Preparation and Properties of Immune-stimulating Complexes Containing Hepatitis B Virus Surface Antigen

By C. R. HOWARD, B. SUNDQUIST, J. ALLAN, S. E. BROWN, S-H. CHEN AND B. MOREIN

Department of Microbiology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K. and The National Veterinary Institute, S-750 07 Uppsala, Sweden

(Accepted 15 June 1987)

SUMMARY

Immune-stimulating complexes (iscoms) have been prepared containing the major S gene products (HBsAg) of the hepatitis B virus genome. Immunization of BALB/c mice with a single dose of hepatitis B iscoms in saline resulted in a high titre antibody response to HBsAg. In contrast, the original HBsAg preparation required an adjuvant to produce equivalent amounts of antibody. Analysis of sera from mice immunized with hepatitis B iscoms revealed antibodies directed against the major a determinants of HBsAg. High secondary antibody responses were observed in immunized animals previously inoculated with a sub-immunogenic dose of HBsAg indicating that hepatitis B iscoms may represent a suitable immunogen for use in individuals in whom a course of immunization with currently licensed hepatitis B vaccines has failed to produce a significant anti-HBs response.

INTRODUCTION

Infection with hepatitis B virus (HBV) is a major public health problem throughout the world, with an estimated 200 million persistent carriers of the virus. The majority of the carriers live in under-developed areas where infection occurs at an early age and there is compelling evidence that infection with hepatitis B virus leads to the subsequent development of primary liver cancer (Zuckerman, 1982). The development of conventional vaccines from virus grown in cell culture has been prevented owing to the failure by numerous investigators to propagate HBV in vitro. Attention has therefore been directed to the use of alternative sources of viral antigens for active immunization. Since hepatitis B surface antigen (HBsAg) leads to the production of neutralizing or protective surface antibody (anti-HBs), purified 22 nm spherical HBsAg particles have been developed as vaccines (Hilleman et al., 1982).

Twenty-two nm HBsAg particles represent non-infectious surplus protein coats of the virus and may be purified from the plasma of asymptomatic human carriers. This vaccine requires extensive purification and inactivation by one or more different procedures and rigorous safety testing to ensure freedom from host proteins and contaminating transmissible agents (Hilleman et al., 1982). Although the safety and efficacy of the plasma-derived vaccine in various population groups at risk of acquiring hepatitis B has been established (Szmuness et al., 1980, 1982), it is expensive and the production of anti-HBs antibodies is poor in patients receiving maintenance haemodialysis and in immunocompromised patients (Stevens et al., 1984; Feuerhake et al., 1984). There is, therefore, an urgent need both to develop safe vaccines from alternative sources of HBsAg and to improve further the immunogenicity of existing hepatitis B vaccine materials.

The two major polypeptides of plasma-derived HBsAg of M, 25 000 (p25) and 30000 (gp30) contain both the a group determinants and sub-type specificities encoded by the viral S gene (for review, see Howard, 1986). Both polypeptides may exist as a 49 000 M, complex under non-reducing conditions and have identical amino acid sequences at both the amino and the carboxyl

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termini, indicating that the larger polypeptide, gp30, represents a glycosylated form of the smaller non-glycosylated p25 peptide. Additional amino-terminal sequences up to 175 amino acids in length may be present, representing transcription from the pre-S region of the genome. Expression of HBsAg in yeast may be achieved by using an autonomously replicating plasmid vector containing the S gene of HBV although the antigen must be released by disruption of the yeast cells (McAleer et al., 1984). Only the non-glycosylated p25 polypeptide is produced in this system although the HBsAg particles purified from yeast cell extracts are fully antigenic and induce a protective antibody response in both chimpanzees and man (McAleer et al., 1984; Jilg et al., 1984).

Solubilization of HBsAg polypeptides and the formation of micelles is readily accomplished with the detergent Triton X-100 regardless of antigen source (Skelly et al., 1979; Howard et al., 1986). These novel structures induce consistently higher levels of anti-HBs in mice and chimpanzees compared to that of the intact HBsAg particle (Skelly et al., 1981; Howard et al., 1982; Sanchez et al., 1982) although they have only low immunogenicity when administered in aqueous form (Tabor et al., 1982). An alternative form of polypeptide complex prepared after detergent solubilization has been described for parainfluenza, measles and rabies viruses (Morein et al., 1984) and for feline leukaemia viruses (Osterhaus et al., 1985) whereby virus polypeptides are presented in an immune-stimulating complex (iscom) with a matrix consisting of the glycoside Quil A. Iscom preparations induce specific antibodies to viral membrane proteins to a higher titre compared with micelle derivatives containing viral polypeptides alone. Enhanced immunogenicity is obtained using aqueous preparations thus eliminating the need of an adjuvant as with the more conventional form. In this paper, we present evidence that iscoms can be prepared containing HBsAg polypeptides and show enhanced immunogenicity compared to standard 22 nm HBsAg preparations.

