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DNA Polymerase in the Core of the Human Hepatitis B Virus Candidate

WILLIAM S. ROBINSON AND RICHARD L. GREENMAN
Department of Medicine, Stanford University, Stanford, California 94305

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Experiments were done to show that the human hepatitis B antigen (HBAg)-associated DNA polymerase is a component of Dane particles and their antigenically distinct cores prepared by Nonidet P-40 detergent treatment of Dane particles. Before detergent treatment, the DNA polymerase was precipitated by serum containing anti-HB surface antigen (anti-HB_s) but not with serum containing anti-HB core antigen (anti-HB_c). After detergent treatment, the enzyme was precipitated by anti-HB_c- and not by anti-HB_s-containing serum. Highly purified 16- to 25-nm HBAg particles blocked only the precipitation of DNA polymerase in untreated HBAg preparations. The 110*S* structure with which the DNA reaction product remains associated in Nonidet P-40-treated preparations was identified as Dane particle core by immunoprecipitation with serum containing anti-HB_c. The DNA polymerase and the radioactive DNA reaction product were used as markers for core in immunoprecipitation tests for anticore. In such assays, 8 of 11 human sera with anti-HB_s activity and all of 10 sera from chronic HBAg carriers were found to contain anti-HB_c activity.

Although the hepatitis B surface antigen (HB_sAg) has properties of a viral antigen, the most common forms carrying the antigen in serum (16- to 25-nm particles) (3) do not appear to be complete virions (1, 4). A larger (42 nm) particle with more complex structure that is more consistent with that of a virus was first described by Dane et al. (4) and is present in much lower concentration in serum than are the 16- to 25-nm particles. The Dane particle has been shown to share HB_sAg with the 16- to 25-nm particles by immune-electron microscopy using sera with anti-HB_s activity (1). Almeida et al. (2) used detergent to prepare core structures from Dane particles and showed by immune-electron microscopy that such cores were aggregated by a human convalescent serum without detectable anti-HB_s activity, thus demonstrating a second antigen antibody system in hepatitis B.

Recent work in this laboratory has demonstrated a DNA polymerase in preparations of hepatitis B antigen (HBAg) concentrated from serum (8). No enzyme activity was found to be associated with highly purified 16- to 25-nm HBAg particles, but after sucrose density gradient sedimentation of concentrated Dane particle-rich HBAg preparations the enzyme activity was found in the same region of the gradient as Dane particles detected by electron microscopy. After treatment of HBAg preparations with a detergent which disrupted the Dane particle and released the inner core, enzyme activity sedimented within the density range of core antigen (HB_cAg) detected by radioimmunoassay, but at a slightly higher density than the average for HB_cAg, suggesting that the enzyme might be associated with a subpopulation of Dane core particles.

The experiments described here provide more conclusive evidence that the enzyme is in fact associated with the Dane particle and its core. Before detergent treatment, the DNA polymerase in HBAg preparations was precipitated by serum containing anti-HB_s but not with serum containing anti-HB_c. After detergent treatment, the enzyme was precipitated by anti-HB_c- and not by anti-HB_s-containing serum.

(The nomenclature used here is that recommended by the Committee on Viral Hepatitis of the National Research Council-National Academy of Sciences [Center for Disease Control Morbidity and Mortality Weekly Report **23**:29, 1974].)

MATERIALS AND METHODS

Materials. [^{3}H]TTP (45 Ci/mmol) and [^{3}H]dCTP (26 Ci/mmol) were purchased from New England Nuclear Corp. Nonidet P-40 (NP-40) was a gift of the Shell Oil Co.

