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Genetic Engineering Applied to the Development of Vaccines

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Genetic engineering applied to the development of vaccines

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[Plate 1]

The simplest application of the modern genetic manipulation methods to vaccine development is the expression in microbial cells of genes from pathogens that encode surface antigens capable of inducing neutralizing antibodies in the host of the pathogen involved. This procedure has been exploited successfully for development of a vaccine against hepatitis B virus (HBV) that is now widely used. Similar approaches have been directed towards formulations for immunization against several other animal and human diseases and some of these preparations are now presently in trials. Of no less importance is the impact of biotechnology in providing reagents for fundamental studies of topics such as the determination of virulence, antigenic variation, virus receptors and the immunological response to viral antigens. The core antigen of HBV is a good example of a product of genetic engineering that is a valuable diagnostic reagent, and that is finding important use in immunological studies of particular pertinence to vaccine development.

Introduction

Man and other animals survive the relentless assault of a large array of microbial pathogens because they have evolved mechanisms that enable them not only to recover from initial attacks and purge themselves of the offender, but also to remember sufficient information about the molecular morphology of an individual aggressor that they are able to repulse further intrusions very quickly and effectively. Through this combination of exposure to a pathogen and clearance and recollection of it, the animal becomes immune to that particular pathogen.

Vaccination is dependent upon the memory of the immune system and seeks to add a particular piece of information to the animal's immunological database without exposing the recipient to the pathogenic effects attending normal infections. Some very effective vaccines against viruses have been made by traditional procedures involving killed preparations of the virus or attenuated or avirulent forms of the virus, which may arise during repeated passage of the wild-type virus through animal hosts or cell lines in culture. Inoculation of an individual with such preparations will normally prime or stimulate the immune system to counter subsequent exposure to the wild-type virus, as this will have the same appearance as the immunizing preparation. In preparing such vaccines killing of the virus culture must obviously be complete and the avirulent strain unable to revert to virulent. Many serious diseases have

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been controlled in this way, and smallpox has been essentially eliminated by virtue of the World Health Organization vaccination programme.

A virion lacking its genome would offer a particularly safe means of vaccination if it could be made, but particles or shapes simulating the viral surface offer an equivalent means of stimulating immunological memory without the potential, however remote, for pathogenic effects inherent in the use of a complete virus. Preparations of particulate envelope proteins, or surface antigens, are often referred to as 'subunit vaccines'. An outstandingly successful subunit vaccine is that developed against hepatitis B virus (HBV) from extensively purified preparations of the surface-antigen particles of the virus from the plasma of infected individuals (Szmuness *et al.* 1980).

The general area of vaccine development was clearly an early and attractive choice for the application of genetic engineering (Murray 1980). The wide range of systems for gene cloning and expression together with the development of rapid methods for nucleotide sequence determination and for oligonucleotide synthesis, and with them opportunities for introduction of mutations at any desired position in a DNA molecule, have provided the means of manipulating genomes with relative ease. In principle these methods could be applied in the development of attenuated live vaccines (which have important practical advantages in ease of application and low cost) once the molecular basis of virulence is understood. This topic is in its infancy, but the recent identification of a single nucleotide change in a non-coding region of the polio type III virus genome that distinguishes virulent and avirulent forms of the virus provides encouragement for such approaches (Evans *et al.* 1985). However, the expression of viral gene products in microbial or animal cells in culture offers an alternative to killed or attenuated viral preparations as a source of raw material for vaccine formulation. This is because recombinant DNA technology has now developed to a stage where cloning and expression of genes are commonplace, and with sufficient application there are probably few proteins that cannot be produced in quite large quantities and in a high degree of purity, although actual levels of expression and problems attending purification of any individual protein vary widely. These procedures have now been used to produce a number of viral and other antigens for vaccine formulations and effective vaccines against hepatitis B made in this way have been approved and are in widespread use.

Surface antigens of viruses have been the principal focus of most attempts to make viral vaccines by genetic manipulation approaches, but antibody formation to other components is well known and the significance of the immunological response to internal viral proteins, particularly in relation to the cell mediated response, is being increasingly recognized. Hepatitis B virus will be used here to illustrate both the successful development of a viral envelope protein into a vaccine against a major disease worldwide (and indirectly against liver cancer), and the importance of other viral antigens in immunity and their potential application in the control of infection.

Hepatitis B virus

The causative agent of hepatitis B, or serum hepatitis, is a roughly spherical virion with a diameter of 42 nm, often referred to as the Dane particle (Dane *et al.* 1970), which is found in the liver and blood of acutely infected individuals. It is transmitted sexually or parentally and poses a major threat to public health worldwide for a carrier state may follow infection and long term sequelae can then include cirrhosis and development of primary liver cancer. The

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extensive literature on clinical and molecular biological aspects of hepatitis B is summarized in a number of recent reviews and symposia volumes (Vyas *et al.* 1978; Szmuness *et al.* 1982; Vyas *et al.* 1984; Murray 1987; Ganem & Varmus 1987; Zuckerman 1988). The virus has a very narrow host range, infecting only man and a few other higher primates, of which the chimpanzee is the best known and has been widely adopted for investigational purposes.

The human virus may be regarded as the prototype of a family of viruses now known as the Hepadna Viridae, for similar viruses have been found in a number of species, but in each case the virus is narrowly confined to a specific host. In the blood of infected individuals the virus is usually accompanied by a large excess of particles of its capsid protein, or surface antigen (HBsAg), which may be in the form of 22 nm diameter spherical particles or as filaments. Treatment of the virus with mild detergent removes the envelope to leave a nuclear capsid or core particle with a diameter of 27 nm; such particles are also found in the liver of infected individuals but not in the blood. HBsAg particles consist of two principal components, one (gp27) being a glycosylated form of the other (p24), but larger polypeptides with amino acid sequences overlapping those of these two smaller components (and having the same carboxy terminus) have now been recognized as authentic minor components of both the spherical and filamentous forms of the surface-antigen particles and of Dane particles (Heermann *et al.* 1984).

The nucleocapsid or core particles have one major protein component, the core antigen (HBcAg), which surrounds the viral genome, a small DNA molecule of rather unusual structure. A DNA-dependent DNA polymerase activity is associated with the virus and the

