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# Hepatitus B virus: specific binding and internalization of small HBsAg by human hepatocytes

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Previously, we identified human liver endonexin II (EII) present on human hepatocyte plasma membrane as a specific hepatitis B surface antigen (HBsAg) binding protein. We also showed the spontaneous development of anti-idiotypic (anti-HBsAg) antibodies in rabbits immunized with EII and in chicken immunized with the  $F(ab')_2$  fragment of rabbit anti-EII IgG. These findings suggest the existence of a receptor-ligand relationship between EII and HBsAg. In the present study, we demonstrate that small HBsAg conjugated to 10 nm

colloidal gold also binds specifically to human hepatocytes. Invagination of the coated pit region at the HBsAg binding sites on the human hepatocyte plasma membrane results in the internalization of the HBsAg-gold particles. The binding and consequently the internalization of HBsAg is inhibited by anti-EII or anti-idiotypic (anti-HBsAg) antibodies. These findings indicate that EII is directly involved in the binding and uptake of hepatitis B envelope proteins.

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Although considerable information on the molecular biology of hepatitis B virus (HBV) has been gained in the past decades, still little is known about the mechanism of attachment and penetration of HBV into human hepatocytes, the natural host cells of HBV. Binding of virus particles to plasma membranes of human hepatocytes is considered an important initial step in viral entry and infection of HBV. The envelope of HBV is composed of three polypeptides: the small, middle and large hepatitis B surface antigens (HBsAg) (Tiollais et al., 1985). These three proteins might play an important role in recognizing specific molecules on the surface of the human hepatocytes. The three HBsAg proteins have the S sequence in common, but differ in their amino-terminal extensions. Middle HBsAg consists of the S and PreS2 sequences, while the large HBsAg consists of the S, PreS2 and PreS1 sequences. Recently, we have identified endonexin II (EII), isolated from human liver tissue, as a specific HBsAg binding protein (Hertogs et al., 1993). Furthermore, we described the spontaneous development of anti-idiotypic (anti-HBsAg) antibodies in rabbits immunized with human liver EII and in chicken

compete with EII for the binding to HBsAg, indicating that the anti-idiotypic antibodies mimic a region of EII that interacts with HBsAg. These findings strongly suggest the existence of a receptor-ligand relationship between HBsAg and EII. In the present study, we report evidence at the ultrastructural level for the specific binding and internalization of small HBsAg by human hepatocytes. The binding and internalization of small HBsAg are inhibited by anti-EII or anti-idiotypic (anti-HBsAg) IgGs. Human livers were obtained from organ donors with the permission of the local committee on human experimentations and informed consent of the families. The hepatocytes were isolated by a two-step collagenase perfusion according to the method of Rijntjes et al. (1986). Viability of the cells, as determined by trypan blue exclusion, was 90%. The cells were seeded at a density of  $2 \times 10^5$  cells/cm<sup>2</sup> as described by Rijntjes *et al*. (1986). Cells were cultured for 3 days prior to use for ultrastructural experiments in six-well dishes (Costar) coated with extracellular matrix obtained from human liver tissue (Rijntjes et al., 1986). Cells that were not used

immunized with the  $F(ab')_2$  fragment of rabbit anti-EII IgG (Hertogs *et al.*, 1994). These antibodies are able to

directly for experiments were cryopreserved as described by Rijntjes *et al.* (1986).