Preparation of DNA polymerase-containing particles from HBAg-positive human plasma. HBAg-P_2 preparations with DNA polymerase activity were prepared from Dane particle-rich human plasma by pelleting and washing particles in an ultracentrifuge and resuspending them in phosphate-buffered saline at 1/20th the original plasma volume as previously described (8). HBAg-S_3 preparations were further purified from P_2 preparations by twice banding DNA polymerase-containing particles in discontinuous 20%-over-65% (wt/vol) sucrose density gradients containing 0.01 M Tris-hydrochloride (pH 7.5), 0.10 M NaCl, and 1 mg of bovine serum albumin per ml (TNB buffer). Centrifugation was for 4 h at 40,000 rpm at 4 C in the Spinco SW40 rotor. The particles from the second sucrose gradient were then pelleted in a Spinco SW65 rotor at 50,000 rpm for 4 h at 4 C and resuspended in TNB at 1/40th the original plasma volume. HBAg-P_2 preparations from plasmas 595239, 644950, and 767932 were kindly provided by John Gerin, and plasma DD was supplied by Girish Vyas. Highly purified (5) 16- to 25-nm HBAg particles from plasmas DD (subtype adw) and Ytr (subtype ayw) were a gift from John Gerin.

Antisera. Serum 1, a convalescent serum from a patient with hepatitis B and first shown by Almeida et al. (2) to contain anti-HB_c activity, was kindly provided by John Gerin. Eleven human sera known to contain anti-HB_s activity were from patients who had had hepatitis B or who had received multiple transfusions with blood products. Goat anti-human gamma globulin (anti-HGG) was kindly provided by Thomas Edgington.

Assay for DNA polymerase. DNA polymerase reactions were carried out as previously described (8), and acid-precipitable radioactivity was determined after spotting samples on paper disks and washing and counting as previously described (10).

RESULTS

Effect of sera with anti-HB_s and anti-HB_c activities on DNA polymerase activity. Two human convalescent sera were tested for ability to inhibit the HBAg-associated DNA polymerase reaction. Anti-HB_c-containing serum 1 was negative for anti-HB_s by passive hemagglutination (Table 1). Serum 12 had a passive hemagglutination titer for anti-HB_s of $1:64 \times 10^6$ (Table 1). Neither serum when incubated in reaction mixtures significantly inhibited the incorporation of [^{3}H]TTP into an acid-insoluble form by the enzyme (Table 2).

Immunoprecipitation of DNA polymerase activity. The same two sera were tested for ability to react with the enzyme-containing particle in a double-antibody precipitation test (9) using goat anti-HGG as the precipitating antibody. Figure 1A shows a titration of anti-HB_s-containing serum 12 with an untreated HBAg-S_3 preparation from plasma DD and with the same HBAg-S_3 preparation treated with NP-40 under conditions shown to produce core structures from Dane particles (8). After removal of immune precipitates by centrifugation, supernatants were assayed for enzyme activity. Under the enzyme reaction conditions used, the reaction rate was shown to be limited by the enzyme concentration. Almost all of the enzyme in the untreated preparation was precipitated in the presence of serum 12 in dilutions less than 1:800, and 50% of the enzyme was precipitated at a dilution of 1:3,200. No enzyme was precipitated by serum 12 or by anti-HGG alone. None of the enzyme in the

TABLE 1. *Anti-HB_c activity in 11 anti-HB_s and 10 HB_sAg-positive human sera*

Serum	Anti-HB_s[a]	^{3}H product immunoprecipitated (%)[b]
1	<1:10	98
Anti-HB_s-positive:		
2	1:10	15
3	1:20	100
4	1:400	0
5	1:800	0
6	1:1,000	45
7	1:2,000	85
8	1:32,000	30
9	1:32,000	0
10	1:128,000	70
11	1:512,000	80
12	1:64,000,000	10
HB_sAg-positive:		
13		98
14		85
15		100
16		100
17		98
18		100
19		100
20		65
21		98
22		98
Normal subjects:		
23	<1:10	0
24	<1:10	0
25	<1:10	0
26	<1:10	0

[a] Highest serum dilution positive for anti-HB_s by passive hemagglutination (11).