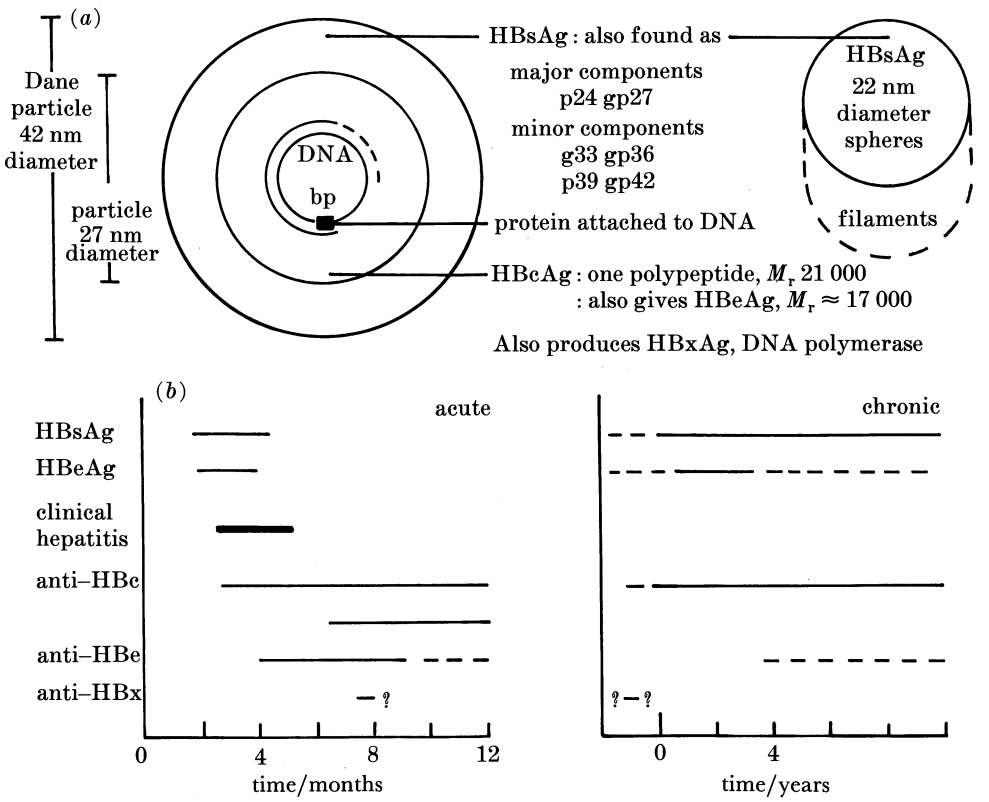

FIGURE 1. (*a*) The components of HBV particles. (*b*) The appearance of HBV antigens, and antibodies to them, in serum during acute and chronic infections. There are wide variations in the time at which the various markers are found in individual cases; this is indicated in part by the broken lines. (From Murray 1987.)

core particles, and another viral antigen, the e antigen (HBeAg), is found in various forms, but has been shown to be a derivative of the core antigen (MacKay *et al.* 1981*a*) The virus and its components are represented diagrammatically in figure 1, which also illustrates the manifestation of infection by the virus in terms of appearance in serum of antigens and antibodies to them in both acute and chronic infections (Murray 1987).

Production of HBV antigens in microbial cells

The limited host range of the virus means that HBV cannot be propagated in laboratory animals and until very recently it could not be produced in cell culture (Sureau *et al.* 1986), which, given the clinical importance of the virus, made it an early candidate for molecular cloning with particular respect to the production of antigens for both diagnostic and vaccine development. Given these objectives, our initial cloning strategy was based upon the insertion of fragments of the viral DNA into the β-lactamase gene carried on the *Escherichia coli* plasmid pBR322 so that immunologically active segments of viral gene products could be detected directly by solid-phase immunochemical methods (Broome & Gilbert 1978). These procedures, which are now well known, rapidly led to the expression of HBcAg in *E. coli* (Burrell *et al.* 1979) and to the generation of a series of cloned HBV DNA fragments from which the nucleotide sequence of the viral genome was determined (Pasek *et al.* 1979; Pugh *et al.* 1986) and which showed the genetic organization of the virus (figure 2). Although the initial yield of HBcAg was not high, it was shown to be highly immunogenic (Pasek *et al.* 1979) and was found to be particulate and when examined in the electron microscope after immunoprecipitation (figure 3*a*, plate 1) it was morphologically virtually identical with HBcAg isolated from the liver of an acutely infected patent (Cohen & Richmond 1982). Further manipulation of the cloned coding sequence for HBcAg gave much higher yields of the antigen in *E. coli* (figure 3*b*) (Stahl, *et al.* 1982) and the product has been successfully developed for diagnostic kits that are now very widely used for detection of antibodies to HBcAg, which not only reflect infection with HBV but also serve as a surrogate marker for hepatitis non-A-non-B (Koziol *et al.* 1986).

HBsAg and vaccination against HBV

Although HBsAg expression was observed in *E. coli* by following the procedures used for HBcAg expression (Burrell *et al.* 1979), yields were very poor; the material was immunogenic (MacKay *et al.* 1981*b*), but in spite of considerable effort in several laboratories effective systems for production of HBsAg in *E. coli* have yet to be made. However, the detailed information from the DNA sequence of HBV enabled the coding sequence for HBsAg to be manipulated in expression vectors that function in the yeast *Saccharomyces cerevisiae*, an organism widely used in large scale fermentation. HBsAg was successfully expressed in yeast strains (Valenzuela *et al.* 1982; Miyanohara *et al.* 1983; Murray *et al.* 1984) and although heterogeneous in cell extracts the product assembles to form particulate structures (figure 3*c*) closely resembling the 22 nm particles of HBsAg found naturally in the plasma of infected individuals. Such preparations proved to be immunogenic and protected chimpanzees against challenge with a high dose of the virus (Murray *et al.* 1984). HBsAg is now in routine production from transformed yeast strains on a large scale, and following extensive trials in human volunteers (Davidson & Krugman 1985; Ichida *et al.* 1988) preparations have now

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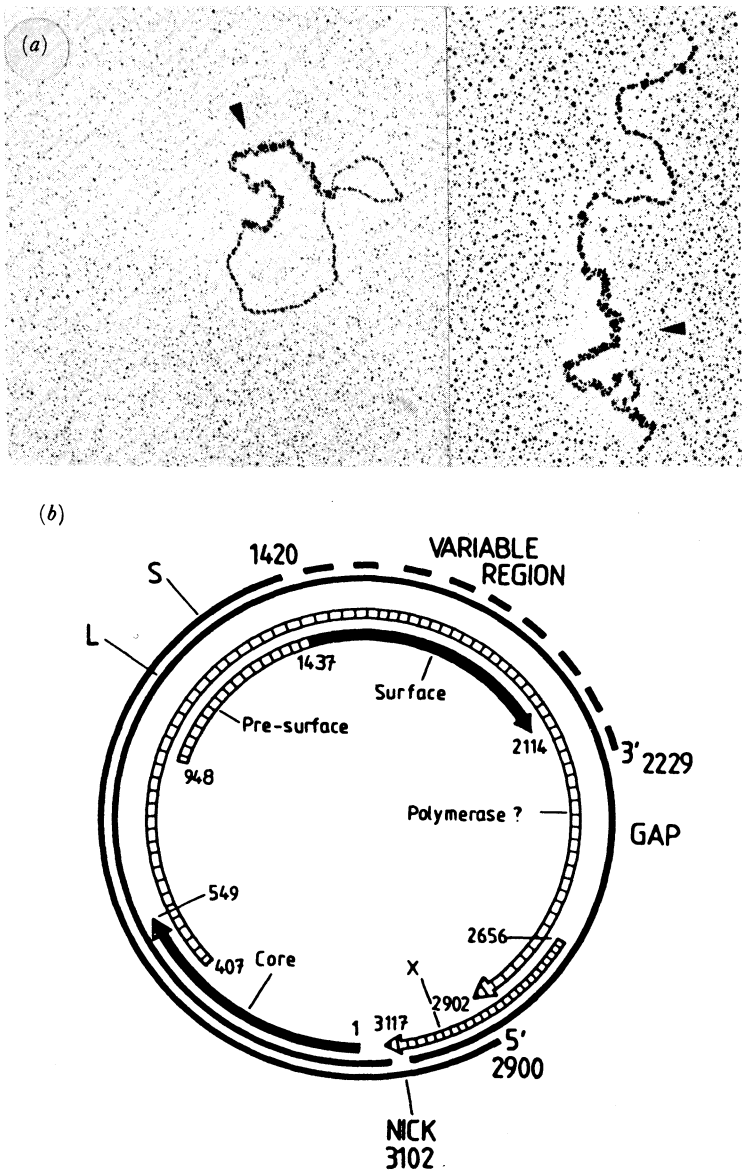