Recombinant yeast derived small HBsAg was kindly provided by Dr Miller (Merck Sharp & Dohme Research Laboratories, Westpoint, Pa., USA) (Emini *et al.*, 1986)). HBsAg (2  $\mu$ g) was labelled with Na<sup>125</sup>I (Amersham) using Iodogen (1,3,6,4-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylgly-

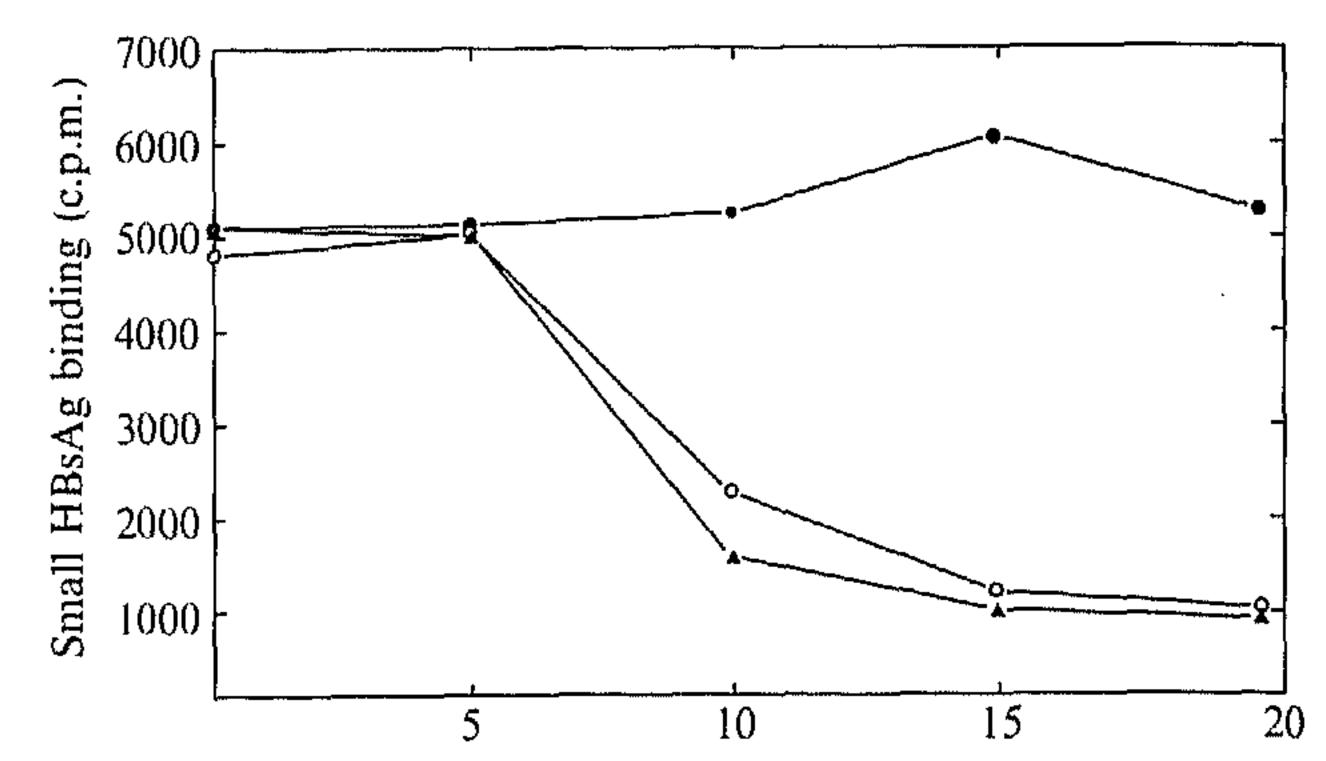
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couril (Pierce) as described previously (Leenders *et al.*, 1990). Specific activity was  $10^7$  c.p.m./µg.

Anti-endonexin II (anti-EII) antibodies were obtained from rabbits immunized with 20 µg recombinant human liver EII in complete Freund's adjuvant and purified as described previously (Hertogs *et al.*, 1994).  $F(ab')_2$ fragments were isolated from the IgGs and used for immunization of chickens for production of antiidiotypic (anti-HBsAg) antibodies as described (Hertogs *et al.*, 1994). IgGs isolated from non-immune serum (NIS) as reported (Hertogs *et al.*, 1994) were used in



control experiments.