[b] After a 3-h incubation of 70-μliter enzyme reactions made up as in Table 2 and including [^{3}H]TTP, [^{3}H]dCTP, 10 μliters of NP-40-treated HBAg-S_3 preparation 644950 and 25 μliters of a 1:50 dilution of each serum, 5 μmol of EDTA was added to stop the enzyme reaction, and goat anti-HGG (50 μliters) was added as in the procedure in Fig. 2. After a further 4-h incubation at 22 C, each sample was centrifuged at 10,000 × *g* for 10 min, and the supernatants were assayed for acid-precipitable radioactive enzyme product.

TABLE 2. *Effect of sera with anti-HB$_s$ and anti-HB$_c$ activity on DNA polymerase activity*[a]

Serum	Acid-precipitable ^{3}H counts/min
Normal human	
1:3	1,840
Anti-HB$_c$ (serum 1)	
1:3	1,975
1:30	1,775
1:300	1,785
Anti-HB$_s$ (serum 12)	
1:3	1,570
1:30	1,710
1:300	1,580
Normal human minus enzyme	
1:3	18

[a] Samples (25 μliters) of HBAg-P$_2$ preparation 767932 were mixed with 25 μliters of the indicated serum dilution. After 3 h of incubation at 22 C, the mixture was made 1% with NP-40 and 0.3% with mercaptoethanol. This mixture was then added to 100 μliters of a solution containing 10 μmol of Tris-hydrochloride (pH 7.5), 4 μmol of $MgCl_2$, 12 μmol of NH_4Cl, 0.05 μmol each of dATP, dCTP, and dGTP, and 26 pmol of [^{3}H]TTP (45 Ci/mmol), giving a final reaction volume of 160 μliters. The reaction mixtures were incubated at 37 C for 180 min, and 100 μliters from each was spotted on a paper disk for washing and counting as previously described (10).

detergent-treated preparation was precipitated by incubation with serum 12 in dilutions as low as 1:50 followed by anti-HGG. This experiment shows that antibody in the anti-HB$_s$-containing serum 12 reacts with the enzyme-containing particle before but not after treatment with NP-40, suggesting that NP-40 dissociates the enzyme from the antigenic determinant which reacts with serum 12.

Figure 1B shows a similar titration of the anti-HB$_c$-containing serum 1 with HBAg-S$_3$ preparations from both plasmas DD and 595239. Only the DNA polymerase activity in the NP-40-treated HBAg preparations and not that in the untreated preparations was precipitated after incubation with serum 1 and then anti-HGG. Neither serum alone precipitated NP-40-treated enzyme. The slopes of the curves for the two detergent-treated preparations are very similar, and their different positions with respect to the abscissa suggest that the two preparations contained different concentrations of enzyme-containing particles. This experiment suggests that the DNA polymerase is associated with Dane particle core structures in NP-40-treated Dane particle-rich HBAg preparations and that before detergent treatment very little enzyme activity is associated with free cores.

To investigate the specificity of the reactions with sera 1 and 12, the ability of highly purified 16- to 25-nm HBAg particles to inhibit the precipitation of DNA polymerase activity by each serum was tested. Figure 1C shows the results of an experiment using an NP-40-treated HBAgS$_3$ preparation from plasma DD with serum 1 and the same HBAg preparation without detergent treatment with serum 12. The highest concentration of purified 16- to 25-nm HBAg particles from plasma DD almost completely inhibited the precipitation of the enzyme in untreated HBAg preparations when incubated with serum 12. Purified 16- to 25-nm HBAg particles from plasma Ytr with an HB$_s$Ag subtype (ayw) different from that of HB$_s$Ag DD (adw) also appeared to inhibit this reaction. The precipitation of the NP-40-treated enzyme preparation by serum 1 was not inhibited at all by the purified HBAg particles from plasma DD. This experiment indicates that the enzyme-containing particles in untreated HBAg preparations were precipitated by anti-HB$_s$ and not by another antibody in serum 12. It also indicates that the precipitation of the enzyme activity in NP-40-treated HBAg preparations by serum 1 was not due to small amounts of anti-HB$_s$ in serum 1 reacting with residual HB$_s$Ag on the NP-40-treated enzyme-containing structures. The enzyme-containing particles in untreated HBAg preparations are thus antigenically like 16- to 25-nm HBAg particles, and after NP-40 treatment they are antigenically distinct.