FIGURE 2. The HBV genome. (*a*) Electron micrographs of DNA extracted from Dane particles and complexed with bacteriophage T4 gene 32 protein, which binds specifically to single-stranded DNA, indicated by the arrowhead. Left, intact molecules, and right, DNA preparations digested with restriction endonuclease *Eco*RI (for which this particular DNA contained one target). These photographs were kindly provided by Dr Hajo Delius. (*b*) The genome of HBV. Heavy lines denote the DNA strands, the broken line showing the region of variable length of the short strand. Arrows represent the four open reading frames (as coding sequences) with the numbers of initiation and termination triplets in the system adopted by Pasek *et al.* (1979).

been officially approved for routine use as a vaccine in humans; several accounts of clinical aspects of these studies are to be found in Zuckerman (1988).

In the context of this symposium it is perhaps appropriate to note that the cloning and expression of HBV genes in microorganisms has not only been of scientific interest and social benefit, but also (like α-interferon) a significant commercial success. The world wide markets for diagnostic reagents for detection of viral hepatitis and vaccines against HBV at present are almost U.S. $500 M annually, and in a recent press release Mr James Vincent, the chief

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executive officer of Biogen Inc. (the sponsor of our research on HBV) stated that 'Biogen's hepatitis B licensing program, which includes both vaccine and diagnostic products, will yield a base $10 million per year in product-driven revenues which will expand as the use of recombinant products increases'. Although it is a personal regret that this project has not been of direct value to a British company, it is gratifying that my University will be a financial beneficiary of it.

HBcAg and its role in immunization

Although the development of vaccines against HBV has centred upon the use of HBsAg, other immunological determinants are carried upon the surface of Dane particles, namely HBeAg, which is a derivative of HBcAg, and the relatively recently demonstrated pre-S epitopes (Neurath *et al.* 1985). HBcAg is seldom found as the free antigen in the serum of infected individuals, but it is manifest as HBeAg, and antibodies of HBcAg appear very early after infection and before those against HBeAg or HBsAg. Further, HBcAg rather than HBsAg is the antigen found on the surface of hepatocytes in patients with chronic infections and is the antigen to which lymphocytes are sensitized, suggesting a role for HBcAg in cell-mediated immunity (Vento *et al.* 1985). Given the ready availability of HBcAg through genetic engineering methods, the potential of this antigen for immunization could be assessed directly even though this had been generally discounted because the antigen is buried within the virus.

Chimpanzees were therefore immunized with HBcAg on aluminium hydroxide adjuvant and challenged with a high intravenous dose of the virus following the anticipated serum conversion (Murray *et al.* 1984, 1987). The results of two experiments are summarized in figure 4. In the first, one of the animals (Rinka) developed a short-lived and low-titre HBsAg antigenaemia, but no detectable HBeAg, and rapidly produced antibodies to both of these antigens at high titre. The other (Gwen) showed no detectable antigenaemia and at first appeared to have been completely protected against HBV, but about five months after the challenge the anti-HBe titre began to increase and anti-HBs was also produced showing that the animal had, in fact, suffered a very mild, serologically undetectable infection. The HBcAg used in this experiment had some HBeAg reactivity and the animal (Gwen) that had been essentially immune to the challenge inoculation had in fact developed a low anti-HBe titre as a result of the immunization with HBcAg, whereas her partner (Rinka) had not and showed the early evidence of a mild infection (figure 4). In the second experiment the HBcAg was again given on aluminium hydroxide adjuvant, but was first treated with sodium dodecyl sulphate to generate an enhanced HBeAg reactivity and one of the animals (Brigitte) was then found to be completely protected against the challenge infection. The second animal (Ling), however, again appeared to have suffered a very mild infection, for a high level of anti-HBs and an enhanced titre of anti-HBe developed very rapidly after inoculation with the virus, even though there had been no HBeAg and only extremely low levels of HBsAg for a very short period (figure 4).

These results, together with similar observations by Iwarson *et al.* (1985) with the same antigen preparations, were taken to indicate a substantial degree of protection against infection (Murray *et al.* 1984, 1987). The expression in *E. coli* of two other HBV genes, those for the X-antigen (Pugh *et al.* 1986) and the DNA polymerase of the virus (McGlynn & Murray 1988) provided reagents for the detection of antibodies to these two viral products in the sera of infected individuals or animals. The appearance of these antibodies is an additional indicator

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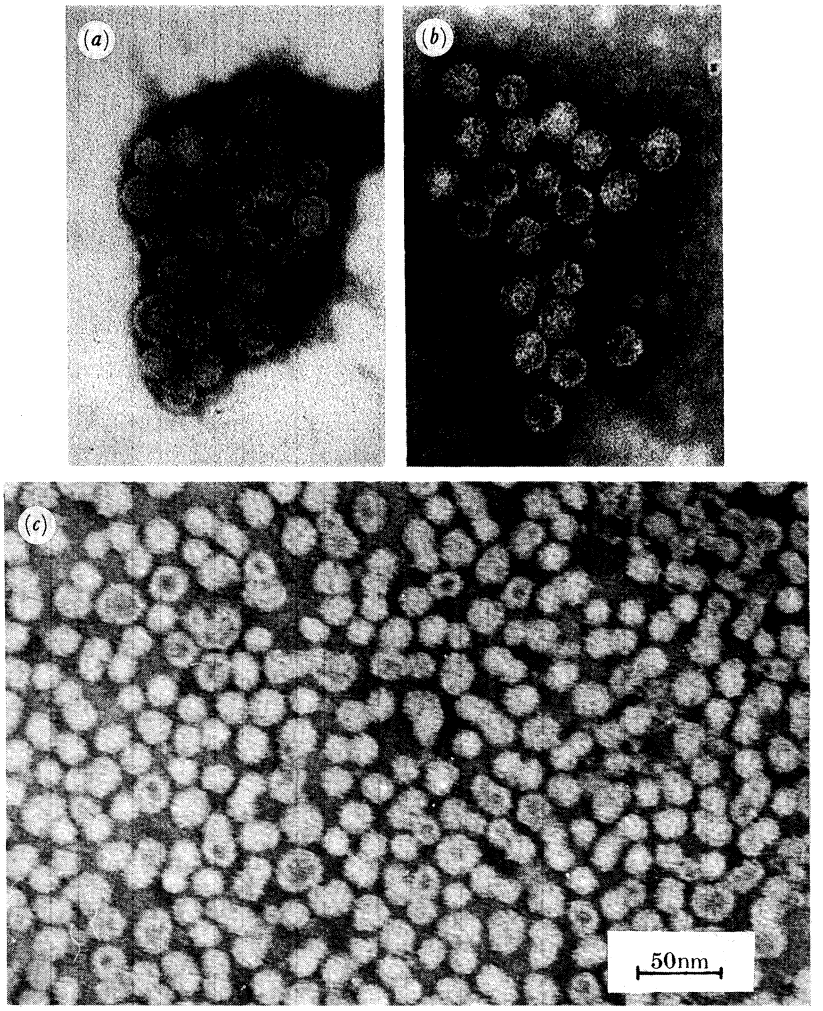

FIGURE 3. (*a*, *b*) Particulate forms of HBcAg: (*a*), isolated from the liver of an acutely infected patient; (*b*), synthesized in *E. coli*. These electron micrographs were kindly supplied by Dr Joan Richmond and are reproduced from Cohen & Richmond (1982), with permission from the authors and Macmillan Journals Ltd. (*c*) HBsAg particles purified from genetically engineered yeast. The electron micrograph was kindly provided by Dr H. Arimura and Dr T. Suyama, Green Cross Corporation, Osaka.