To perform binding studies of radiolabelled HBsAg and human hepatocytes, cryopreserved hepatocytes were thawed as described (Leenders et al., 1990) and resuspended in WEM containing 0.5% BSA. Viability of the hepatocytes after thawing was 50% as determined by trypan blue exclusion. About 100000 cells were incubated for 1 h at 4 °C to reduce non-specific binding. This was followed by incubation for 1 h in WEM-0.5% BSA with serial dilutions of rabbit anti-EII, chicken anti-HBsAg or non-immune serum (NIS) IgGs as control. Radiolabelled HBsAg (100 ng) was added and cells were incubated for an additional 2 h in the presence of the IgGs. Cells were subsequently washed three times with WEM and bound HBsAg was determined in a gamma-counter. Nonspecific binding was determined by incubation with radiolabelled ligand in the presence of a 20-fold excess of unlabelled HBsAg. All experiments were performed in

#### IgG in medium (%, v/v)

Fig. 1. Specific binding of radiolabelled small HBsAg to intact human hepatocytes in the presence of serial dilutions of non-immune serum ( $\bullet$ ), rabbit anti-EII ( $\bigcirc$ ) and chicken anti-idiotypic (anti-HBsAg) ( $\blacktriangle$ ) IgGs.

glutaraldehyde in PBS at 4 °C. To investigate whether chicken anti-idiotypic (anti-HBsAg) IgGs were able to block the binding and uptake of HBsAg–gold particles by human hepatocytes, the following experiment was performed: HBsAg was preincubated for 0.5 h with a 1:100 dilution of chicken anti-HBsAg (0.8  $\mu$ g/ml) or NIS (1.4  $\mu$ g/ml) IgGs at room temperature before HBsAg–gold was added to the cell cultures.

Fixed hepatocytes were harvested, resuspended in 2% agarose at 60 °C and pelleted. The cell pellet was cut into 1 mm<sup>3</sup> pieces and postfixed with 1% osmium tetroxide for 20 min at 4 °C. The cell blocks were dehydrated with ethanol and embedded in Epon. Ultrathin sections were counterstained with uranyl acetate and lead citrate and were examined with a Philips EM 301 at 80 kV. Sections were examined 'blind' by two independent investigators. To investigate whether anti-EII or anti-idiotypic (anti-HBsAg) antibodies were also able to inhibit the specific binding of radiolabelled small HBsAg to human hepatocytes, binding studies were performed in the presence of rabbit anti-EII, chicken anti-idiotypic (anti-HBsAg) or NIS IgGs. As shown in Fig. 1, binding of small HBsAg to human hepatocytes was specifically inhibited by both anti-EII and anti-HBsAg antibodies as compared to the incubations with NIS IgGs. Incubation with a 1:10 dilution of the anti-EII ( $5 \mu g/ml$ ) did not completely inhibit binding of HBsAg to the hepatocytes. However, when a 1:5 dilution (10  $\mu$ g/ml) of the rabbit anti-EII IgG preparation was used in the incubation, specific binding of HBsAg was totally abolished (Fig. 1). Chicken and

duplicate.

HBsAg was coupled to colloidal gold and used as a tracer for binding studies and uptake of HBsAg by primary cultures of human hepatocytes. The preparation of the 10 nm gold solution and the coupling of the gold to the HBsAg were done according to the method described by Slot & Geuze (1985). The gold particles were coupled to 200  $\mu$ g HBsAg at pH 7.8. Gold conjugated HBsAg was separated from free HBsAg and unbound gold particles by centrifugation on a 10% glycerol cushion in phosphate-buffered saline (PBS) with 0.1% BSA for 1 h at 25000 g at 4 °C. The HBsAg-gold pellet was resuspended in PBS and used directly for binding and internalization studies.

