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Immunology

Hepatitis B virus core antigen: Synthesis in *Escherichia coli* and application in diagnosis

(recombinant DNA/serology/animal virus)

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ABSTRACT **Fragments of hepatitis B virus DNA cloned in plasmid pBR322 carrying the gene for the viral core antigen have been placed under the control of the *lac* promoter of *Escherichia coli*. Several of the new recombinants direct higher levels of synthesis of the antigen, but the degree of enhancement varies with the different structures of the plasmids and hence the mRNAs produced. The antigen in crude bacterial lysates is a satisfactory diagnostic reagent for antibodies to the core antigen in serum samples.**

DNA from hepatitis B virus (HBV) has been cloned and propagated in *Escherichia coli* as a series of fragments produced by digestion with restriction endonucleases and as entire molecules after joining to plasmid or λ phage vectors (1, 2, 3, 4). Some of the fragments of HBV DNA cloned into the *Pst* I restriction site in the β-lactamase gene carried by plasmid pBR322 direct the synthesis of HBV core antigen (HBcAg) in *E. coli* (1, 5), the product being readily detectable by solid-phase radioimmunoassay (6) and having the capacity to induce specific antibody synthesis in rabbits (5).

To increase the level of expression of HBcAg in bacteria, various plasmids have been constructed in which the HBcAg gene has been fused to the beginning of the β-galactosidase gene under control of the *lac* UV5 promoter. Some of these constructions direct the synthesis of HBcAg at much higher levels than the parent plasmid, pHBV139a. Bacterial extracts prepared from cells harboring one of these newly constructed plasmids have been used successfully as a diagnostic reagent for the detection of antibodies to HBcAg (anti-HBc) in human sera by solid-phase immunoassay methods.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* strain K-12 HB101 was obtained from H. W. Boyer and *E. coli* strain K-12 DS410 (7) was obtained from J. Reeve.

Enzymes. Restriction enzymes were purchased from New England BioLabs and Boehringer Mannheim A. G. Exonuclease BAL 31 was purchased from Bethesda Research Labs. DNA polymerase I (Klenow fragment) and polynucleotide kinase were purchased from Boehringer Mannheim A. G. and T4 DNA ligase was the gift of S. Scherer.

DNA Preparations. pEX150, a pBR322 derivative containing the *lac* UV5 promoter and the sequence coding for the first eight amino acids of β-galactosidase, was obtained from H. Schaller. Plasmid pHBV139a has been described (1, 5) and was prepared as described by Clewell (8); the HBV sequences were recovered from it by digestion with *Pst* I and separation of the two fragments by electrophoresis in an 8% polyacrylamide gel (9). *Eco*RI and *Hin*dIII linkers were purchased from Collaborative Research Inc. and phosphorylated at their 5′ termini in reactions with polynucleotide kinase (10). Recombinant DNA molecules were made by incubation of DNA fragments with T4 DNA ligase under appropriate conditions (11) and recovered by transformation as described by Lederberg and Cohen (12).

Exonuclease Digestion. The ends of the hepatitis DNA fragment from pHBV139a were digested with the double-strand-specific exonuclease, BAL-31 (13), at 30°C for 4 min in a mixture of 60 μg of pHBV139a DNA digested with *Pst* I and 6.6 units of BAL-31 in 600 μl of 20 mM Tris·HCl, pH 8/12 mM $CaCl_2$/12 mM $MgCl_2$/600 mM NaCl/1 mM EDTA; the reaction was stopped by extraction with phenol.

Preparation of Bacterial Extracts. Cells were harvested in late logarithmic growth phase and stored at −20°C. Thawed cells (from 2 liters of culture mixture) were suspended in 6 ml of 25% (wt/vol) sucrose in 50 mM Tris·HCl, pH 8.0, and 1 ml of lysozyme (5 mg/ml) in 250 mM Tris·HCl, pH 8.0, was added. After 5 min at 0°C, 2.5 ml of 0.25 M EDTA, pH 8.0, was added and, after a further 5 min at 0°C, 10 ml of 1% Triton X-100, 0.4% Na deoxycholate in 50 mM Tris·HCl, pH 8.0/62.5 mM EDTA was added. After 10 min at 0°C with occasional shaking, 1 ml of 1 M $MgCl_2$ and 0.2 ml of pancreatic DNase solution (10 mg/ml) were added; this mixture was incubated at 37°C for 1 hr and then clarified by centrifugation at 10,000 rpm for 10 min. Protein concentration was measured by the Lowry method. The clear supernatant was dispensed and stored at −20°C.