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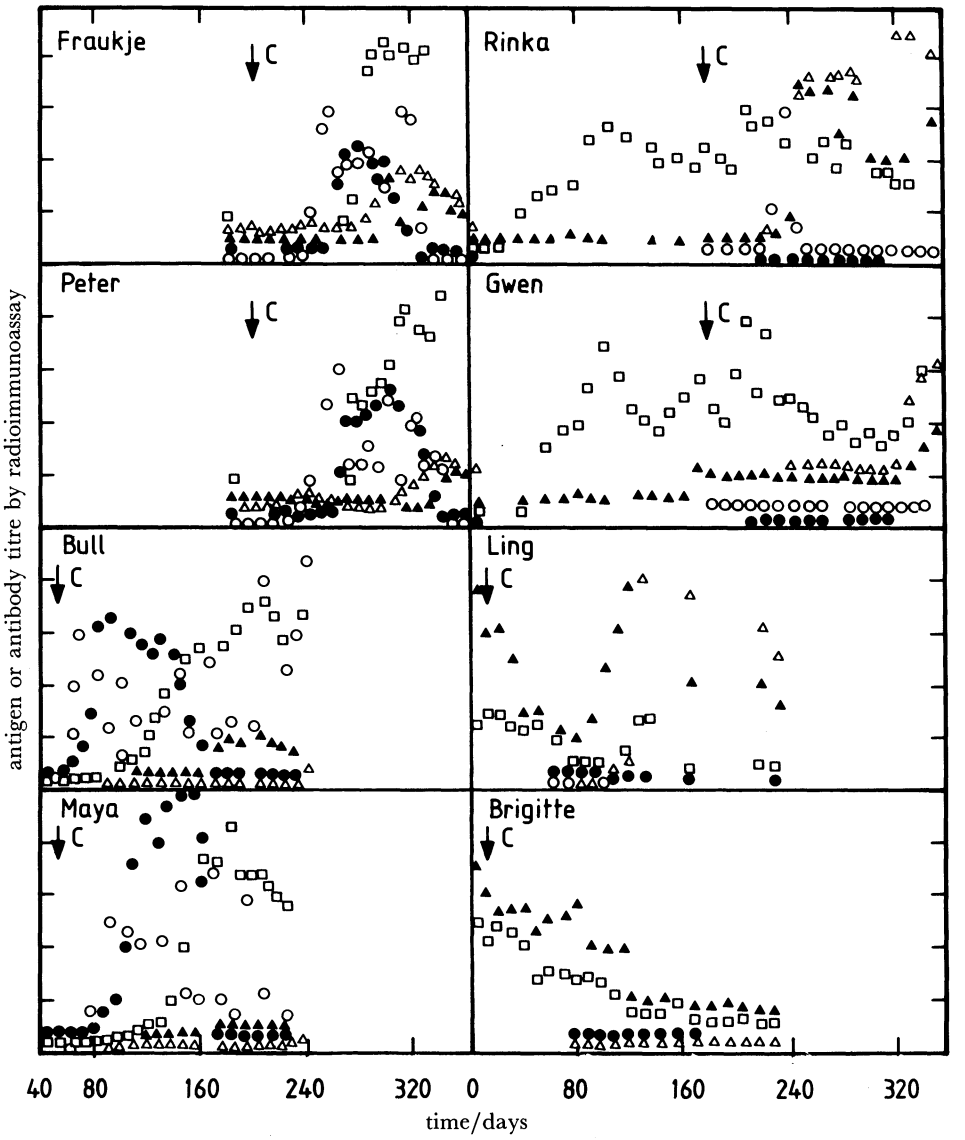

FIGURE 4. Serological analysis on four chimpanzees immunized with HBcAg (right-hand panels) and four unvaccinated control animals (left-hand panels). □, Anti-HBc; ○, HBsAg; △, anti-HBs; ●, HBeAg; ▲, anti-HBe. See also table 1. (Arrow C indicates challenge with HBV.)

of viral replication following infection, and analysis of the sera of both immunized and control animals for these markers before and after inoculation with the virus shows that they were present only in the four control animals that exhibited the normal pattern of infection (table 1). This would suggest that in the four animals immunized with HBcAg there was little or no replication of the virus administered as the challenge, even in the two (Rinka and Ling) that generated high anti-HBs and anti-HBe responses (Murray 1988).

It is particularly interesting to examine these results in the light of the recent observations of Milich *et al.* (1987*a*, *b*) on the immune response of inbred strains of mice to HBcAg and to synthetic oligopeptides contained within the amino acid sequences of HBcAg. A single inoculation of HBcAg in Freund's complete adjuvant induced quite different antibody responses in various strains of mice and so a series of ten synthetic oligopeptides (figure 5) was

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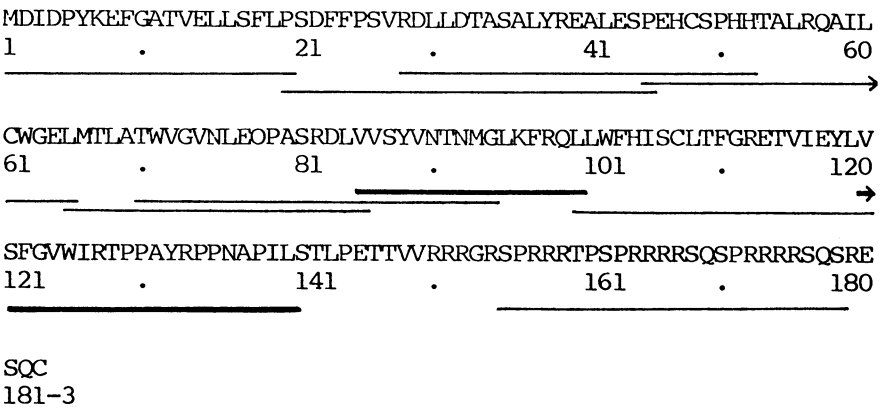

FIGURE 5. The amino acid sequence of HBcAg in the single-letter code. Sequences underlined are those of the synthetic peptides used by Milich *et al.* (1987*b*), heavy underlying indicates those most active in T-cell priming.