After culture for 3 days, human hepatocytes were incubated for 1 h at 4 °C in WEM containing 1 % BSA and subsequently for 1 h with rabbit anti-EII or NIS IgGs diluted 1:10 in WEM-1% BSA (final concen-

trations were, respectively, 5  $\mu$ g/ml and 14  $\mu$ g/ml). The HBsAg–gold pellet was diluted 1:10 in WEM–1% BSA and added to the cells. After incubation for 2 h at 4 °C in the presence of the antibodies, hepatocytes were washed three times with ice-cold WEM and kept for 0, 5, 10 and 30 min respectively at 37 °C. The cells were washed three times with ice-cold PBS and fixed for 0.5 h with 2%

rabbit NIS IgGs gave similar results in the binding studies. The results were compared to those obtained in experiments with rabbit NIS IgGs.

The specific binding and uptake of HBsAg by human hepatocytes was studied using electron microscopy. If EII is involved in the binding/uptake process, incubation of hepatocytes with rabbit anti-EII IgGs prior to

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Table 1. Mean number of gold particles attached per cell under different experimental conditions\*

IgGs used in the incubation	Gold particles bound per cell (±sp)	Percentage of control
NIS Anti-EII Anti-HBSAg	28 + 6.6 12.5 + 4.95 3.5 + 1.7	100%

\* Per experimental group 60 cells were analysed by two independent investigators and the average number of gold particles per cell was determined in three experiments (Wilcoxson test, P < 0.005).

IgGs (14  $\mu$ g/ml). After incubation at 4 °C the HBsAggold particles were found to be attached to the plasma membrane of the hepatocytes (Fig. 2A). In Fig. 2D binding of HBsAg-gold to a typical coated pit region is shown. Warming the cells at 37 °C resulted in invagination of this region (Fig. 2B, E). After incubation for 5 min at 37 °C endocytotic vesicles containing gold particles could be observed (Fig. 2C, F), indicating that internalization took place within a few minutes. After incubation at 4 °C no endocytotic vesicles containing gold particles could be detected. When cells were incubated for 30 min at 37°C, virtually no gold conjugated HBsAg could be detected on the surface of the plasma membrane, indicating that bound HBsAg was internalized. Rat hepatocytes, which served as negative control, did not bind and internalize HBsAg (result not shown). Electron microscopy is an elegant method for visualization of the binding and uptake of a ligand by its specific receptor protein. In this study, HBsAg conjugated to 10 nm colloidal gold was used to investigate whether EII is involved in the binding and uptake of HBsAg by human hepatocytes. Binding of HBsAg-gold to human hepatocytes could be inhibited specifically by incubation with rabbit anti-EII or with anti-idiotypic (anti-HBsAg) IgGs. The results obtained from binding studies using radiolabelled ligand, shown in Fig. 1, were consistent with those obtained with HBsAg-gold par-

HBsAg-gold administration or preincubation of HBsAggold conjugate with anti-idiotypic (anti-HBsAg) antibodies before addition to the cells should inhibit this binding. After incubation at  $4^{\circ}$ C, cells were washed and fixed for electron microscopic analysis. The number of gold particles per cell was determined (Table 1). Cells incubated with gold conjugated HBsAg in the presence of NIS IgGs displayed the highest labelling density  $(28 \pm 6.6 \text{ gold particles/cell})$ . After preincubation of the hepatocytes with anti-EII IgGs ( $5 \mu g/ml$ ) the number of gold particles per cell was significantly reduced by 66%  $(12.5 \pm 4.95$  gold particles/cell). When the HBsAg-gold was preincubated with anti-idiotypic antibodies  $(8 \mu g/ml)$ , binding to the hepatocytes was inhibited by 87% (3.5 + 1.7 gold particles/cell) as compared to results obtained when experiments were performed using NIS

