 h, after which 24  $\mu$ l 1 M Tris was added. Biotinylated rHBsAg (b-rHBsAg) was purified by gel filtration on a Sephadex G25 column using PBS. Fractions of 1 ml were collected and the two b-rHBsAg peak fractions, as determined by ELISA, were pooled.

■ **ELISA.** Maxisorb 96-well plates (Nunc) were coated with rHBsAg or b-rHBsAg in PBS. The wells were blocked with 0.1% BSA in PBS, followed by washing three times (0.1% Triton X-100). HBsAg-specific monoclonal antibodies (MAbs) (1  $\mu$ g/ml) or streptavidin-horseradish peroxidase were added and plates were incubated for 1 h at room temperature. MAbs were detected with goat anti-mouse or goat anti-human antibodies labelled with peroxidase. After three washes, 3,3',5,5'-tetramethylbenzidine (Sigma) was added and, 30 min later, the reaction was stopped with 1 N H<sub>2</sub>SO<sub>4</sub>.

■ **Antibodies.** Mouse anti-human CD3-FITC (clone SK7), CD14-FITC (clone MP9), CD19-FITC (clone 4G7), IgG1-FITC isotype control and streptavidin-phycoerythrin (Strep-PE) antibodies were purchased from Becton Dickinson. Mouse anti-human CD14-FITC (clone My4) and IgG2b-FITC isotype control antibodies were purchased from Immunotech. Human anti-HBsAg clones F47B and F9H9 were a kind gift from Lia Sillekens (Centraal Laboratorium van de Bloedbank, Amsterdam). Human MAb anti-a was developed in the laboratory. Mouse anti-d and anti-y were a kind gift from DiaSorin.

■ **Cells.** Human PBMCs were isolated from buffy coats using Ficoll-Hypaque (density = 1.077 g/ml, Nycomed Pharma) centrifugation. Cells were stored in liquid nitrogen. Phorbol 12-myristate 13-acetate treatment (25 ng/ml) (PMA, Sigma) was performed for 4 h in cRPMI (RPMI 1640 containing 10% foetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin and

20  $\mu$ M  $\beta$ -mercaptoethanol). THP-1 cells were grown in cRPMI. To induce differentiation, 100 nM 1,25-dihydroxyvitamin D3 (1,25-VitD3, Calbiochem) was added for 24 or 48 h. Cultured cells were detached mechanically or by using non-enzymatic cell dissociation buffer (Sigma), washed twice with 2% non-heat-inactivated human AB serum (HS, Bio-Whittaker) in Hank's Balanced Salt Solution (Gibco BRL) (2% HS-HBSS) and stained as described below.

■ **Staining of cells.** PBMCs were thawed and washed twice with 2% HS-HBSS. Approximately 10<sup>6</sup> cells were incubated with b-rHBsAg in 200  $\mu$ l 2% HS-HBSS for 1 h on ice. After two washes with the same solution, cells were incubated with Strep-PE and/or FITC-labelled antibodies in 2% HS-HBSS for 1 h on ice. After two washes, cells were resuspended in 1 ml 2% HS-HBSS or PBS containing propidium iodide (PI) and analysed on a FACScan flow cytometer (Becton Dickinson). Dead cells, which incorporated PI, were gated out of analysis. At least 5000 cells were counted per analysis. Fluorescence (530 nm for FITC and 580 nm for PE) was measured. Median fluorescence was determined in each case. Signals were acquired in a logarithmic mode for FL1 (FITC) and FL2 (PE). Threshold levels were set according to negative (Strep-PE only) and isotypic controls.

■ **LPS treatment of THP-1 cells and PBMCs.** THP-1 cells (5  $\times$  10<sup>5</sup>) were treated for 24 h with 100 nM 1,25-VitD3. After washing, the cells were incubated in cRPMI either with or without 10 or 50 ng/ml LPS (*Escherichia coli* 0111:B4, Sigma), to which 0, 0.1, 1, 10 or 50  $\mu$ g/ml rHBsAg was added. In separate experiments, 10<sup>6</sup> PBMCs were incubated in cRPMI either with or without 10 or 50 ng/ml LPS to which 0, 0.1, 1, 10, 25 or 50  $\mu$ g/ml rHBsAg was added. Cell supernatants were collected after 24 h and tested for the presence of IL-1 $\beta$  and TNF $\alpha$ .

■ **IL-2 treatment of PBMCs.** Approximately 10<sup>6</sup> PBMCs were incubated in cRPMI either with or without 1000 U/ml IL-2 (Eurocetus), after which, 0, 1, 10, 25 or 50  $\mu$ g/ml rHBsAg was added. Cell supernatants were collected after 24 h and tested for the presence of IL-8.