TABLE 1. THE OCCURRENCE OF HBV ANTIGENS AND ANTIBODIES TO THE ANTIGENS IN CHIMPANZEE SERUM SAMPLES BEFORE (b) AND AFTER (a) INTRAVENOUS INOCULATION WITH A CHALLENGE DOSE ($10^{3.5}$ I.D.$_{50}$) OF HBV SEROTYPE *ayw*

(I.D.$_{50}$ is the minimum dose required to produce a serologically detectable infection in 50% of a group of animals inoculated with the batch of virus.)

unvaccinated (control) animals

chimpanzee...	Fraukje		Peter		Bull		Maya	
antigen or antibody	b	a	b	a	b	a	b	a
HBsAg	−	+	−	+	−	+	−	+
HBeAg	−	+	−	+	−	+	−	+
anti-HBc	−	+	−	+	−	+	−	+
anti-HBs	−	+	−	+	−	+	−	+
anti-HBe	−	+	−	+	−	+	−	+
anti-HBx	−	+	−	+	−	+	−	+
anti-HBp	−	+	−	+	−	+	−	−

animals vaccinated with HBV antigens

	HBsAg (yeast)				HBcAg (*E. coli*)				HBcAg + HBeAg (*E. coli*)			
chimpanzee...	Dolf		Ianthe		Gwen		Rinka		Brigitte		Ling	
antigen or antibody	b	a	b	a	b	a	b	a	b	a	b	a
HBsAg	−	−	−	−	−	−	−	+	−	−	−	(±)
HBeAg	−	−	−	−	−	−	−	−	−	−	−	−
anti-HBc	−	−	−	−	+	+	+	+	+	+	+	+
anti-HBs	+	+[a]	+	+[a]	−	+[b]	−	+[c]	−	−	−	+[c]
anti-HBe	−	−	−	−	+	+[b]	−	+[c]	+	+[a]	+	+[c]
anti-HBx	−	−	−	−	−	−	−	−	−	−	−	−
anti-HBp	−	−	−	−	−	−	−	−	−	−	−	−

[a] No observable increase in antibody titre at any time after challenge with the virus.
[b] Antibody appeared or increased in titre, but not until some five months after challenge with the virus.
[c] Very early appearance of high-titre antibody or rapid and early elevation of antibody titre after challenge.

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used to search for sequences that would stimulate T-cell proliferation in groups of mice of defined H2 haplotype which had been primed with HBcAg. Two of the peptides (heavy underlining in figure 5) were similar to HBcAg itself in their effect on both antibody production *in vivo* and T-cell proliferation *in vitro* in specific strains of mice and some of the other peptides elicited these effects to a lesser extent in other strains. Further, it was found that these particular peptides could substitute for HBcAg in priming for the proliferation of T cells on subsequent stimulation with HBcAg or the peptide. With two strains, B10.S and Balb/c, the analysis was extended by using HBV (which cannot replicate in the mouse) for challenge after priming with HBcAg or peptides 85–100 or 120–140, and this led to the production of antibodies to HBsAg (including pre-S epitopes) as well as anti-HBc. However, when HBV was replaced as the challenge inoculation by a mixture of HBcAg and HBsAg particles, no antibodies were generated against any of the HBsAg or pre-S epitopes, although anti-HBc was produced as usual (Milich *et al.* 1987*b*), thus demonstrating the necessity for physical linkage of the challenging epitope to the molecule or particle used for priming.

These exciting results obtained in mice clearly have significance for vaccine development if they can be extrapolated to man, for a range of immunological targets can be physically linked to HBcAg and this antigen can be produced readily in *E. coli* (Stahl *et al.* 1982). The results of our experiments with chimpanzees immunized with HBcAg described above (figure 4) certainly encourage this view. One of the four animals was completely protected and another suffered an infection too mild for detection, but which left the characteristic imprint of HBV upon the animal's immune system in that anti-HBs and anti-HBe appeared at the normal titre after infection. The other two animals (Rinka and Ling) showed early and strong responses in anti-HBs production and anti-HBe elevation which it is attractive to attribute to priming of T-helper cells by the HBcAg inoculations, by analogy with the observations of Milich *et al.* (1987*b*) in mice. None of the four animals developed antibodies to the X antigen or polymerase, which may be regarded (like HBsAg) as markers of viral replication and which were produced by the control animals (table 1). That only two of the four immunized animals responded in this way may reflect differences in histocompatibility types among outbred animals and the use of the less potent adjuvant, aluminium hydroxide.

Gene fusions and derivatives of HBcAg

Expression of the coding sequence of HBcAg in *E. coli* gives a highly immunogenic product (Pasek *et al.* 1979) that assembles to form particles that are virtually indistinguishable from natural core particles (Cohen & Richmond 1982). In manipulating the original recombinant plasmids for elevation of expression levels in *E. coli*, sequences corresponding with the *N*-terminus of the antigen were changed so that the first two amino acids of HBcAg were replaced by the first eight residues of *E. coli* β-galactosidase and a linker sequence, Glu-Phe-His (Stahl *et al.* 1982). This change had no observable adverse effect upon assembly of the molecules into particles or the immunogenicity of particles, which encouraged the view that HBcAg may enhance the antigenicity of other polypeptide sequences linked to it as a result of genetic fusions.

Our early attempts to fuse the coding sequence for HBsAg in its entirety or in halves at or near the 3′ end of the coding sequence for HBcAg resulted in the expression of products that displayed HBcAg antigenicity in radioimmunoassays, but showed no cross reactivity with anti-

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HBs in either the Abbott 'Ausria' test or in double antibody radioimmunoprecipitation assays (S. Stahl and K. Murray, unpublished work). Following the demonstration that HBcAg synthesized in *E. coli* could be converted into HBeAg (MacKay *et al.* 1981*a*), the products of a series of deletions from the HBcAg coding sequence were examined with respect to their antigenic, immunogenic and biophysical characteristics (S. Stahl, P. Wingfield, B. Fearns, R. S. Tedder and K. Murray, unpublished work). The protamine-like carboxy-terminal region of the antigen could be removed without impairing assembly of particles and a number of gene fusions were therefore made that would yield a range of different epitopes fused to the carboxy terminal region of a truncated form of HBcAg; some of these are described as their translation products in figure 6.