**METHODS**

*HBsAg.* HBsAg particles were obtained from yeast cells carrying the HBV S gene in a suitable vector (Valenzuela et al., 1982; McAleer et al., 1984). The antigen was purified from cell extracts as described by Howard et al. (1986) and treated with 3 M-potassium thiocyanate in order to promote interchain disulphide bonds (Wampler et al., 1985). The final HBsAg preparation was obtained by extensive diafiltration against phosphate-buffered saline (PBS) and sterilized by passage through a 0.2 µm membrane; it consisted of S determinants only.

**Preparation of hepatitis B iscoms.** Iscoms were prepared in a single step, essentially as described previously for paramyxoviruses and rabies virus (Morein et al., 1984). One mg of HBsAg in 0.4 ml TN buffer was solubilized in 2% Triton X-100 for 18 h at 37 °C. A small quantity of radioiodinated antigen was added as a trace marker. The mixture was then layered onto a 10 to 40% (w/w) discontinuous sucrose gradient in TN buffer containing 0.05% Quil A (obtained as Spikoside from Iscotec, Sweden) and centrifuged at 80000 g for 16 h at 20 °C. Fractions containing the p25 component separated from solubilized HBsAg particles were pooled, dialysed against 0.05 M-ammonium acetate pH 7.0 and lyophilized. The final product was analysed by SDS-PAGE and examined by electron microscopy. The concentration of protein in the iscom preparation was measured as described by Bradford (1976).

Alternatively the following preparation method was used: 1 mg of HBsAg in 0.5 ml TN buffer was solubilized in 1% of the dialysable detergent N-decanoyl-N-methyl-glycoside (Mega-10) (Hildreth, 1982; Hanatani et al., 1984) for 18 h at 37 °C. After incubation the mixture was supplemented with 0.05% Quil A, 0.25 mg phosphatidylcholine and 0.25 mg cholesterol. The mixture was then dialysed against ammonium acetate buffer and lyophilized.

**Immunization of mice.** Antigen samples were inoculated intraperitoneally into 6- to 8-week-old female BALB/c mice (Olac Ltd., Banbury, U.K.) using a fixed volume of 0.2 ml. Blood samples were obtained as required by puncture of the retro-orbital plexus.

**Measurement of antibodies.** Antibody responses to HBsAg were routinely measured by solid phase radioimmunoassay (AUSAB, Abbott Laboratories, North Chicago, Ill., U.S.A.) using a 1 in 10 dilution of sera in PBS. Results were recorded as a ratio of bound radiolabel with respect to a negative control sample. A ratio of ten was previously determined to be equivalent to 50 mIU using a standard reference preparation of human anti-HBs antibodies.

Affinities of antibodies to selected HBsAg determinants were determined as described by Brown et al. (1984) using two different antigens labelled with 125I. These were cyclical synthetic peptides representing amino acids 124 to 137 (C124) and 139 to 147 (C139) respectively of the major p25 HBsAg polypeptide, ayw.
RESULTS

Production of hepatitis B iscoms

Solubilization of the major p25 component of HBsAg particles expressed in yeast followed by sucrose gradient centrifugation in the presence of Quil A resulted in a band of material approximately halfway into the gradient as identified by the presence of radioactive marker protein. Electron microscopy of this fraction revealed the presence of iscom-like particles 40 to 60 nm in diameter. Occasionally, some smaller particles were present which may have represented either residual HBsAg particles not solubilized by Triton X-100 treatment or reaggregated p25 monomers that had failed to complex with the Quil A micelles.

Analysis of iscom preparations obtained after solubilization of HBsAg particles with Mega-10 showed iscom particles more uniform in size with a mean diameter of 40 nm (Fig. 1). These preparations contained less intact or reaggregated material compared to Triton X-100 solubilization of HBsAg and did not require prior gradient centrifugation for detergent removal. Iscom preparations obtained after Mega-10 treatment of HBsAg particles were used for all subsequent animal experiments.