■ **Cytokine determination.** The concentrations of IL-1 $\beta$ , TNF $\alpha$  and IL-8 in the cell supernatants were determined using commercially available kits (Bioscience) according to the manufacturer's instructions.

## Results

### Effect of biotinylation on the antigenicity of rHBsAg

rHBsAg was biotinylated and purified as described. Because the three lysine residues that can be biotinylated all lie in the major antigenic region of the S protein, the recognition of b-rHBsAg by four HBsAg-specific MAbs was investigated (Fig. 1). Biotinylation did not influence the binding of MAb F47B (Stricker *et al.*, 1985), which recognizes an epitope in the carboxy-terminal end of the S protein (Paulij *et al.*, 1999), a region that is predicted to form a membrane-spanning domain (Stirk *et al.*, 1992). Binding of MAb anti-d was reduced strongly after biotinylation. This is not unexpected as the lysine residue at position 122 is the key determinant for the d serotype. Binding of MAb F9H9 was reduced strongly and that of anti-a was reduced only slightly. This diminished reactivity is probably due to biotinylation at residue 141, which lies within the a determinant.

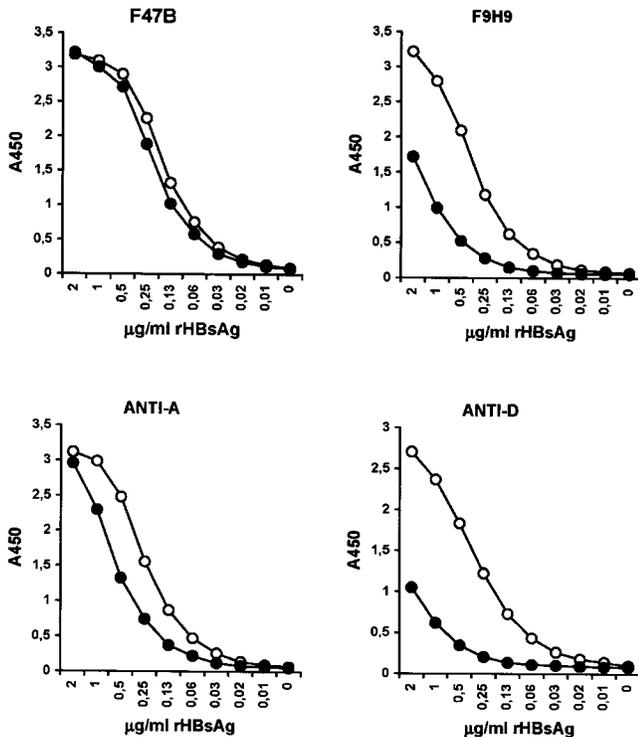


Fig. 1. Biotinylation of rHBsAg interferes with the binding of MABs specific for the major antigenic region. ELISA plates were coated with twofold dilutions of rHBsAg (○) or b-rHBsAg (●). After blocking with BSA, human MABs F47B, F9H9 and anti-a and the mouse MAB anti-d were allowed to bind to the particles. MABs were detected with either goat anti-mouse or goat anti-human antibodies labelled with peroxidase.

### Biotinylated rHBsAg binds to CD14<sup>+</sup> PBMCs

In preliminary binding experiments, it was observed that attachment of b-rHBsAg was enhanced in the presence of 1–2% non-heat-inactivated HS (see below). Therefore, all binding experiments were performed under these conditions. PBMCs were incubated with 10 µg/ml b-rHBsAg in 2% HS-HBSS, followed by an incubation with MABs specific for monocytes, T or B cells. b-rHBsAg was detected with Strep-PE. Using this concentration of b-rHBsAg, very strong binding to CD14<sup>+</sup> cells and some attachment to CD19<sup>+</sup> cells were observed. Binding of b-rHBsAg to CD3<sup>+</sup> cells did not occur (Fig. 2). Although biotinylation clearly altered the attachment of some MABs, it did not prevent the interaction with the monocytes of PBMCs. Additional experiments showed that when using 1–2 µg/ml b-rHBsAg, only monocytes stained positive (data not shown). Based on these results, it was decided to use approximately 1–2 µg/ml b-rHBsAg in most binding experiments.