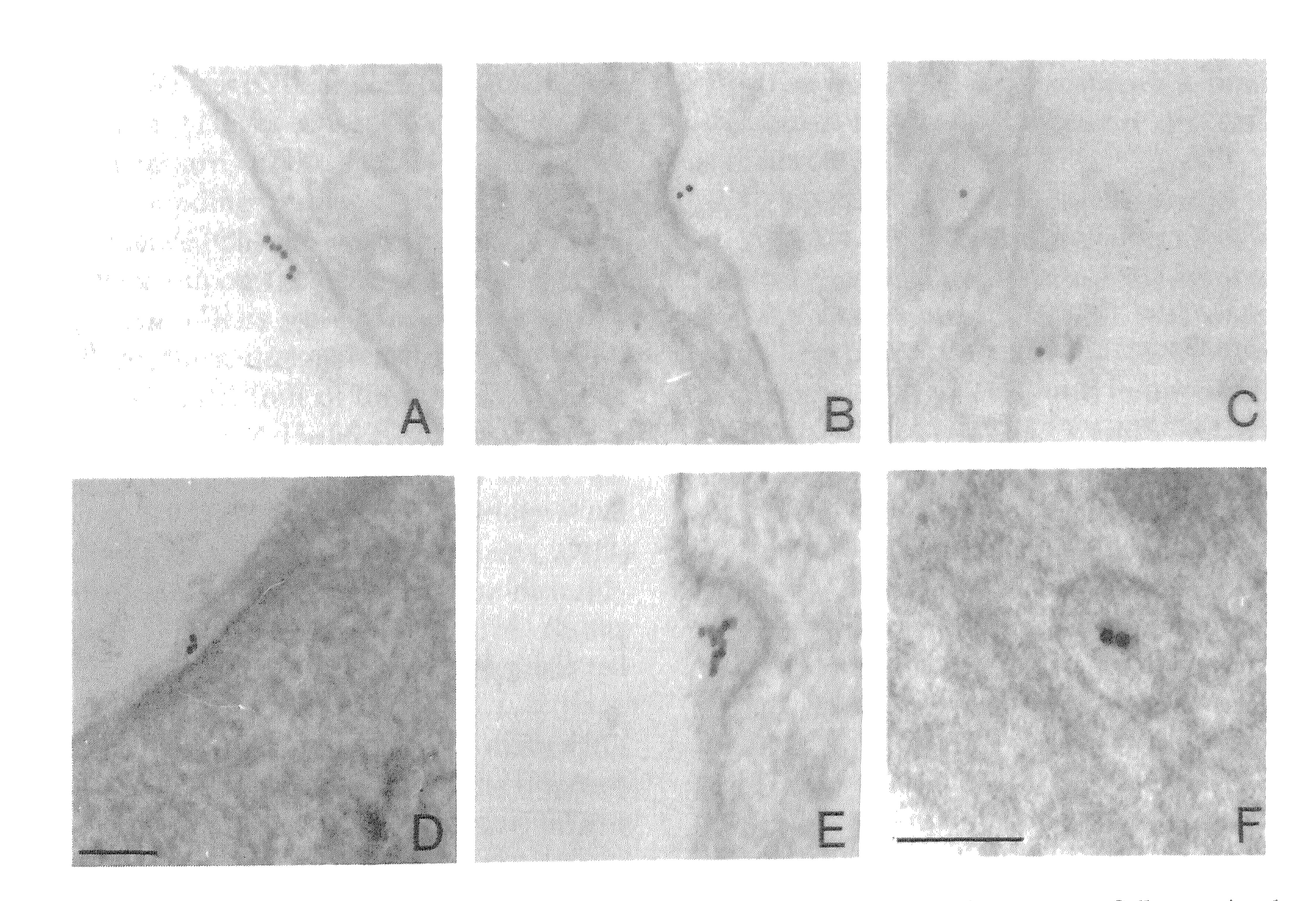


Fig. 2. Binding and internalization of HBsAg conjugated to 10 nm colloidal gold by human hepatocytes. Cells were incubated with HBsAg–gold for 2 h at 4 °C and subsequently warmed to 37 °C for 0 (panels A and D), 5 (panels B, C and E) or 10 (panel F) min. The bars represent  $10^{-2} \mu m$ . The bar in panel D is for panels A–D; the bar in panel F is for panels E and F.