SDS–PAGE analysis of hepatitis B iscoms showed the presence of the p25 HBsAg polypeptide as the major component (Fig. 2a) and this polypeptide was further identified by immunoblotting with a rabbit antiserum to HBsAg (Fig. 2b).

Adjuvant effect of Quil A

The effect of Quil A as an adjuvant compared with alum was examined using yeast recombinant-derived HBsAg (Table 1). A single dose of the antigen produced high levels of anti-HBs antibodies by 28 days. Lower levels were found in animals receiving antigen and Quil A,
Fig. 2. (a) SDS-polyacrylamide gel electrophoresis of hepatitis B iscoms prepared from yeast HBsAg (lane 1) compared to the standard preparation (lane 2). HBsAg purified from plasma (lane 3) is shown for comparison. Polypeptides were revealed by silver staining. (b) Immunoblot analysis of HBsAg-containing iscoms (lanes 1 and 2) compared to either yeast-derived HBsAg (lanes 3 and 4) or plasma-derived HBsAg (lanes 5 and 6). Polypeptides were resolved using a rabbit anti-HBs serum (lanes 1, 3 and 5). No bands were seen using a normal rabbit serum as a control (lanes 2, 4 and 6). (c) Identification of plasma-derived HBsAg polypeptides with mouse antibodies to hepatitis B iscoms (lane 1). An equivalent dilution (1:40) of a normal mouse serum was included as a control (lane 2).

<table>
<thead>
<tr>
<th>HBsAg concentration (µg)*</th>
<th>Adjuvant</th>
<th>AUSAB ratio†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 28</td>
<td>Day 47</td>
</tr>
<tr>
<td>4-0</td>
<td>Alum (800 µg)</td>
<td>56-5</td>
</tr>
<tr>
<td>0-4</td>
<td>Alum (800 µg)</td>
<td>30-1</td>
</tr>
<tr>
<td>4-0</td>
<td>Quil A (10 µg)</td>
<td>26-2</td>
</tr>
<tr>
<td>0-4</td>
<td>Quil A (10 µg)</td>
<td>17-9</td>
</tr>
</tbody>
</table>

* BALB/c mice injected intraperitoneally with 200 µl volumes. Each inoculum was given to at least five animals.
† Ratio of sample c.p.m. to negative control serum c.p.m. obtained using a commercial radioimmunoassay for the detection of anti-HBs antibodies.
Immune-stimulating complexes containing HBsAg

Fig. 3. Dose–response experiment in BALB/c mice inoculated with 0.2 μg (■), 1 μg (▲), or 5 μg (●) of hepatitis B iscoms in saline. Control animals receiving a 5 μg dose of the original HBsAg preparation in saline failed to seroconvert to HBsAg.

Fig. 4. Total amount of antibody binding sites in fixed volumes of antisera obtained in the dose–response experiment with 0.2 μg (■), 1 μg (▲) or 5 μg (●) hepatitis B iscoms (see Table 2). Two peptides were used for these measurements, either a cyclic peptide representing amino acids 124 to 137 (C124; (a)) or a cyclic peptide representing amino acids 139 to 147 (C139; (b)).

Table 2. Measurement of affinity (K*) of anti-HBs responses in BALB/c mice inoculated with hepatitis B iscoms using synthetic peptides

<table>
<thead>
<tr>
<th>Day 34</th>
<th>Day 62</th>
<th>Day 93</th>
<th>Day 146</th>
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<tbody>
<tr>
<td>Peptide C139</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (5 μg)</td>
<td>1.3</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>II (1 μg)</td>
<td>1.8</td>
<td>2.5</td>
<td>2.6</td>
</tr>
<tr>
<td>III (0.2 μg)</td>
<td>2.7</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Peptide C124</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (5 μg)</td>
<td>0.19</td>
<td>0.34</td>
<td>0.56</td>
</tr>
<tr>
<td>II (1 μg)</td>
<td>0.26</td>
<td>0.36</td>
<td>0.91</td>
</tr>
<tr>
<td>III (0.2 μg)</td>
<td>0.36</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

* K, Affinity in litres per mole, × 10⁻⁶.

although these differences were less by day 47. All mice seroconverted at either the 4 μg or 0.4 μg dose level. Antibody was not detected in animals receiving up to 5 μg of HBsAg in the absence of adjuvant over the same period of observation. Although the anti-HBs response increased steadily over the 100 days of observation as measured by solid phase radioimmunoassay, the anti-a response in BALB/c mice declined rapidly after 28 days (C. R. Howard & S. E. Brown, unpublished observations).