Immunoprecipitation of the DNA polymerase reaction product. It was previously shown that after a DNA polymerase reaction with NP-40-treated Dane particle-rich HBAg preparations, the DNA reaction product remained associated with a 110*S* structure postulated to be the core of the Dane particle (8). To test this, an experiment was done to determine whether anti-HB$_c$-containing serum 1 would react with the 110*S* structure. Figure 2A shows the sedimentation in a sucrose density gradient of the radioactive product of a DNA polymerase reaction using HBAg-S$_3$ preparation 595239. The radioactive material with a peak in fraction 13 has a sedimentation coefficient around 110*S*. As previously described (8), the material in this band sediments heterogeneously. The small amount of radioactive material with a peak in fraction 3 sedimented to the top of a dense solution of CsCl layered on the bottom of the tube. The addition of normal human serum to the reaction mixture followed by anti-HGG after the reaction did not effect the sedimentation of the 110*S* material (Fig. 2B). The addition of human serum 9, which contained anti-HB$_s$ at

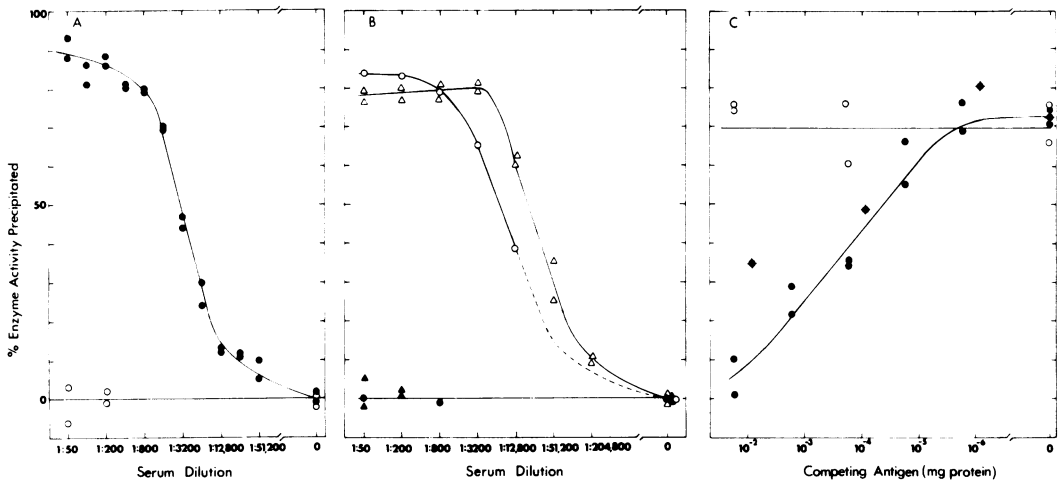

FIG. 1. *Precipitation of DNA polymerase activity with anti-HB$_s$- and anti-HB$_c$-containing sera. Samples (25 μliters) of the HBAg-S$_3$ preparations were mixed with either 10 μliters of water (closed symbols) or 10 μliters of 2.5% NP-40 (open symbols) and incubated at 37 C for 1 h. A 50-μliter amount of the indicated dilution of antiserum (dilutions beyond 1:50 were made in 1:50 normal human serum) was then added to each and the incubation was continued for 1 h at 22 C. A 75-μliter amount of a 1:6 dilution of anti-HGG was added, and the incubation was continued for 3 h at 4 C. After centrifugation at 2,000 × g for 10 min, 100 μliters of each supernatant was removed and made 1% with NP-40 and 0.3% with mercaptoethanol and assayed for DNA polymerase activity as described in Table 2, except that the [^{3}H]TPP concentration was increased fourfold and 168 pmol of [^{3}H]dCTP (26 Ci/mmol) was used in a reaction volume of 150 μliters. (A) An