### Attachment of b-rHBsAg to monocytes is influenced by serum components

Serum proteins have been shown to bind to HBsAg and are thought to deliver HBsAg to a cellular membrane protein

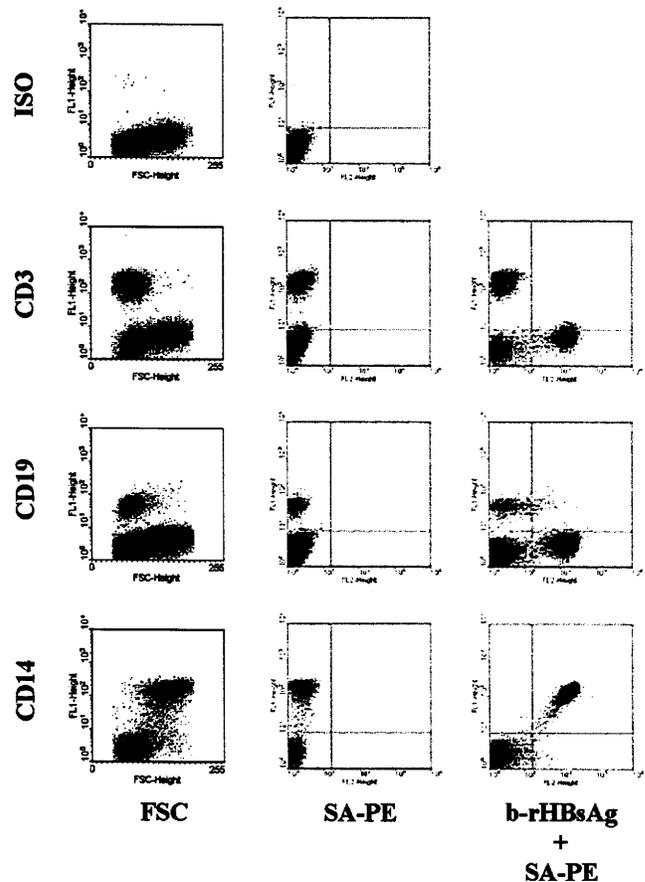


Fig. 2. Attachment of b-rHBsAg to CD14<sup>+</sup> PBMCs. Cells were incubated for 1 h with 10 µg/ml b-rHBsAg in 200 µl 2% HS-HBSS, washed twice with the same buffer and incubated with anti-CD14-FITC (clone P9), anti-CD19-FITC or anti-CD3-FITC and Strep-PE.

(Imai *et al.*, 1979; Neurath *et al.*, 1992; Budkowska *et al.*, 1993; Gagliardi *et al.*, 1994; Mehdi *et al.*, 1994). Therefore the effect of different serum concentrations on the attachment of b-rHBsAg to CD14-expressing cells was investigated. As shown in Fig. 3(a), attachment of b-rHBsAg to monocytes was clearly enhanced at low serum concentrations (1–3%). At higher concentrations (7–10%), binding was slightly inhibited compared to the serum negative control. These effects were not observed when BSA was used at similar protein concentrations (Fig. 3a). Incubation of serum at 56 °C for 30 min inactivated the factor responsible for the enhanced binding of b-rHBsAg to PBMCs (Fig. 3b), thus demonstrating its thermolability. Heat inactivation did not destroy the factor responsible for the reduction in attachment. These results demonstrate that different serum components were responsible for the enhancement and inhibition.

### Non-biotinylated rHBsAg prevents binding of b-rHBsAg to monocytes

To demonstrate the specificity of the observed interaction, PBMCs were incubated with different amounts of non-