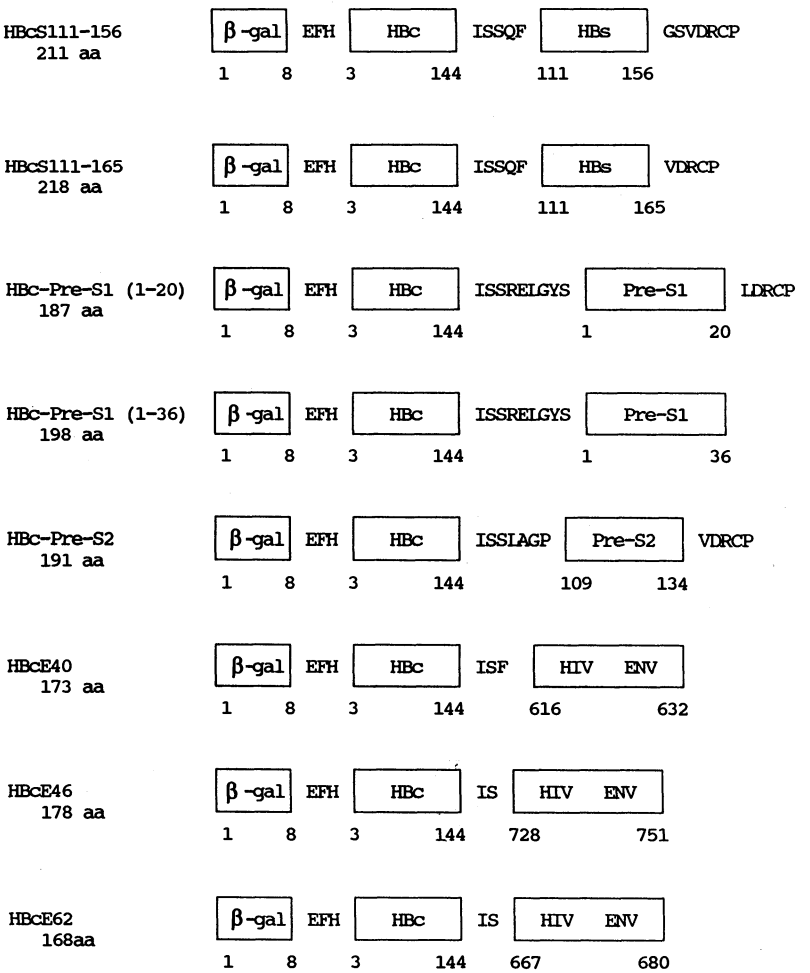

FIGURE 6. Predicted amino acid sequences of HBcAg fusion proteins. The names and total amino acids of the proteins are on the left. The numbers below the boxes indicate the positions of the amino acids in the original protein. HIV ENV, HIV envelope protein (GP160).

All of the polypeptides shown in figure 6 were produced in reasonably good yield in *E. coli* and formed particles that simplified their recovery from cell extracts. They exhibited strong antigenic activity in their cross reactions with anti-HBc and anti-HBe, and were good immunogens. In addition to high titres of anti-HBc, sera from immunized rabbits were shown to have high titres of antibody to the epitope contained in the added carboxy-terminal region

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in the case of the pre-S1 and pre-S2 constructions. The fusions of the HBsAg segments to HBcAg were particularly interesting, they showed no antigenic behaviour in standard radioimmune or ELISA tests for HBsAg, but they nevertheless elicited an antibody response in rabbits (reminiscent of the products of direct expression of HBsAg sequences in *E. coli* (MacKay *et al.* 1981*b*)), giving sera that displayed high titres of antibodies against HBsAg either on solid phase or in solution (Stahl & Murray, 1989).

The HBcAg derivatives carrying the short sequences from the envelope protein of human immunodeficiency virus (HIV) were examined for their immunogenicity in Balb/c mice and one of them (HBcAg-E46) stimulated production of antibodies against both HBcAg and the HIV peptide. The sequence of HIV envelope carried by this fusion protein is 728–751; antibodies raised against a synthetic peptide comprising amino acid residues 735 to 732 have been found to precipitate the native gp160 protein of HIV-1 (Kennedy *et al.* 1986) and to neutralize the virus (Chanh *et al.* 1986; Dalgleish *et al.* 1988).

The results of these experiments, and a somewhat similar one by Clarke *et al.* (1987) in which a peptide corresponding to amino acid residues 137–162 of the coat protein of foot and mouth disease virus (FMDV) was fused to the amino terminus of HBcAg via an appropriate genetic construction, give credence to the anticipation that fusion of antigenic peptide sequences to HBcAg is likely to constitute a general method for potentiation of the immune response to the additional antigenic or immunogenic site. Where this also enhances priming of T-cell memory, the fusion proteins should have potential for development of vaccines and offer the prospect of producing single molecules that serve as an effective multivalent vaccine against HBV and other infectious agents.

Alteration of immunological specificity by mutation

There are a number of serological variants of HBsAg. Most isolates display the dominant *a* epitope and two sets of subtype epitopes described as *d* or *y* (Le Bouvier 1971) and *w* or *r* (Bancroft *et al.* 1972), which are believed to be to a large extent mutually exclusive so that the four serotypes generally encountered are *adw*, *adr*, *ayw* and *ayr*. Most of the antibodies induced by HBsAg are against the *a* epitope, and subtypic antisera are said to contribute little to protective immunity (Murphy *et al.* 1974; McAuliffe *et al.* 1980).

Several publications describe the use of synthetic peptides representing predicted antigenic regions of the HBsAg sequence to generate antisera which were then used in serological reactions with HBsAg of defined serotype. These experiments, which are summarized in figure 7, indicate that the dominant *a* epitope lies between residues 138 and 149 and that the region between residues 110 and 139 is involved in specification of the *d* or *y* subtype. However, modification of the conformation of HBsAg by reduction or treatment with detergent greatly reduces its immunoreactivity, suggesting that distinct peptide domains collectively constitute discontinuous epitopes (Benjamin *et al.* 1984; Dreesman *et al.* 1973; Sukeno *et al.* 1972; Vyas *et al.* 1972). Furthermore, some *y*-specific and *d*-specific monoclonal antibodies fail to cross react with these oligopeptides (Swenson *et al.* 1988) indicating that *d*- and *y*-antigenicity may, like *a*-antigenicity (Tedder *et al.* 1984) be governed by more than one epitope.

To identify residues that are critical for antigenicity and to define the *a*, *y*, and *d* epitopes more precisely we have made a number of mutants in HBsAg at residues that we believed, on the basis of a comparison of amino acid sequences of HBsAg of different serotypes, to correlate

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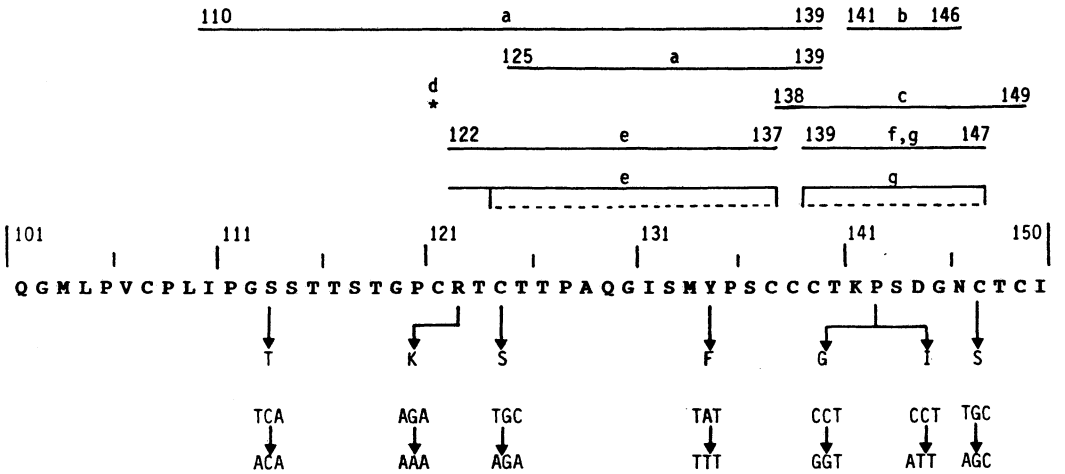

FIGURE 7. The major antigenic region of HBsAg. Lines above the sequence denote synthetic peptides used to locate the *a* epitope region: a, Gerin *et al.* 1983; b, Hopp and Woods 1981; c, Prince *et al.* 1982; e, Dreesman *et al.* 1982; f, g, Bhatnagar *et al.* 1982 and Brown *et al.* 1984. d is a point mutation with little effect upon *a* reactivity (Antoni & Peterson 1988). The amino acid substitutions and the mutations used to make them are indicated below the sequence.

with the display of the appropriate antigenic subtype. These mutants are defined in figure 7; four of them, at residues 124 (cysteine), 142 (proline) and 147 (cysteine) were made for studies of the *a* epitope, and the others, involving residues 113 (serine), 122 (arginine) and 134 (tyrosine), were made to explore the *y* versus *d* subtype determination.