HBAg-S$_3$ preparation from plasma DD was incubated with dilutions of serum 12. (B) HBAg-S$_3$ preparations from plasma DD (circles) and 595239 (triangles) were incubated with dilutions of serum 1. (C) The designated amounts of purified 16- to 25-nm HBAg particles from plasma DD (closed circles) or Ytr (crossed circles) were incubated with 50-μliter samples of a 1:1,600 dilution of serum 12 for 1 h at 22 C before addition to 25-μliter amounts of the HBAg-S$_3$ preparation from plasma DD and completion of the experiment as described above. Purified 16- to 25-nm particles from plasma DD were similarly preincubated with samples of serum 1 at 1:3,200 before addition to NP-40-treated samples of HBAgS$_3$ from plasma DD (open circles).*

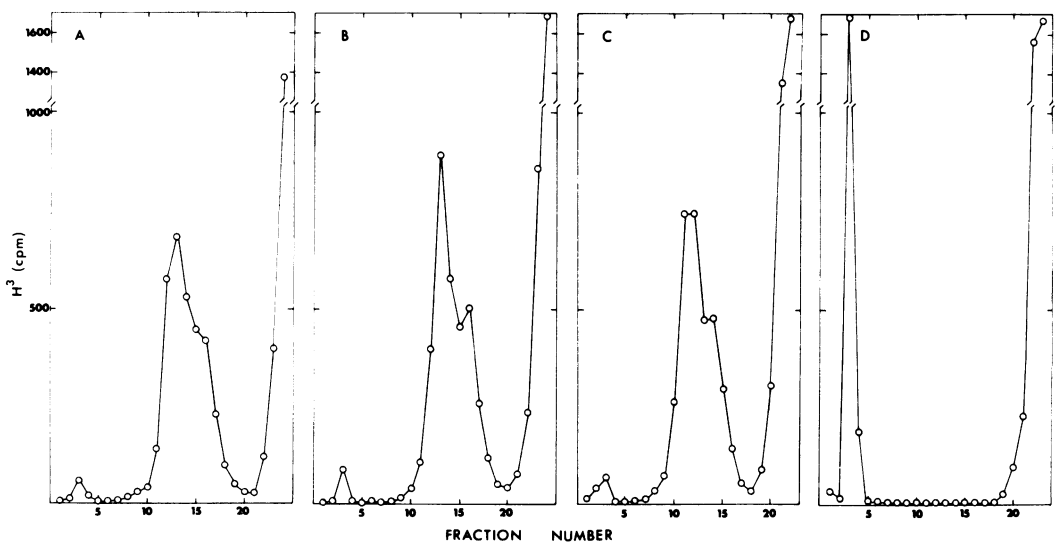

FIG. 2. *Precipitation of DNA polymerase ^{3}H reaction product with anti-HB$_s$- and anti-HB$_c$-containing sera. Samples (25 μliters) of an HBAg-S$_3$ preparation from 595239 were mixed with 25 μliters of a 1:50 dilution of calf serum (A), normal human serum (B), serum 9 (C), and serum 1 (D), and 2.5 μliters of 10% NP-40 and 2.5 μliters of 3% mercaptoethanol were added to each. A DNA polymerase reaction with [^{3}H]TTP as described in Table 2 was carried out with each mixture. After incubation at 37 C for 3 h, 6 μliters of anti-HGG was added to reaction mixtures B, C, and D, and all were incubated at 22 C for 30 min. Each was then layered on top of a 5 to 20% sucrose gradient containing 0.01 M Tris-hydrochloride (pH 7.5) and 0.1 M NaCl overlying 1 ml of a CsCl solution of density 1.45 g/ml and centrifuged in a Spinco SW40 rotor at 36,000 rpm for 85 min at 4 C. Acid-precipitable ^{3}H was determined in fractions collected from the bottom of the tube.*

1:32,000 by passive hemagglutination in such an experiment, similarly did not alter the sedimentation of the 110S material (Fig. 2C). However, when serum 1 was used in a similar experiment, 100% of the 110S material was precipitated and appeared at the top of the CsCl layer in fraction 3 (Fig. 2D). This experiment strongly suggests that the 110S structure is core material from the Dane particle.