Radioimmunoassay. Solid-phase radioimmunoassay was carried out on bacterial colonies (6) (the cells were lysed by direct application of bacteriophage λ*vir*). Bacterial lysates were diluted serially and assayed for HBcAg in microtiter wells coated with anti-HBc IgG (14). To detect the presence of anti-HBc, equal volumes of test serum and HBcAg extract (40 μl) were added to the coated microtiter well and left overnight at room temperature. The wells were then washed, incubated with ^{125}I-labeled anti-HBc (≈10^5 cpm) at 45°C for 60 min, washed extensively, and assayed in a γ-ray spectrometer.

Immunodiffusion. Experiments were carried out with 0.9% agarose gels in 0.01 M Tris·HCl, pH 7.2/0.001% EDTA/0.1 M NaCl in Petri dishes 5 cm in diameter. The samples (20 μl) were placed in wells 5 mm in diameter spaced 4 mm apart. HBcAg

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Abbreviations: HBV, hepatitis B virus; HBcAg, HBV core antigen; anti-HBc, antibody against HBcAg; HBsAg, hepatitis B surface antigen; anti-HBs, antibody against HBsAg.

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prepared from human liver was provided by B. J. Cohen and P. P. Mortimer, and human plasma positive for anti-HBc was from the Hepatitis Reference Laboratory of the University of Edinburgh. After diffusion overnight at room temperature (≈20°C), the gels were washed with 0.1 M NaCl, stained with 0.1% Coomassie brilliant blue in methanol/acetic acid/water (45:5:50), destained in the same solvent mixture, and photographed.

DNA Sequence Analysis. Plasmid DNA was treated with *Eco*RI and labeled at the 3′ ends with [α-^{32}P]dATP and DNA polymerase I (Klenow fragment). Sequence analysis was by the method of Maxam and Gilbert (9, 15).

Analysis of Polypeptides Synthesized in Minicells. *E. coli* strain DS410 (7) was transformed with recombinant plasmids carrying the gene for HBcAg and minicells were prepared and separated from nucleated cells by sedimentation through 10–30% (wt/vol) sucrose gradients (16, 17). Minicells (2×10^9) were suspended in 100 μl of M9 minimal salts medium/0.4% glucose containing cycloserine (20 μg/ml; ref. 17) and incubated at 37°C for 30 min in the presence of 0.5 μl of Difco methionine assay medium (Difco Inc., Detroit, Michigan) containing 0.5 μl of L-[^{35}S]methionine (100 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels). The minicells were then harvested by centrifugation, suspended in 40 μl of gel electrophoresis sample buffer (18) and heated at 100°C for 1 min before electrophoresis (22 μl) in a 12.5% (wt/vol) polyacrylamide gel in a buffer containing 0.1% $NaDodSO_4$.

Immunoprecipitation of Polypeptides Synthesized in Minicells. Proteins were labeled with L-[^{35}S]methionine as described above. After centrifugation, minicells were suspended in 10 μl of TNE buffer (10 mM Tris·HCl, pH 8/100 mM NaCl/1 mM EDTA) and incubated with 20 μl of lysozyme (2 mg/ml) solution for 10 min at 0°C. Nonidet P-40 (Fluka A.G.) was then added to 0.5%. Extracts were preadsorbed with 25 μl of a 50% suspension of *Staphlococcus aureus* protein A-Sepharose (Pharmacia) in TNE buffer. Samples were centrifuged (2 min, Hettick Mikroliter centrifuge), and supernatants were mixed with human anti-HBc IgG and incubated for 30 min at 37°C. Then, 60 μl of 50% protein A-Sepharose was added and incubation was continued for 15 min at room temperature and 2 hr at 4°C. The immune complexes were washed four times by centrifugation and resuspension in TNE buffer/2 M urea/0.05% Nonidet P-40. Finally, precipitates were centrifuged, suspended in 32 μl of gel sample buffer, and heated for 1 min at 100°C before electrophoresis as described above.