Titration of the wild-type and mutant HBsAg preparations (obtained from appropriately transformed yeast strains) with anti-HBs showed that replacement of cysteine 124 or 147 by serine drastically reduced cross reactivity with the antibodies, with the change at 147 virtually eliminating it. The proline at 142 was shown to be essential for full antigenicity, but replacement by glycine impaired cross reactivity with antibody to a lesser extent than replacement by isoleucine. All four changes also influenced (reduced) the antigen's ability to interact with a *y*-monospecific antibody and in addition virtually eliminated cross reactivity with a subtype-specific monoclonal antibody (P. G. Ashton-Rickardt and K. Murray, unpublished work). These results emphasize the immunological importance of this region of the HBsAg polypeptide and reinforce the view that the epitopes are conformational.

The HBsAg used in these studies, defined as wild type, was derived from a coding sequence cloned from a virus isolated from serum exhibiting the complex serotype *adyw* (Burrell *et al.* 1979), but in antibody precipitation reactions the antigen showed a clear specificity for a monospecific anti-*y* serum and no interaction with a monospecific anti-*d* serum. It also induced antibodies (in mice) that cross reacted only with HBsAg of the *y* sub-type.

The mutations introduced at residues 113 (serine to theonine), 124 (arginine to lysine) or 134 (tyrosine to phenylalanine) were not by themselves sufficient to change the interaction with monospecific antibodies, but double mutants at residues 113 and 122 or 113 and 134 and a triple mutant at residues 113, 122 and 134 all cross reacted with similar efficiency with both *y*- and *d*-monospecific antisera. When antibodies raised in mice against all six mutants were exposed to *y*- and *d*-specific antigens in immunoprecipitation competition assays, those raised against the three single mutants all cross reacted efficiently with both antigens. Antibodies to the two double mutants also cross reacted with both antigens, but the avidity for the *d* subtype

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Table 2. Antigenic and immunogenic characteristics of HBsAg and its mutants

The results are expressed as interpolated values from titration experiments in which the antigen or antibody under test competed with its counterpart in double-antibody radioimmuno-precipitation (DARIP) assays with ^{125}I-labelled HBsAg of *y* or *d* subtype and monospecific antisera of the same subtype.)

	cross reactivity with monospecific antibody[a] subtype		cross reactivity of induced antibody[b] subtype	
antigen or mutant	*y*	d	*y*	*d*
wild type	0.21 μg ml^{-1}	no inhibition	1:400	no inhibition
y-113	0.22	no inhibition	1:125	1:11
y-122	0.23	no inhibition	1:22	1:22
y-134	0.22	no inhibition	1:100	1:40
y-113, 122	0.23	0.23 μg ml^{-1}	1:32	1:562
y-113, 134	0.22	0.27	1:32	1:40
y-113, 122, 134	0.26	0.25	no reaction	> 1:1000

[a] Concentration of HBsAg giving 50% maximum inhibition of subtype-specific antigen–antibody interaction.
[b] Serum dilution at 50% maximum subtype-specific antigen capture.

antigen was enhanced, and in the case of the triple mutant the induced antibodies showed no reaction with the *y* subtype antigen, but reacted very strongly with the *d* antigen.

These results, which are summarized in table 2, show that even without determination of the detailed structure of an antigen, site-directed mutagenesis can be used to change the immunological specificity of a protein. The examples described here are largely of academic interest, but a more practical application might involve mutant antigens that induce antibodies with higher affinities for a virus, or with a different or wider specificity to provide immunity against antigenic variants. The results also underscore the importance of precise conformation in dictating the selectivity and specificity of immunological reactions, and they further emphasize the distinction between antigenic and immunogenic epitopes.

Conclusion

The work described on hepatitis B virus provides an example of the successful application of modern biotechnological approaches to the development of an effective and commercially viable vaccine against a serious viral disease that constitutes a major public health problem worldwide. Similar approaches are in principle applicable to other vaccines; for example, immunization against rabies with a viral glycoprotein expressed in a recombinant vaccinia virus was shown to protect rabbits and mice against the virus (Wiktor *et al.* 1984) and much effort has been directed to the development of a subunit vaccine against FMDV. Work is proceeding in many laboratories on a number of viral, bacterial and parasitic diseases, much of which was described at a Nobel Symposium in 1987 and published in *Vaccine* (Lindberg *et al.* 1988) and there is, of course, widespread activity in the very difficult quest for vaccines against HIV.

This Discussion Meeting will highlight some of the successful developments and applications of biotechnology in the eight years that have elapsed since the publication of the Spinks Report. At a similar Discussion Meeting held in June 1979 one of us described some of the then current developments in genetic engineering (Murray 1980) and concluded that although the range of methods and systems for genetic manipulation and amplification of gene expression had

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already found useful application in medicine and was having a major impact on fundamental cell biology, persistent lack of investment in research and training in the underlying basic sciences had left the United Kingdom poorly placed to exploit these new developments. This is still the case today. Spinks helped; the New Blood Lectureships and the Biotechnology Lectureships provided a welcome infusion to our Universities, but the unremitting cuts in funds for academic research must result in lost opportunities in an increasingly competitive world.