Immunoprecipitation testing for anticore. The ultracentrifuge method for detecting immunoprecipitation of ^{3}H-labeled core was too tedious for testing large numbers of samples, and a more conventional immunoprecipitation test was found to give identical results. We took advantage of the observation that almost all of the acid-precipitable DNA polymerase reaction product remains associated with the 110S structure (shown in Fig. 2 to be core) after an NP-40-treated enzyme reaction (8). Thus, the [^{3}H]DNA product could be precipitated directly from the NP-40-treated enzyme reaction mixture by human serum containing anti-HB_c and anti-HGG. Table 1 shows the results of such a test for anti-HB_c with serum 1, which has been shown in other tests to contain anti-HB_c and no anti-HB_s activity, 11 sera (designated 2 through 12) with different titers of anti-HB_s activity, 10 sera (designated 13 through 22) from chronic HBAg carriers, and 4 sera from normal control subjects. Each serum was tested at a 1:50 dilution in a double-antibody precipitation test. The ^{3}H-labeled core was almost completely precipitated in the presence of serum 1 at a 1:50 dilution. Three of the eleven anti-HB_s-positive sera at a 1:50 dilution (4, 5, and 9) had no detectable anti-HB_c activity, and the other eight showed at least some anti-HB_c activity. The relative amount of anti-HB_c did not correlate with the anti-HB_s titer. Each of the 10 sera from chronic HBAg carriers at a 1:50 dilution was found to contain anti-HB_c activity. Eight of the sera resulted in precipitation of 98% or more of the ^{3}H-labeled core, and the other two gave 85 and 65% precipitation of the radioactivity, respectively. The four control sera from normal subjects had no anti-HB_c activity at a 1:50 dilution. Sera 1 through 12 were also assayed for anti-HB_c activity by the ultracentrifuge method shown in Fig. 2, and results almost identical to those in Table 1 were obtained.

DISCUSSION

Several lines of evidence now strongly indicate that the DNA polymerase found in Dane particle-rich HBAg preparations is a component of Dane particles and antigenically distinct cores prepared from Dane particles by detergent treatment. (i) Before detergent treatment, the DNA polymerase was precipitated by serum containing anti-HB_s and not by serum containing anti-HB_c (Fig. 1). The reaction with anti-HB_s-containing serum was blocked by highly purified 16- to 25-nm HBAg particles which have HB_sAg on their surface. (ii) After treatment with detergent NP-40, which disrupts Dane particles, the enzyme activity was precipitated by anti-HB_c- and not by anti-HB_s-containing serum (Fig. 1). The reaction with anti-HB_c was not blocked by purified 16- to 25-nm HBAg particles. (iii) After sucrose gradient sedimentation of HBAg preparations, the enzyme activity was found in the same position in the gradient as 42-nm Dane particles detected by electron microscopy (8). (iv) After NP-40 treatment, the DNA polymerase activity was found at the same position in a sucrose gradient as 28-nm Dane particle core structures detected by electron microscopy and radioimmunoassay (8). (v) No enzyme activity has been found in purified 16- to 25-nm HBAg particles (5, 8).

The observation that neither the serum with anti-HB_s activity nor that with anti-HB_c activity inhibited the enzyme activity (Table 2) suggests that either neither serum contains antibody against the enzyme itself or the enzyme is an internal component of the core and not available to large molecules such as antibody as previously postulated (8).