RESULTS AND DISCUSSION

Cloning of HBV DNA fragments in the *Pst* I site of pBR322 via oligo(dG·dC) tails gave recombinants that directed the synthesis of HBcAg in cultures of *E. coli* (1). In these clones, the β-lactamase gene is fused to the hepatitis DNA such that ribosomes translating the β-lactamase mRNA would read through the transcript of the oligo(dG·dC) region and arrive in phase at a termination codon ≈75 nucleotides later in the HBV DNA. Three base pairs after this termination codon is the codon for initiation of the HBcAg gene, from which translation presumably is reinitiated (5). To increase the level of expression of HBcAg in *E. coli*, additional plasmids have been constructed in which synthesis of HBcAg is controlled by the *lac* UV5 promoter instead of the β-lactamase promoter.

The construction of these plasmids is summarized in Fig. 1. The HBV component of pHBV139a was recovered by the action of *Pst* I and digested with exonuclease BAL 31 (13) such that an average of 100 base pairs were removed from each end of the fragment. *Eco*RI or *Hin*dIII linkers were ligated to these truncated DNA molecules, and this DNA was inserted into pEX150,

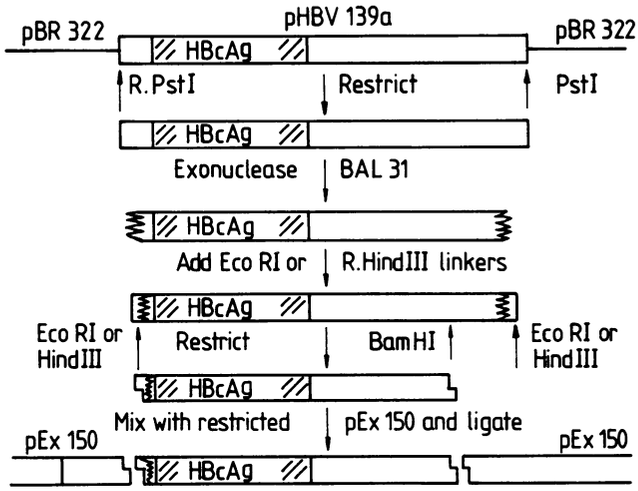

FIG. 1. Scheme for construction of plasmids that have the HBcAg gene fused to the *lac* UV5 promoter.

a derivative of pBR322 containing a restriction fragment that has the UV5 mutation of the *lac* promoter and a sequence corresponding to the first eight amino acids of the β-galactosidase gene followed by an *Eco*RI and a *Hin*dIII restriction site (19). The construction was such that fragments could be inserted in only one orientation (Fig. 1). Transformants of *E. coli* HB101 harboring these plasmids were analyzed for the expression of HBcAg by the disc radioimmunoassay of Broome and Gilbert (6). Twelve of the transformants that expressed HBcAg, pR1-1 through pR1-11 and pH80, were selected for further analysis.

Relative Levels of HBcAg Expression. The amount of HBcAg produced by several of the transformants was compared by applying a microtiter-well version (14) of the solid-phase radioimmunoassay to serial dilutions of bacterial lysates having similar initial total protein concentrations (20 mg/ml). The results are shown in Fig. 2 as plots of efficiency of anti-HBc binding against dilution; the binding efficiency is the ratio of ^{125}I-labeled anti-HBc bound relative to that in a suitable negative control (sample/negative ratio). The HBcAg activity in the extract of cells carrying pR1-11 was ≈100 times that in extracts