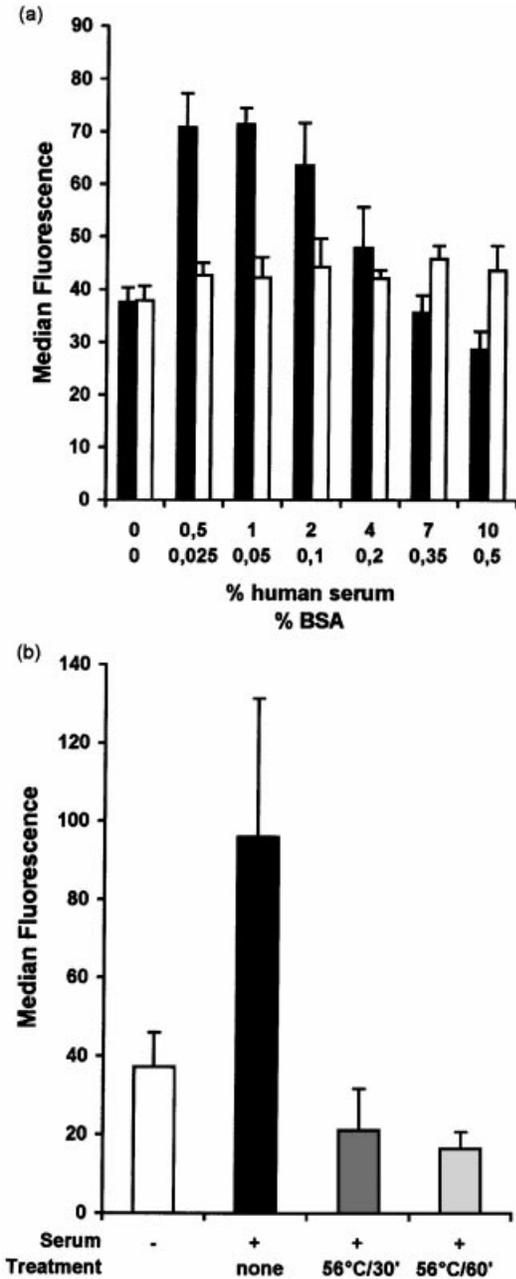


Fig. 3. (a) Attachment of b-rHBsAg to monocytes is enhanced by low concentrations of serum and reduced at higher concentrations. PBMCs were incubated for 80 min on ice with b-rHBsAg in HBSS containing different concentrations of HS (black bars) or BSA (white bars). After two washes, b-rHBsAg was detected with Strep-PE. Median fluorescence was determined. Data shown represent the average of three separate experiments. Error bars represent SD. (b) Heat-inactivation of serum destroys the factor that enhances binding of b-rHBsAg to monocytes. PBMCs were incubated for 80 min on ice with b-rHBsAg in HBSS without HS (white bar), with 2% HS (black bar) or with 2% HS that was heat inactivated for either 30 (dark grey bar) or 60 (light grey bar) min. After two washes, b-rHBsAg was detected with Strep-PE. Median fluorescence was determined. Data shown represent the average of three separate experiments. Error bars represent SD.

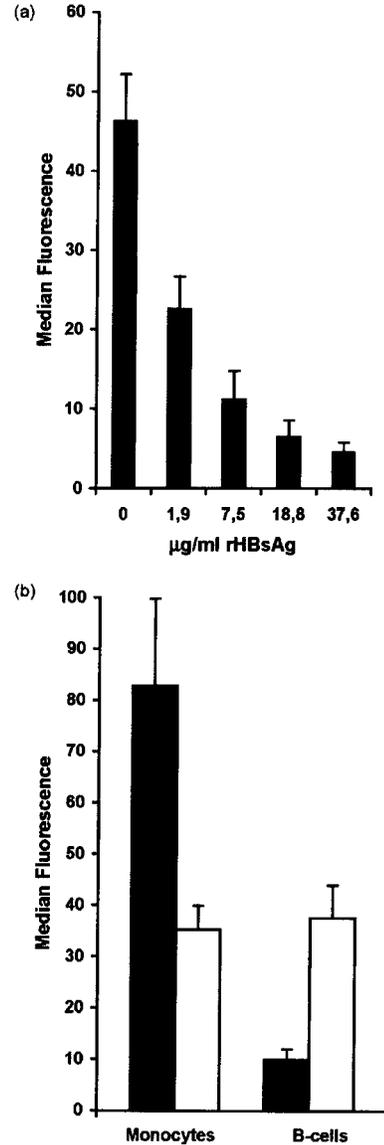


Fig. 4. (a) Non-biotinylated rHBsAg competes with attachment of b-rHBsAg to monocytes. PBMCs were incubated with different amounts of rHBsAg in 200 µl 2% HS-HBSS. After 1 h, b-rHBsAg was added and the cells were incubated for another hour. After two washes, cells were stained with Strep-PE. Median fluorescence was determined. The data shown represent the average of three separate experiments. Error bars represent SD. (b) MAb F47B reduces attachment of b-rHBsAg to monocytes and induces attachment to B cells. b-rHBsAg was incubated either with (white bars) or without (black bars) 5 µg/ml MAb F47B. After 1 h, PBMCs were added and the cells were incubated for 90 min. After two washes, cells were stained with Strep-PE, washed twice and analysed. Median fluorescence was determined. Data shown represent the average of three separate experiments with a total of six samples per experiment. Error bars represent SD.

biotinylated rHBsAg. After 1 h, b-rHBsAg was added to this mixture. As shown in Fig. 4(a), unlabelled rHBsAg blocked the binding of b-rHBsAg. Inhibition was also observed when non-biotinylated rHBsAg from lots DVP93/1 and DVP93/2 was used (data not shown).

### MAB F74B inhibits attachment of b-rHBsAg to monocytes

If the binding of b-rHBsAg to monocytes is specific, antibodies to b-rHBsAg should be able to block the interaction. To test this prediction, b-rHBsAg was incubated with different concentrations of F47B, F9H9, anti-a and anti-d antibodies. Only with MAb F47B was a dose-dependent reduction in binding observed (data not shown). This was not unexpected, as biotinylation interferes strongly with the binding of MABs F9H9 and anti-d to rHBsAg. Although recognition of b-rHBsAg by MAb anti-a was reduced only slightly, doses of up to 20  $\mu\text{g}/\text{ml}$  anti-a did not inhibit attachment of b-rHBsAg to PBMCs. Additionally, with MAb F47B, a dose-dependent binding of b-rHBsAg to 20–35 % of lymphocytes was detected, which were identified as B cells (data not shown). Maximal inhibition of b-rHBsAg binding to the monocytes and maximal attachment to B cells was obtained already with 5  $\mu\text{g}/\text{ml}$  MAB F47B (Fig. 4b).