Dose responses to hepatitis B iscoms

The immunogenicity of the hepatitis B iscom preparations was compared to the standard yeast HBsAg particles in BALB/c mice immunized once with hepatitis B iscoms in saline (Fig. 3). Significant levels of antibody were detected 34 days after immunization in those animals inoculated with either 1 μg or 5 μg of viral protein and these titres increased over a 5 month period of observation. Animals inoculated with 0.2 μg did not produce a positive antibody response as assessed by solid phase radioimmunoassay. In contrast, animals immunized with equivalent amounts of intact 20 nm yeast-derived HBsAg particles in saline did not seroconvert and remained negative throughout the duration of the experiment. The maturation of the antibody response was measured using synthetic peptides mimicking HBsAg/a determinants (Fig. 4 and Table 2). Binding to both C139 and C124 peptides were obtained using sera drawn
Fig. 5. Effect of priming of BALB/c mice with a subimmunogenic dose of a standard HBsAg preparation prior to inoculation with hepatitis B iscoms in saline. Animals were primed with 0.05 μg of HBsAg and inoculated 40 days later with a single 5 μg dose of either standard HBsAg particles (C) or hepatitis B iscoms (○), both in saline.

Table 3. Measurement of anti-HBs responses*

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<thead>
<tr>
<th></th>
<th>Antibody binding sites (pmol/10 μl)</th>
<th>Affinity (K, 1/mol × 10^{-6})</th>
</tr>
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<tbody>
<tr>
<td>Iscoms</td>
<td>60.1 ± 14.2†</td>
<td>1.60 ± 1.07</td>
</tr>
<tr>
<td>Standard HBsAg</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* BALB/c mice were primed with a subimmunogenic dose of HBsAg and inoculated 40 days later with either hepatitis B iscoms or with standard HBsAg particles in saline. Sera were taken 61 days after the second inoculation and responses were measured with the C139 peptide only.
† Values are given ± the standard deviation.

from animals immunized with either 1 or 5 μg of hepatitis B iscoms; animals receiving the lower dose (0.2 μg) also showed reactivity for both peptides up to 93 days for the C139 peptide and 62 days with peptide C124. Antibodies induced by the iscom preparations bound all the major polypeptides present in plasma-derived HBsAg particles (Fig. 2c). Collectively, these results indicated that iscom preparations containing the yeast-derived p25 molecule induced antibodies which cross-reacted with native HBsAg determinants.

**Effect of priming on immune response to HBsAg**

In order to assess the properties of hepatitis B iscoms for inducing secondary antibody responses, groups of BALB/c mice were immunized with a sub-immunogenic dose of intact HBsAg particles adsorbed to alum 40 days previously. Previous experiments had shown that BALB/c mice do not generate circulating antibodies if given a single dose of 0.05 μg HBsAg or less (unpublished observations). On inoculation with hepatitis B iscoms, primed animals developed a rapid and high titred anti-HBs response in contrast to the continuing absence of antibodies detectable by radioimmunoassay in primed animals given the intact HBsAg preparation (Fig. 5). Measurement of antibodies by binding to synthetic peptides also showed the development of antibodies specific for the a determinants of HBsAg in animals receiving the iscom preparation (Table 3). Animals positive for anti-HBs were monitored over a period of 18
months. Antibody levels remained high with an average positive : negative ratio of 39.8. This was substantially higher compared to the anti-HBs titres found in mice immunized with a single dose of plasma-derived hepatitis B vaccine adsorbed to alum (C. R. Howard, unpublished observations).