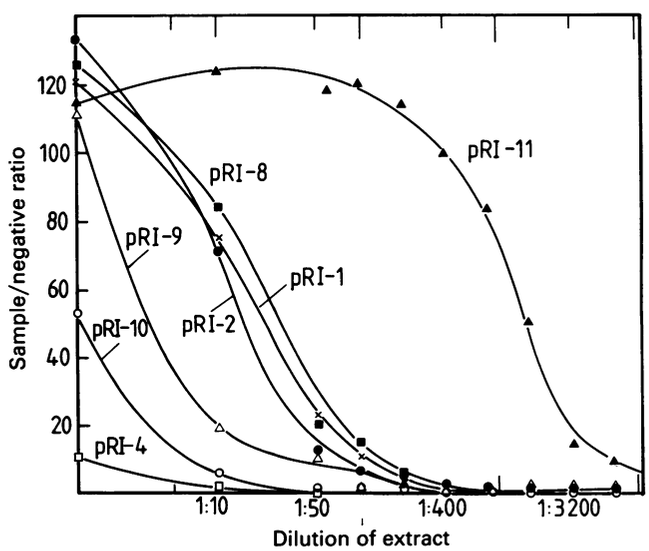

FIG. 2. Relative levels of expression of HBcAg in extracts of bacteria carrying the plasmids described in Fig. 3. □, pRI-4, ○, pRI-10; △, pRI-9; ●, pRI-2; x, pRI-1; ■, pRI-8; ▲, pRI-11.

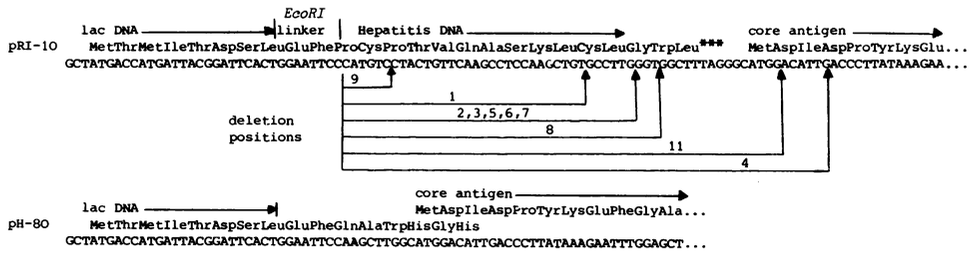

FIG. 3. Sequences of plasmid constructions expressing HBcAg. The region shown extends from the start point of translation of the β-galactosidase gene to ≈10 amino acids into the HBcAg gene. pRI-1, pRI-2, pRI-3, pRI-4, pRI-5, pRI-6, pRI-7, pRI-8, pRI-9, and pRI-11 can be considered deletions of pRI-10, the extents of which are indicated by vertical arrows. ***, Termination codon.

of cells with pR1-1, pR1-2, and pR1-8, which were similar to each other and almost an order of magnitude more active than an extract from pR1-9. The plasmid pR1-10 gave activity levels slightly lower than pR1-9 but higher than those induced by pR1-4, which was similar to the parent plasmid pHBV139a. It is of interest to examine these large differences in efficiency of antigen activity elicited by the various recombinant plasmids in terms of differences in structure of the plasmids.

The nucleotide sequences at the region of fusion of the HBV DNA to the β-galactosidase gene determined by the method of Maxam and Gilbert (9, 15) are shown in Fig. 3. In clones pR1-4 and pR1-11, the β-galactosidase coding sequence is fused directly to the coding sequence for HBcAg to give a gene that codes for a fusion protein containing the first eight amino acids of β-galactosidase followed by the core antigen. In the other nine pR1 clones, the hepatitis DNA sequence is fused to that of β-galactosidase such that ribosomes translating the β-galactosidase gene arrive in phase at the termination codon preceding the start codon for the core antigen, in a situation analogous to that of the original clones expressing HBcAg in the β-lactamase gene (5). Presumably, the ribosomes then restart translation at the initiation codon of the HBcAg gene.

Clone pH80, which expressed relatively low levels of HBcAg as indicated by the disc radioimmunoassay assay (6), has a structure such that a ribosome proceeding from the initiation codon of the β-galactosidase gene would enter the core antigen gene in the wrong reading frame and so encounter a termination codon (Fig. 3). Apparently, the ribosome is then able to move back to the initiation codon of the core antigen gene, or a new ribosome binds here and starts synthesis of the core antigen. This process appears to be less efficient than that in which the termination codon precedes the initiation codon.