### Divalent cations reduce binding of b-rHBsAg to monocytes

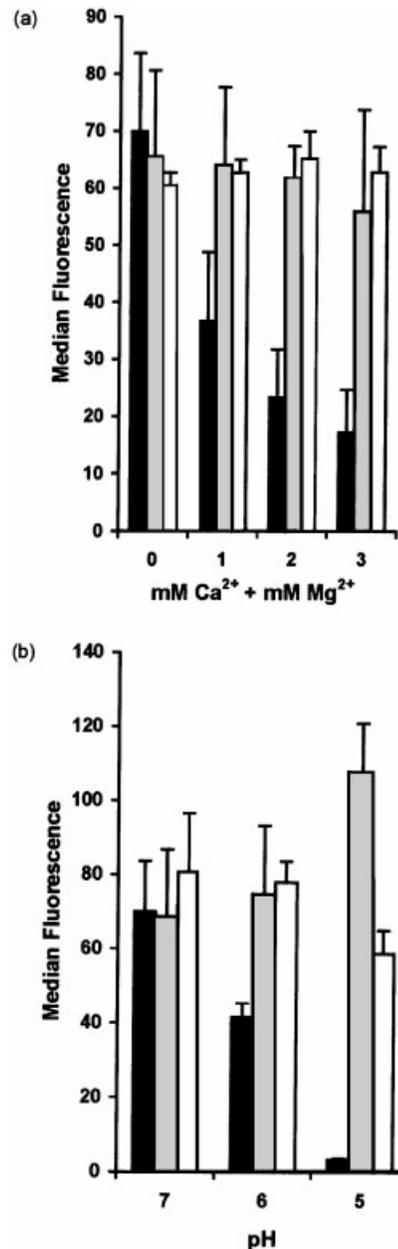
Divalent cations that are present at millimolar concentrations in serum can often modulate the interaction between ligands. Therefore, the effect of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on the binding of b-rHBsAg to PBMCs was investigated. The addition of increasing amounts of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  caused reduced binding of b-rHBsAg (Fig. 5a); the addition of 5 mM EDTA to the mixture restored attachment. The addition of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  after binding had no effect (Fig. 5a). Reduced binding was also observed when only  $\text{Ca}^{2+}$  or only  $\text{Mg}^{2+}$  was added (data not shown). These experiments were all performed in 2% HS in 50 mM Tris-HCl and 150 mM NaCl instead of HBSS to prevent acidification when adding 5 mM EDTA.

### Low pH reduces binding of b-rHBsAg to monocytes

Conflicting results about the influence of low pH on HBsAg attachment to cells have been reported (Komai & Peeples, 1990; Mehdi *et al.*, 1996). Therefore, the effect of pH on particle binding was measured at pH 7, 6 and 5. As shown in Fig. 5(b), b-rHBsAg binding was strongly reduced when the pH was lowered. Pre-incubation of the cells or b-rHBsAg at the same pH and for the same time did not have any effect (Fig. 5b).

### Effect of monocyte differentiation state on the attachment of b-rHBsAg

THP-1 cells, a pre-monocytic cell line, differentiate towards a monocytic cell type by treatment with 1,25-VitD<sub>3</sub>. This differentiation is easily detected by the expression of CD14. Binding of b-rHBsAg to this pre-monocytic cell line and 1,25-VitD<sub>3</sub>-differentiated THP-1 cells was investigated. CD14 expression was detected using two different specific MABs, clones P9 and My4. Both antibodies were used because they



**Fig. 5.** (a)  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  interfere with attachment of b-rHBsAg to monocytes. PBMCs were incubated with b-rHBsAg in the presence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (black bars) or  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and 5 mM EDTA (grey bars) in 2% HS in 50 mM Tris-HCl and 150 mM NaCl, pH 7.4. After two washes with the same buffer, cells were stained with Strep-PE. Median fluorescence was determined. Data shown represent the average of two separate experiments. Error bars represent SD.  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  added after attachment of b-rHBsAg (white bars) does not reverse binding. (b) Low pH prevents attachment of b-rHBsAg to monocytes. PBMCs were incubated with b-rHBsAg in 2% HS-HBSS or 2% HS in 10 mM citrate buffer and 150 mM NaCl, pH 6 and 5 (black bars). After one wash step with the same buffer and one wash step with 2% HS-HBSS, cells were stained with Strep-PE, washed twice and analysed. Median fluorescence was determined. Data shown represent the average of two separate experiments. Error bars represent SD. Pre-treatment of PBMCs (grey bars) or b-rHBsAg (white bars) with the same buffers does not prevent binding.