DISCUSSION

The outer surface coat of HBV contains a complex of determinants known collectively as surface antigen (HBsAg). The presence of antibody to the a determinants correlates with protection and it has been shown that a synthetic peptide in cyclic form representing amino acids 139 to 147 (C139) of the p25 and higher mol. wt. HBsAg polypeptides is bound with high affinity by a major proportion of specific antibodies to HBsAg in the sera of healthy persons immunized either with the currently licensed hepatitis B vaccine (Brown et al., 1984) or with a yeast-derived recombinant hepatitis B vaccine (Brown et al., 1986). These observations indicate that this peptide represents the dominant epitope of HBsAg to which protective antibodies are directed, and that the measurement of antibody binding to C139 is a useful procedure for the evaluation of sera positive for anti-HBs. In the present study, mice immunized with an iscom preparation of the p25 HBsAg polypeptide produced antibody which bound to the C139 peptide (Table 2) with an affinity similar to that measured previously in human and animal sera containing antibodies to hepatitis B vaccines (Brown et al., 1984, 1986). The highest affinity values were obtained with animals in the group receiving 0.2 μg of the iscom preparation, although the response was short-lived in this group. Affinity values measured using peptide C124 were generally much lower but followed a similar pattern of development. The lower K values obtained with peptide C124 may reflect that the conformation of this peptide does not so closely approximate the structure of the native determinant as that mimicked by the C139 peptide. It is interesting that BALB/c mice immunized with hepatitis B iscoms continued to produce antibody which bound to this peptide over a 146 day period of observation (Table 2, Fig. 4). In separate experiments, mice inoculated with plasma-derived antigen adsorbed to alum showed a decline in antibody recognizing the C139 peptide (data not shown). The longevity of the response in animals receiving hepatitis B iscoms may in part have been due to the more effective presentation of certain HBsAg/a antigen epitopes in this species.

The 25 000 Mr HBsAg polypeptide is frequently translated from its mRNA contiguous with either a 55 amino acid sequence at its amino terminus (pre-S2) or, occasionally, an extended 119 residue peptide (pre-S1). Experiments using inbred mouse strains have shown that the presence of pre-S determinants may stimulate the appearance of antibody to S determinants (Milich et al., 1985). This complication was avoided in the present study by the use of recombinant, yeast-derived HBsAg expressed by a vector which did not include pre-S gene sequences. The observed responses to iscoms on primary immunization (Fig. 3) and in primed mice (Fig. 5) compared to the standard HBsAg preparation was thus more likely to be due to antigen presentation and/or the adjuvant effect of Quil A, rather than a reorganization of pre-S sequences on the surface of the iscoms. In this context, positive antibody responses have been obtained in mice immunized with standard HBsAg 22 nm particles adjuvanted by the addition of Quil A as opposed to adsorption to alum (Table 1). Although anti-HBs levels were initially lower in those animals receiving antigen and Quil A, approximate levels were obtained by day 47. These results indicated that Quil A may substitute for alum as an adjuvant for HBsAg, although the antibody response was slower to develop and did not lead to enhanced immunogenicity unless the protein was reorganized into an iscom-like structure.

The marked duration of anti-HBs at constant titre suggested that long-lasting immunity may be possible using hepatitis B iscoms as an immunogen. The finding that antibody recognized peptide C139 more than 100 days after a single inoculation was encouraging, as responses to this sequence begin to decline in animals receiving the standard plasma-derived hepatitis B vaccine (C. R. Howard & S. E. Brown, unpublished observations). The reason for this difference is not clear although antigen presentation may be prolonged by the close association of viral protein and the glycoside components of Quil A.
Although the safety and efficacy of hepatitis B vaccines consisting of 22 nm HBsAg particles has been established in various population groups at risk of acquiring hepatitis B (Szmuness et al., 1980, 1982), there is an urgent need to improve the immunogenicity of existing vaccines to reduce the number of those individuals who do not respond to immunization, and thus to improve vaccine efficacy. Patients receiving maintenance haemodialysis and immunocompromised individuals in whom production of specific antibody is poor would benefit from such a vaccine. The rapid rise in anti-HBs levels in mice primed with a subimmunogenic dose of intact HBsAg and subsequently given an iscom preparation (Fig. 5) suggested that administration of antigen in iscom form enhanced secondary antibody responses to HBsAg. Clinical trials would be required to establish whether such an enhancement would increase the rate of seroconversion in hitherto non-responding recipients whose immune response to HBsAg may have nevertheless been primed by administration of the vaccine.

Several different approaches to the development of novel hepatitis B vaccines have been suggested (Howard & Zuckerman, 1984), including the use of micelle procedures whereby the immunogenicity of vaccine preparations containing antigen derived from either plasma or recombinant technology may be improved (Skelly et al., 1981; Howard et al., 1986). The use of iscoms containing selected HBsAg sequences may enhance the immunogenicity of hepatitis B vaccines in a manner which avoids the use of a alum adjuvant, and warrants further investigation.

The assistance of Mr Tapio Nikkela in the preparation of the electron micrograph is gratefully acknowledged. This work was supported in part by the British Technology Group. We are indebted to Dr W. J. McAleer, Merck Sharp and Dohme Research and Development Laboratories, for the supply of recombinant HBsAg.

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Immune-stimulating complexes containing HBsAg


*(Received 18 February 1987)*