A number of explanations can be advanced for the various levels of HBcAg resulting from the different plasmid constructions, most of which relate to differences in nucleotide sequence of the mRNA. Variations in HBcAg synthesis may result from differences in the secondary structure of the mRNA that lead to alterations in efficiency of translation, as proposed by Iserentant and Fiers (20). The variations may also be due simply to differences in the nucleotide sequence preceding the initiation codon of the HBcAg gene, which alter the efficiency of ribosome binding and initiation of protein synthesis. In the case of pRI-4 and pRI-11, the large apparent difference in HBcAg in the extracts may result from different levels of expresssion of the two HBcAg genes or could reflect a difference in efficiency of interaction between antigen and antibody resulting from the altered NH_2 termini of the two antigens, which differ in only two amino acids.

Not all of the cells in the population transformed with the new recombinant plasmids made after digestion of the original cloned HBV DNA fragment with exonuclease BAL 31 (Fig. 1) gave positive results in the disc radioimmunoassay for HBcAg production but the analyses described were carried out on those selected on the basis of positive results in this test. It is likely that other transformants carry plasmids covering a broader range of deletions than those indicated in Fig. 3 and, in consequence, show little or no synthesis of HBcAg or give a derivative that does not interact efficiently with anti-HBc.

Characterization of HBcAg. Gel exclusion chromatography of the bacterial HBcAg indicated that the material was aggregated with a M_r >40,000. Extracts from cells carrying pRI-11, the recombinant that gave the highest level of HBcAg expression, were examined by immunodiffusion against human anti-HBc and gave precipitin lines coincident with those formed by HBcAg derived from human liver (21) (Fig. 4). This confirms the immunological identity of the core antigens derived from these two sources.

The polypeptides produced in minicells containing various plasmids that direct HBcAg production were examined by gel electrophoresis. In some of these strains, a new polypeptide was detected that has a mobility somewhat greater than that of β-lactamase, which appears as characteristic doublet attributed to the precursor and mature forms of the enzyme, (i.e., after cleavage of the signal sequence) (Fig. 5). The mobility of this new polypeptide is consistent with its assignment as the native HBcAg polypeptide (M_r, 21,000) as should be the case with clone RI-8 or a fusion peptide containing the first eight amino acids for β-galactosidase along with the HBV core antigen sequence (M_r, 22,000) as in the case of clone RI-11. Fig. 5 shows that the polypeptide attributed to HBcAg from pRI-11 can be precipitated from the extract by anti-HBc.

Stability. The stability of the HBcAg in crude bacterial extracts was assessed by radioimmunoassay on samples after freeze-drying or storage at −20°C for 8 months. Little or no deterioration of the core antigen occurred under these condi-

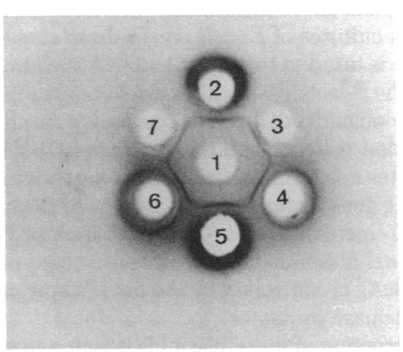

FIG. 4. Comparison by immunodiffusion of human HBcAg with that from an extract of bacteria carrying plasmid pRI-11. Wells: 1, human anti-HBc-positive plasma; 2 and 5, HBcAg extracted from human liver; 4 and 6, bacterial extract; 3 and 7, same preparation as in 4 and 6 but diluted 1:2.