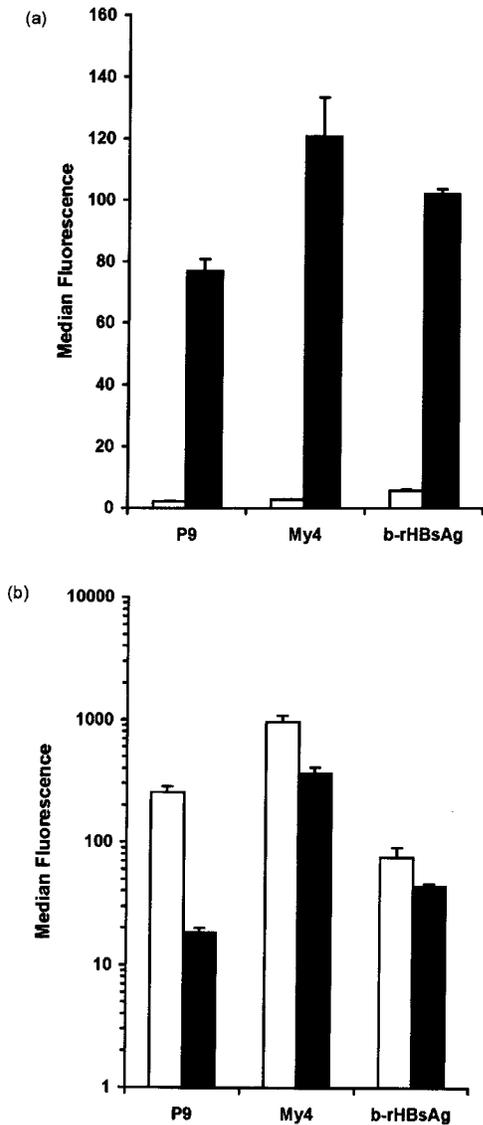


Fig. 6. (a) 1,25-VitD3-differentiated THP-1 cells express CD14 and bind b-rHBsAg. Non-differentiated THP-1 cells (white bars) or 48 h differentiated cells (black bars) were incubated with anti-CD14-FITC clone P9, clone MY4 or b-rHBsAg. Data shown represent the average of two separate experiments. Error bars represent SD. (b) PMA treatment reduces binding of b-rHBsAg. Non-treated cells (white bars) and PMA-treated cells (black bars) were stained with anti-CD14-FITC clone P9, clone MY4 or b-rHBsAg. Data shown represent the average of two separate experiments. Error bars represent SD.

recognize different forms of CD14, which may differ in expression levels on monocytes and monocytic cell lines (Pedron *et al.*, 1995). Undifferentiated THP-1 cells showed no detectable expression of CD14 and did not bind b-rHBsAg (Fig. 6a). 1,25-VitD3-differentiated THP-1 cells expressed CD14 molecules that were recognized by both antibodies. These differentiated cells did bind b-rHBsAg (Fig. 6a), which demonstrates that only monocytes in a certain maturation state bind rHBsAg.

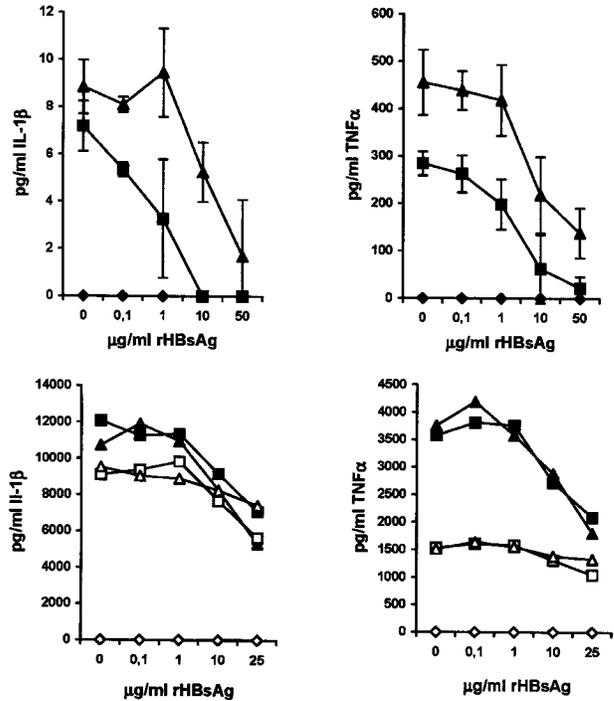


Fig. 7. rHBsAg reduces LPS-induced IL-1 $\beta$  and TNF $\alpha$  secretion. 1,25-VitD3-differentiated THP-1 cells (upper panels) or PBMCs (lower panels) from donor 1 (black symbols) and donor 2 (white symbols) were incubated with 0 ( $\diamond$ ,  $\blacklozenge$ ), 10 ( $\square$ ,  $\blacksquare$ ) or 50 ( $\triangle$ ,  $\blacktriangle$ ) ng/ml LPS and different concentrations of rHBsAg. Cell supernatant was collected after 24 h and cytokine concentrations were determined.