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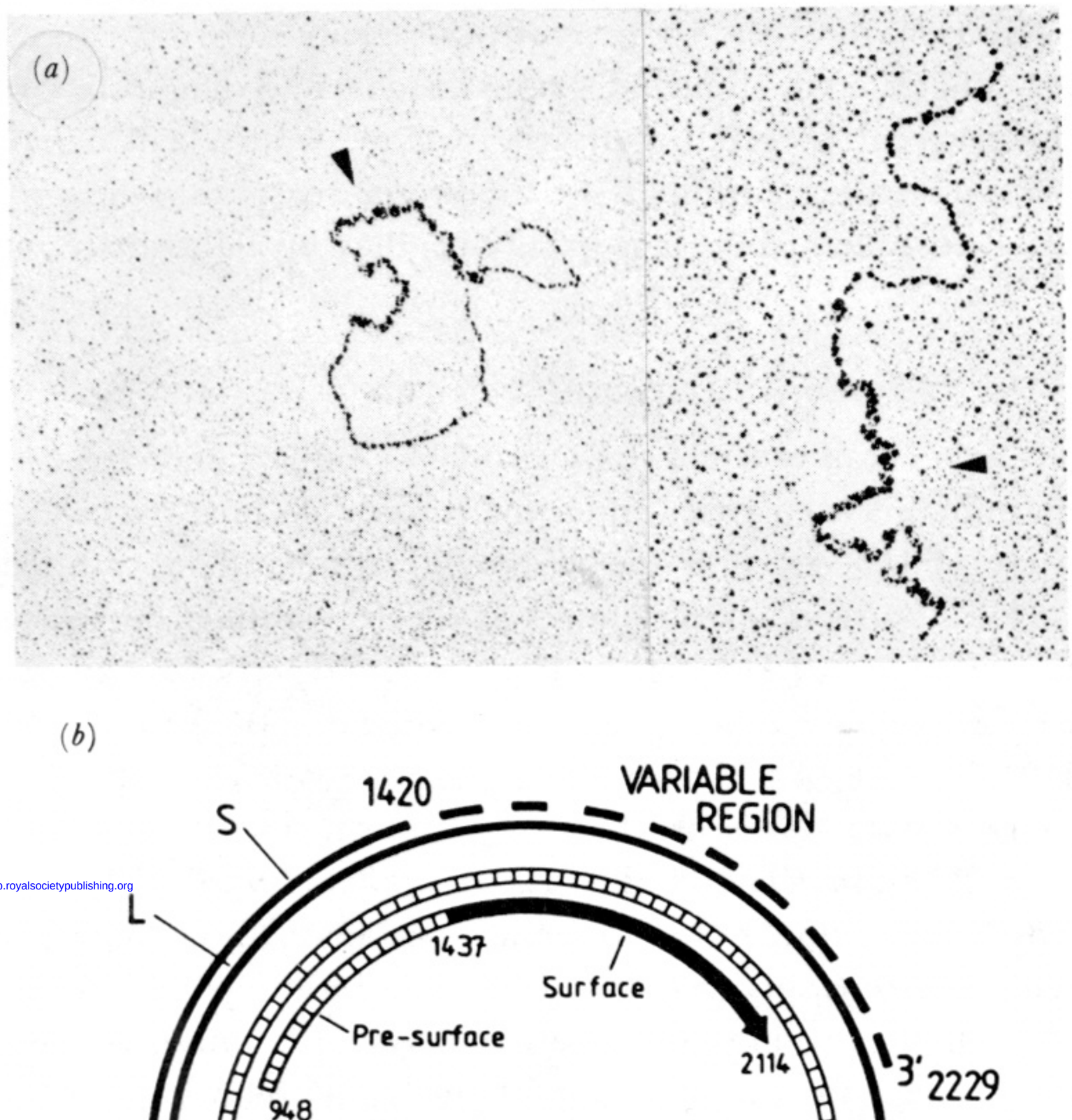

FIGURE 2. The HBV genome. (*a*) Electron micrographs of DNA extracted from Dane particles and complexed with bacteriophage T4 gene 32 protein, which binds specifically to single-stranded DNA, indicated by the arrowhead. Left, intact molecules, and right, DNA preparations digested with restriction endonuclease *Eco*RI (for which this particular DNA contained one target). These photographs were kindly provided by Dr Hajo Delius. (*b*) The genome of HBV. Heavy lines denote the DNA strands, the broken line showing the region of variable length of the short strand. Arrows represent the four open reading frames (as coding sequences) with the numbers of initiation and termination triplets in the system adopted by Pasek *et al.* (1979).

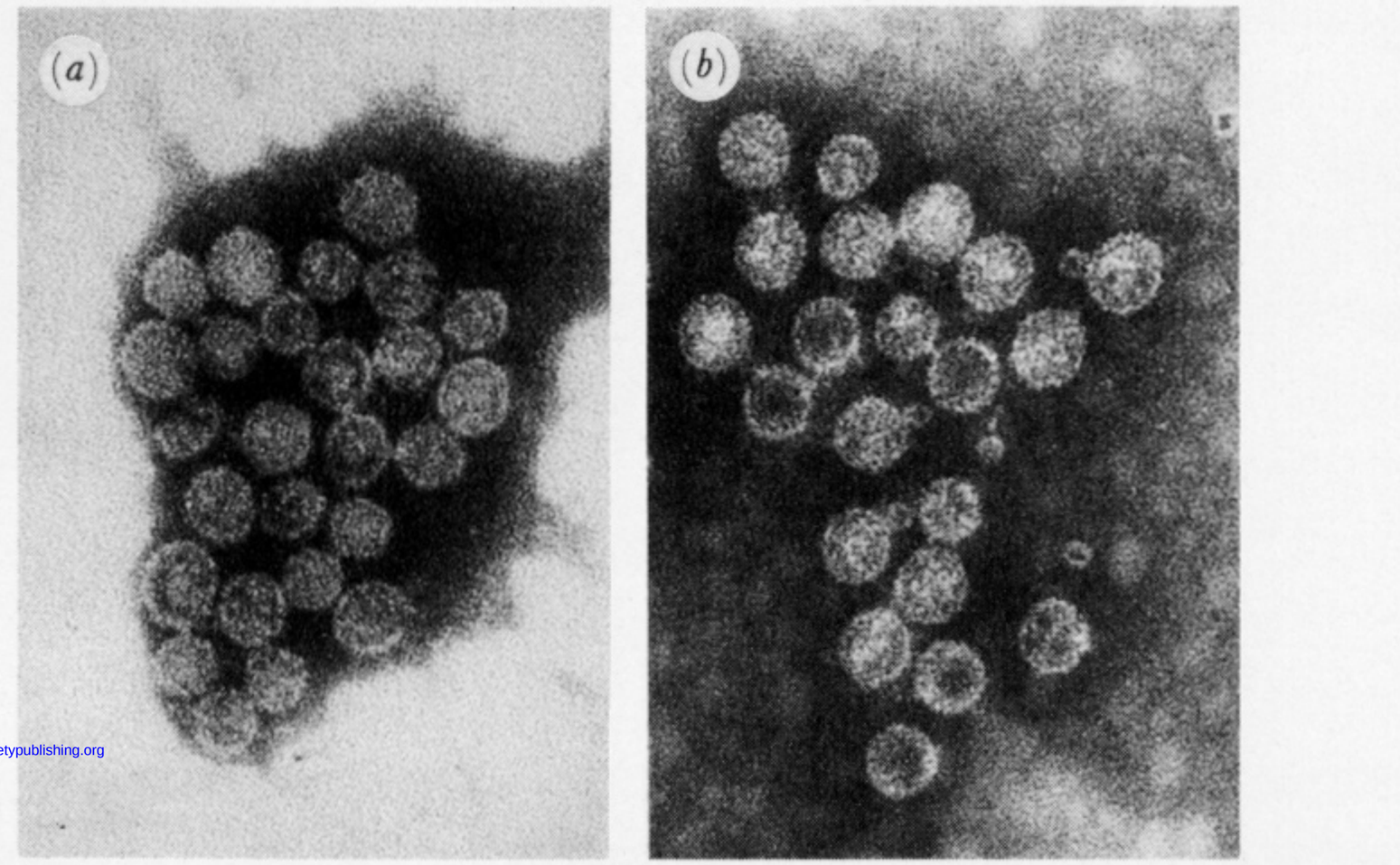

FIGURE 3. (*a*, *b*) Particulate forms of HBcAg: (*a*), isolated from the liver of an acutely infected patient; (*b*), synthesized in *E. coli*. These electron micrographs were kindly supplied by Dr Joan Richmond and are reproduced from Cohen & Richmond (1982), with permission from the authors and Macmillan Journals Ltd. (*c*) HBsAg particles purified from genetically engineered yeast. The electron micrograph was kindly provided by Dr H. Arimura and Dr T. Suyama, Green Cross Corporation, Osaka.

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