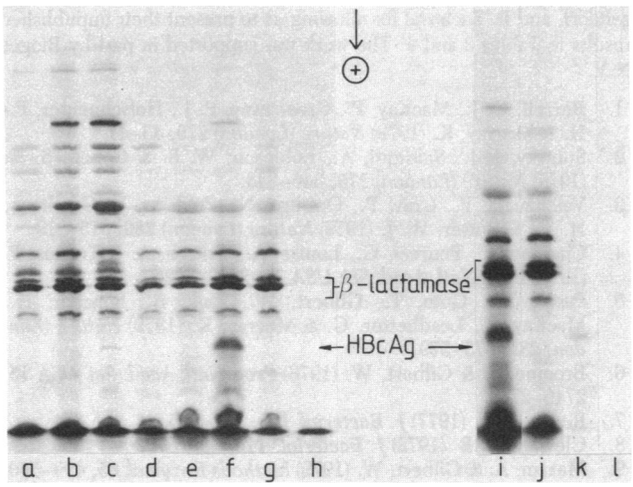

FIG. 5. Synthesis of HBcAg in bacterial minicells. Peptides synthesized by minicells produced by *E. coli* strain DS410 containing various plasmids carrying the HBcAg gene were analyzed by electrophoresis through 12.5% acrylamide/$NaDodSO_4$ gels. Tracks: a, pRI-1; b, pRI-2; c, pRI-8; d, pRI-9; e, pRI-10; f, pRI-11; g, pBR322; h, no plasmid; i, pRI-11; j, pBR322; k and l, same samples as in i and j, respectively, but after precipitation with anti-HBc.

tions, the two extracts having 83% and 78%, respectively, of the activity of the original lysate.

Use of Bacterial HBcAg in a Solid-Phase Immunoassay for Detection of Anti-HBc. Crude bacterial extracts prepared from cells transformed with pRI-11 were used as a diagnostic reagent for the detection of anti-HBc in human sera by solid-phase radioimmunoassay. To establish the optimum concentration of bacterial HBcAg for use in the assay system, serial dilutions of bacterial extracts (up to 2×10^{-3}) were tested against a known anti-HBc-positive serum diluted 10^2–10^6.

The validity of the test was assessed with a panel of 40 sera of known hepatitis B surface antigen (HBsAg) and antibody (anti-HBs) status (Table 1). The presence of anti-HBc in these sera was detected by solid-phase radioimmunoassay (14) with the bacterial lysate (either a crude extract or the fraction precipitated with ammonium sulphate at 55% saturation), with HBcAg derived from human liver (21), and with a commercial CORAB test kit (Abbot Laboratories). All three systems discriminated clearly between anti-HBc-positive and -negative sera (Table 1) and there were no discrepancies among the three sets of results.

Having established the validity of the assay based on the bacterial HBcAg preparations with these panel sera, an extended study was carried out with 500 sera of various HBV status, all of which were negative for HBsAg when tested with a commercial solid-phase radioimmunoassay kit (Travenol). A double-antibody radioimmunoprecipitation method (22) was used for determination of anti-HBs; 224 of these sera were assayed for anti-HBc with both the bacterial HBcAg and the CORAB reagents, the remainder were tested with bacterial HBcAg only. In the 224 sera, discrepancies between the anti-HBc status were observed with the two reagents in five cases (2.2%). Three of these five sera were repeatedly positive for anti-HBc when tested with the bacterial HBcAg but negative by the CORAB test, while the other two sera were positive by the CORAB test but negative with bacterial HBcAg. In all five cases, the levels of anti-HBc indicated were very low and close to the cut-off point of the assay systems.

Table 1. Radioimmunoassay for anti-HBc activity of 40 sera of known HBV status

HBV status of serum	% inhibition of ^{125}I-labeled anti-HBc binding		
	Bacterial HBcAg	Human HBcAg	CORAB test
HBsAg positive (n = 31)	93 (75–97)	93 (79–97)	94 (83–97)
Anti-HBs positive (n = 4)	94 (94–95)	93 (92–94)	94 (93–96)
HBsAg, anti-HBs negative (n = 5)	3.5 (0–23)	2.2 (0–20)	5.9 (0–23)

Results are relative to a control serum negative for anti-HBc and represent mean (range).