### Effect of PMA treatment on the attachment of b-rHBsAg

PMA has several effects on monocytes, like changes in cell shape, endocytosis and shedding of membrane proteins. PMA also induces differentiation of (pre)-monocytes into a more mature state. To monitor PMA-induced changes in monocytes, anti-CD14 antibodies, clones P9 and My4, were used to detect changes in CD14 levels; an established feature of CD14 is that monocytes rapidly shed CD14 when treated with PMA. Similar to previous reports (Pedron *et al.*, 1995), PMA treatment removed CD14 molecules recognized by clone P9 almost completely, while a two- to threefold reduction in CD14 molecules recognized by clone My4 was obtained (Fig. 6b). More importantly, a 50% reduction in attachment of b-rHBsAg to the PMA-treated monocytes was observed.

### Effect of non-biotinylated rHBsAg on the function of monocytes

Because rHBsAg interacts specifically with monocytes, the effect of rHBsAg on the function of these cells was investigated (Fig. 7). Monocytes were activated with LPS or IL-2. First, THP-1 cells were incubated for 24 h with 100 nM 1,25-VitD3, washed and incubated either with or without LPS in the

**Table 1.** rHBsAg reduces IL-2-induced IL-8 production

The IL-2-induced increase in levels of IL-8 was determined by first subtracting IL-8 concentrations of non-stimulated cells from those of stimulated ones. These values were compared to one of the non-rHBsAg-treated PBMC sample levels, set at 100%.

| PBMC donor | rHBsAg $\mu\text{g/ml}$ | IL-2       |       | IL-2-induced increase (%) |
|------------|-------------------------|------------|-------|---------------------------|
|            |                         | –          | +     |                           |
|            |                         | IL-8 pg/ml |       |                           |
| a          | 0                       | 2659       | 4946  | 100                       |
|            | 1                       | 3006       | 4403  | 61                        |
|            | 10                      | 2307       | 4095  | 78                        |
|            | 25                      | 2049       | 3018  | 42                        |
| b          | 0                       | 4993       | 16306 | 100                       |
|            | 1                       | 4393       | 9995  | 88                        |
|            | 10                      | 4750       | 9710  | 44                        |
|            | 50                      | 2347       | 6694  | 38                        |
| c          | 0                       | 13122      | 25889 | 100                       |
|            | 1                       | 6631       | 18188 | 91                        |
|            | 10                      | 5280       | 13187 | 62                        |
|            | 50                      | 4019       | 14576 | 83                        |
| d          | 0                       | 906        | 1428  | 100                       |
|            | 1                       | 941        | 1419  | 92                        |
|            | 10                      | 997        | 1296  | 57                        |
|            | 25                      | 999        | 1168  | 32                        |

presence of different concentrations of rHBsAg. Culture supernatant was collected after 24 h of incubation and tested for the presence of IL-1 $\beta$  and TNF $\alpha$ . rHBsAg alone did not induce any detectable cytokine production, while LPS induced the secretion of both cytokines (Fig. 7). High levels of TNF $\alpha$  were produced, while lower concentrations of IL-1 $\beta$  were detected. The highest cytokine levels were obtained with 50 ng/ml LPS. In the presence of rHBsAg, LPS-induced cytokine production decreased in a dose-dependent manner.

The effect of rHBsAg on LPS-induced cytokine production (IL-1 $\beta$  and TNF $\alpha$ ) was also studied with PBMCs from different donors. As shown in Fig. 7, PBMCs from donor 1 produced higher concentrations of TNF $\alpha$  compared to the PBMCs from donor 2. Nevertheless, the dose-dependent reduction in TNF concentrations in the presence of rHBsAg was more pronounced with PBMCs from donor 1. Similar results were obtained for IL-1 $\beta$ . Contrary to the reduced secretion of these pro-inflammatory cytokines, levels of an anti-inflammatory cytokine, IL-10, increased or remained unaffected (data not shown).