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ticles (Table 1). The use of HBsAg-gold is therefore a suitable method for studying the specific binding and internalization of HBV envelope proteins.

In the present study we showed that invagination of the coated pit region occurred at the HBsAg-gold binding sites, after warming the cells (Fig. 2). Moreover, the coated pit region was pinched off which led to the formation of endosomes containing HBsAg-gold. The process of invagination and formation of endocytotic vesicles takes place within a few minutes after warming the cells. Rabbit anti-EII and anti-idiotypic (anti-HBsAg) IgGs could specifically inhibit the binding of HBsAg-gold to human hepatocytes and subsequent formation of endocytotic vesicles containing HBsAggold. Since it is generally accepted that coated pits play an important role in receptor mediated endocytosis of specific ligands (Heuser et al., 1988; Lin et al., 1992), our results strongly suggest that human liver endonexin II is actively involved in receptor mediated endocytosis of small HBsAg. At this moment the effect of anti-EII and anti-HBsAg IgGs on infection of primary cultures of human hepatocytes with hepatitis B virus is under investigation in order to establish the possible role of EII in viral infection.

#### References

- EMINI, E. A., ELLIS, R. W., MILLER, J. W., MCALEER, W. J., SCOLNICK, E. M. & GERETY, R. J. (1986). Production and immunological analysis of recombinant hepatitis B vaccine. *Journal of Infection* 13(suppl. A), 3-9.
- HERTOGS, K., LEENDERS, W. P. J., DEPLA, E., DE BRUIN, W. C. C., MEHEUS, L., RAYMACKER, S. J., MOSHAGE, H. & YAP, S. H. (1993).
  Endonexin II, present on human liver plasma membranes, is a specific binding protein of small hepatitis B virus (HBV) envelope protein. Virology 197, 549-557.
- HERTOGS, K., DEPLA, E., CRABBE, T., DE BRUIN, W., LEENDERS, W., MOSHAGE, H. & YAP, S. H. (1994). Spontaneous development of anti hepatitis B virus envelope antibodies (anti-idiotypic) in animals immunized with human liver endonexin II or with Fab fragments of anti-human liver endonexin II IgGs: evidence for the relationship between small HBsAg and endonexin II. Journal of Virology 68, 1516-1521.

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- HEUSER, J. E. & ANDERSON, R. G. W. (1988). Hypertonic mechanism inhibit receptor mediated endocytosis by blocking the clathrin coated pit formation. *Journal of Cellular Biology* **108**, 389-400.
- LEENDERS, W. P. J., GLANSBEEK, H. L., DE BRUIN, W. C. C. & YAP, S. H. (1990). Binding of the major and large HBsAg to human hepatocytes and liver plasma membranes: putative external and internal receptors for infection and secretion of hepatitis B virus. *Hepatology* 12, 1050–1056.
- LIN, H. C., SÜDHOF, T. C. & ANDERSON, R. G. W. (1992). Annexin VI is required for budding of clathrin-coated pits. *Cell* 70, 283–291.
- RIJNTJES, P. J. M., MOSHAGE, H. J., VAN GEMERT, P. J. L., DE WAAL, R. & YAP, S. H. (1986). Cryopreservation of adult human hepatocytes: the influence of deep freezing on the viability, cell seeding, survival, fine structures and albumin synthesis in primary cultures. Journal of Hepatology 3, 7–18.
- SLOT, J. W. & GEUZE, H. J. (1985). A new method for the preparing of gold probes for multiple labelling cytochemistry. *European Journal of Cellular Biology* 38, 87–93.
- TIOLLAIS, P., POURCEL, C. & DEJEAN, A. (1985). The hepatitis B virus. Nature 317, 489-495.

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