Table 2. Radioimmunoassay for anti-HBc activity of routine serum samples

HBV Status	No.	%
Anti-HBs, -HBc negative	448	89.4
Anti-HBs, -HBc positive	42	8.4
Anti-HBs positive, anti-HBc negative	3	0.6
Anti-HBs negative, anti-HBc positive	7	1.4

n = 500.

These results show that, even with a crude preparation of bacterial HBcAg, the incidence of false positive results is low. The sera tested are grouped according to antibody status in Table 2 which shows that seven (1.4%) of the sera that were negative for both HBsAg and anti-HBs were in fact positive for anti-HBc. Tests of this type for anti-HBc are thus clearly of value in routine diagnostic examinations, for sera that are positive for anti-HBc in the absence of other HBV markers are of particular interest clinically. Modification of the solid-phase assay procedure to test specifically for anti-HBc of the IgM subclass has been achieved in an enzyme-linked immunosorbent assay by Deinhardt *et al.* as described below.

Two other series of diagnostic tests have been carried out with the bacterial HBcAg. At the Virus Reference Laboratory of the Public Health Service in London, B. J. Cohen and P. P. Mortimer (personal communication) carried out a survey of HBV infection in unselected patients attending a clinic for sexually transmitted disease. Samples of 2,154 sera were examined for anti-HBc by competitive radioimmunoassay (23) using HBcAg derived from *E. coli* harboring plasmid RI-II and from human liver (21). Their results are summarized in Table 3. The two sera giving discrepant results were weakly positive for anti-HBc in a competitive radioimmunoassay; with commercial radioimmunoassay kits (Abbott), both sera were positive for anti-HBc and negative for HBsAg but one was positive for anti-HBs. At the Max-von-Pettenkofer-Institut in Munich, F. Deinhardt, M. Roggendorf, and R. Zachoval (personal communication) evaluated HBcAg produced in *E. coli* in tests measuring anti-HBc of the IgM class. In this study, the HBcAg content of a crude extract of *E. coli* carrying pRI-II was determined by a solid-phase enzyme-linked immunoabsorbant assay (24) and a commercial radioimmunoassay (Abbott) and HBcAg was de-

Table 3. Comparison of anti-HBc results obtained with HBc from *E. coli* and from liver

HBcAg from liver	HBcAg from *E. coli*	
	Anti-HBc positive	Anti-HBc negative
Anti-HBc positive	256	2
Anti-HBc negative	0	1,896

n = 2,154.

Table 4. Comparison of HBcAg from *E. coli* and liver in tests for anti-HBc IgM

	Hepatitis status of patient				
	Acute	Convalescent	Past	Chronic	None
Sera, no.	22	10	20	19	10
E. coli HBcAg					
Positive, no.	22	5	1	5	0
Sample/negative*	7.1	3.3	0.9	3.1	0.9
Liver HBcAg					
Positive, no.	22	6	1	4	0
Sample/negative*	5.5	3.1	1.1	3.4	0.9

* Sera were used at 1:1000 dilution. Sample/negative ratios ≥2.1 were considered positive.

tected up to a dilution of 10^{-5} in both assays; this compares with a maximal titer of 1:160 for semipurified HBcAg from human liver. The HBcAg from *E. coli* (bacterial HBcAg) was then evaluated further in a diagnostic assay that detects antibodies of the IgM class to HBcAg (anti-HBc IgM) (24, 25). The bacterial HBcAg could be used in this test system to a dilution of 10^{-4} whereas the HBcAg from liver could only be diluted to 1:20. The results obtained by Deinhardt *et al.* with both HBcAg preparations on 71 sera from patients with acute, convalescent (sera taken 4–6 months after onset of disease), past (sera taken >1 year after onset of disease), or chronic hepatitis, and 10 sera from persons without markers of acute or past hepatitis B are given in Table 4. Both tests detected anti-HBc IgM in all sera from patients with acute hepatitis B and with the same frequency in sera obtained during convalescence or chronic hepatitis. However, the sample/negative ratio (optical density of sample/optical density of negative control) was generally higher with bacterial HBcAg. Although the number of sera examined so far is small, the assays for anti-HBc IgM carried out with bacterial HBcAg appear at least as sensitive as those with HBcAg derived from liver.

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