The effect of rHBsAg on IL-2-induced cytokine production was studied with PBMCs from four donors. As shown in Table 1, non-stimulated PBMCs already produced IL-8, the levels of which increased by adding IL-2. In the presence of rHBsAg, IL-8 production of both stimulated and non-stimulated PBMCs decreased in a dose-dependent manner.

## Discussion

Plasma-derived or recombinant HBsAg particles have been used widely to identify cellular receptors for HBV attachment and entry. Several receptors have been put forward but, so far, convincing data to support these claims are lacking. Serum proteins that bind to HBsAg were identified as well. These are thought to bridge virus and cellular receptors. Again, data to support these possibilities are lacking. The possible modulation by HBsAg of cellular and immunological responses during HBV infection has received much less attention. It has been speculated that the large number of HBsAg particles may induce T cell anergy and may prevent antibody-mediated neutralization of HBV. *In vitro* studies have demonstrated a reduced capacity of PBMCs from chronically infected persons to produce cytokines upon stimulation with LPS (Muller & Zielinski, 1990, 1992). Moreover, ten years ago it was shown that HBsAg can suppress the production by human macrophages of different cytokines induced by different agents such as LPS, vesicular stomatitis virus and granulocyte-macrophage colony-stimulating factor. A role for endoribonuclease V as a post-transcriptional inactivator of cytokine transcripts was demonstrated (Jochum *et al.*, 1990). Taken together, these results suggest that HBsAg interacts with one or more receptors on antigen-presenting cells, like monocytes, macrophages and dendritic cells. Attachment of HBsAg to mono-

cytes was reported previously, an interaction that was shown to occur via pre-S1 (Pontisso *et al.*, 1991; Neurath *et al.*, 1992). Data presented here clearly demonstrate that monocytes express a receptor, which is recognized by – S protein only – HBsAg expressed in yeast. Moreover, using THP-1 cells and PMA-treated monocytes, it is shown that this receptor is present in a more mature differentiation state only. Binding to macrophages, obtained by culturing monocytes for 7 days, was observed as well (data not shown). A protein present in HS enhances attachment of rHBsAg to the plasma membrane of monocytes. This protein is rapidly inactivated by incubation at 56 °C, a procedure routinely performed with sera used for tissue culture. Binding of rHBsAg is partially inhibited by an S protein-specific MAb (F47B), while F47B-dependent binding to B cells is observed. When the same experiment was performed with heat-inactivated serum, attachment of rHBsAg to monocytes and B cells was observed, but only in the presence of MAb F47B (data not shown). This observation suggests that the complete inhibition of binding to monocytes is masked, probably by the alternative attachment of rHBsAg–antibody complexes to Fc receptors. The binding of rHBsAg to B cells most probably results from such interactions as well. Binding of b-rHBsAg was decreased in the presence of  $\text{Ca}^{2+}/\text{Mg}^{2+}$  and  $\text{H}^+$  (low pH).

To find a possible biological function for this rHBsAg–receptor interaction, the effect of rHBsAg on LPS- and IL-2-induced activation of monocytes was investigated. One of the most potent activators of monocytes is LPS, which induces the secretion of several cytokines, such as IL-1 $\beta$ , IL-6, IL-12 and TNF $\alpha$ . rHBsAg particles themselves did not induce any of these cytokines, while LPS-induced secretion of IL-1 $\beta$  and TNF $\alpha$  was reduced in the presence of rHBsAg. Using macrophages and plasma-purified HBsAg, identical results for TNF $\alpha$  have been reported previously. However, in contrast to our results, human macrophages produced IL-1 $\beta$  in response to HBsAg (Jochum *et al.*, 1990). A second potent activator of monocytes is IL-2, which, among several other activities, increases the secretion of cytokines like IL-8, IL-6 and TNF $\alpha$ . As shown previously (Bosco *et al.*, 1997), blood monocytes already secreted IL-8 when cultured without IL-2. This production was downregulated by rHBsAg. More importantly, IL-2-induced IL-8 secretion was reduced in the presence of rHBsAg.

Viruses have long been viewed as simple genetic parasites that use the host cellular machinery to propagate themselves. However, it has become clear that the co-existence of these pathogens and their hosts have shaped the immune system and resulted in a surprising diversity of virus strategies to manipulate different cellular and immune regulatory systems. Viruses have targeted cellular cytokine production and cytokine receptor-signalling pathways, apoptotic pathways, cell growth and activation pathways, MHC-restricted antigen presentation pathways and humoral immune responses (Alcami *et al.*, 2000; Tortorella *et al.*, 2000). Our results suggest

strongly that monocytes express a receptor that is recognized by HBsAg. Engagement of this receptor, through interaction with a serum protein, suppresses the activity of monocytes. These observations suggest that HBV produces HBsAg in excess amounts to interfere with the normal function of antigen-presenting cells.

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