It was previously shown (8) that the DNA product of the DNA polymerase reaction remained associated with a 110S structure after its synthesis. Although associated with the 110S structure, the new DNA was not susceptible to digestion by DNase as it was after extraction with sodium dodecyl sulfate or phenol. This suggested that the enzyme product was in an internal or protected position within the 110S structure and not available to DNase. The experiment in Fig. 2 showing that the 110S structure is precipitated by anti-HB_c- and not by anti-HB_s-containing serum indicates that the 110S structure is the Dane particle core, and therefore the core contains both the DNA polymerase and the DNA enzyme product.

Immunoprecipitation of the [^{3}H]DNA-core complex can be used as a specific assay for anti-HB_c in sera. In such an assay, 8 of 11 human sera with anti-HB_s activity and all of 10 sera from chronic HBAg carriers were found to contain anti-HB_c activity. Purcell et al. (R. H. Purcell, J. L. Gerin, J. D. Almeida, and

P. V. Holland, submitted for publication), using a radioimmunoassay, and Hoofnagle et al. (7), using complement fixation, have also found anti-HB_c activity in some anti-HB_s-positive sera and in almost all sera from chronic carriers of HBAg. The assays used here for anti-HB_c appear to be significantly more sensitive than the complement fixation method of Hoofnagle et al. (7), who obtained a titer of 1:800 for anti-HB_c in the serum first described by Almeida et al. (2) (serum 1). A 1:24,000 dilution of the same serum (serum 1) resulted in precipitation of 50% of the ^3H-labeled core in HBAg preparation 595239, and detectable precipitation was observed in dilutions up to 1:200,000 (Fig. 1B). The sensitivity could easily be increased by increasing the specific activity of the ^3H-labeled core. The presence of anti-HB_c in the sera of all chronic HBAg carriers suggests that such patients are producing HB_cAg as well as HB_sAg. This idea is also supported by the finding of the core-associated DNA polymerase in unconcentrated sera of 50% of HBAg carriers (6).

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LITERATURE CITED

1. Almeida, J. D. 1971. Electron microscopic observations and speculations on Australia antigen. Postgrad. Med. J. **47**:484-487.
2. Almeida, J. D., D. Rubenstein, and E. J. Stott. 1971. New antigen-antibody system in Australia-antigen-positive hepatitis. Lancet **2**:1225-1227.
3. Bayer, M. E., B. S. Blumberg, and B. Werner. 1968. Particles associated with Australia antigen in sera of patients with leukemia, Down's syndrome and hepatitis. Nature (London) **218**:1057-1059.
4. Dane, D. S., C. H. Cameron, and M. Briggs. 1970. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet **1**:695-698.
5. Gerin, J. L. 1972. Isolation and physiochemical characterization of HBAg, p. 205-219. *In* G. N. Vyas, H. A. Perkins, and R. Schmidt (ed.), Hepatitis and blood transfusion. Grune and Stratton, New York.
6. Greenman, R. L., P. M. Kaplan, and W. S. Robinson. 1974. Evidence for the presence of a DNA polymerase in the core of the Dane particle of hepatitis B. Clin. Res. 22:125A.
7. Hoofnagle, J. H., R. J. Gerety, and L. F. Barker. 1973. Antibody to hepatitis-B-virus core in man. Lancet **2**:869-873.
8. Kaplan, P. M., R. L. Greenman, J. L. Gerin, R. H. Purcell, and W. S. Robinson. 1973. DNA polymerase associated with human hepatitis B antigen. J. Virol. **12**:995-1005.
9. Plow, E. F., C. Hougie, and T. S. Edgington. 1971. Neoantigenic expressions engendered by plasmin cleavage of fibrinogen. J. Immunol. **107**:1496-1500.
10. Robinson, W. S., and H. L. Robinson. 1971. DNA polymerase in defective Rous sarcoma virus. Virology **44**:457-462.
11. Vyas, G. N., and N. R. Shulman. 1970. Hemagglutination assay for antigen and antibody associated with viral hepatitis. Science **170**:332-333.