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Prospects for New Viral Vaccines [and Discussion]

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Prospects for new viral vaccines

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Animal virology has made outstanding contributions to preventive medicine by the development of vaccines for the control of infectious disease in man and animals. Cost–benefit analysis indicates substantial savings in health care costs from the control of diseases such as smallpox, poliomyelitis, yellow fever and measles.

Areas for further development include vaccines for influenza (living, attenuated virus), the herpes group (varicella: cytomegalovirus), respiratory syncytial virus, rotavirus and hepatitis A, B, and non A/non B.

The general options for vaccine formulation are discussed with particular emphasis on approaches with the use of viral genetics to 'tailor make' vaccine viruses with defined growth potential in laboratory systems, low pathogenicity, and defined antigens.

Current progress with the development of an inactivated hepatitis B vaccine is reviewed as a case study in vaccine development. The impact of recent experiments in cloning hepatitis B virus DNA in *E. coli* on the production of a purified viral polypeptide vaccine is assessed.

Dixon (1962), in his book on smallpox, recounts how, around 1796, Edward Jenner submitted a paper to the Royal Society, describing the inoculation of James Phipps, aged 8, with exudate from a cowpox lesion on the hand of Sarah Nelmes, a milk maid, and the subsequent resistance of the boy when variolated with smallpox virus some seven weeks later.

This one-subject, volunteer trial, without benefit of control *placebo* group, represents the first assessment in man of an attenuated living vaccine based on the host range principle; variolation with smallpox had, of course, been practised for a number of years.

Jenner was evidently uneasy about the statistical aspect, but commented that the inclusion of more data would swell the paper to an unnecessary bulk. It seems that the paper was not accepted, and, subsequently, in his 'Inquiry' Jenner (1798) described some 18 persons vaccinated with cowpox and later challenged with smallpox.

Now, in 1979, some 160 years later, the World Health Organization is about to declare the world free from smallpox. This is a remarkable achievement, not by the Jennerian principle of mass vaccination, once thought to give immunity for life, but by a campaign of case detection and vaccination of contacts with a potent and stable freeze-dried vaccine (Fenner 1977), the principle of 'ring containment', which has served us so well during the periodic importations of smallpox into the United Kingdom.

There are many morals to be drawn from the controversial history of Jennerian vaccination. As a preface, I have to mention the obvious one that prospects for new (or old) viral vaccines are not simply a matter of the sort of ingenious technologies that I am going to describe, but as much, if not more, a matter of social and political will and organization, and of climates of

public opinion. No medical procedure can be guaranteed absolutely safe; it is a matter of weighing advantages against risks. There is, for example, a connection between rising expectations on vaccine safety, the ease of litigation for real or supposed vaccine-associated complications, and the declining number of pharmaceutical firms undertaking vaccine manufacture (Melnick 1977; Sabin 1978).

TABLE 1. SOME ESTABLISHED VACCINES AGAINST HUMAN VIRUS DISEASES

smallpox (vaccinia)	measles
poliomyelitis (Salk/Sabin)	mumps
yellow fever (and other arboviruses)	rubella
adenovirus (e.g. in military groups)	influenza (inactivated)

TABLE 2. SOME ESTABLISHED VIRAL VACCINES USED IN VETERINARY MEDICINE (FROM BUXTON & FRASER (1977))

foot and mouth disease	canine distemper
feline enteritis	Marek's disease
Newcastle disease	blue tongue
canine hepatitis	rabies

Since those early days of Jenner's experiments (and of Pasteur's, with rabies, in the new microbiological era that followed soon after) animal virology has made outstanding contributions to the prevention of infectious disease in man and animals by prophylactic vaccination (see table 1). Thus, for example, the use of inactivated (Salk) poliovirus vaccine in Finland, Sweden and the Netherlands, and the living attenuated vaccine (Sabin) in the U.S.A., U.K., U.S.S.R. and elsewhere has dramatically decreased the prevalence of paralytic poliomyelitis. Similarly, table 2 gives a few examples from the 30 or more viral vaccines used in veterinary medicine (Buxton & Fraser 1977).

It is easy to see the benefits of these advances in human and social terms; it is perhaps less easy to quantitate them in terms of cost–benefit. Melnick (1977) recently analysed the use of poliovirus, rubella, measles and adenovirus vaccines in the latter way, comparing the cost of diagnosis, hospitalization and treatment of established case of infection with the cost of vaccine development and administration. He concluded that the cost–benefit was clear (positive) and drew attention, for example, to the survey of Witte & Axnick (1975) that reported a saving of $1.3 billion during 10 years by the prophylactic use of measles vaccine.

On the other hand, although inactivated influenza virus vaccine is listed in table 1, and has been available for more than 30 years, and, although there is no doubt that a vaccine of an antigenic composition related to a prevalent strain will protect against true influenza, the general use of influenza vaccine seems to have made little impact on the periodic visitations of 'influenza', much of which represents infection by agents other than influenza virus. Probably, there has been less effect on morbidity and mortality than from the use of sulphonamides and penicillin to treat the secondary bacterial infection after 'influenza' (Sabin 1978).

Some reasons can be advanced for the success of viral vaccines. By comparison with bacteria, the surface antigen mosaic of a virus is simple. The antigens provoking a protective, neutralizing antibody, or a specific cellular immune response, may consist of a few, highly immunogenic, proteins or glycoproteins making up the capsid of a naked icosahedral virus, or the envelope proteins of a membrane-maturing virus. Vaccine prophylaxis appears to be more effective

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against viruses with a single, stable or limited range of serotypes and those in which pathogenesis involves a viraemic phase in spread to the target organ; this maximizes the opportunity of antibody to interfere with adsorption, penetration and uncoating of virus in the host cell, and perhaps, for cell-mediated responses to reject and destroy the virus infected cells at the portal of entry or at intermediate replication sites on the way to the target organ.

On the other hand, diseases caused by viruses that infect at mucosal surfaces, such as the multiple serotypes of rhinoviruses, and particularly those, such as influenza A, that show frequent antigenic change (minor = 'drift' and major = 'shift'), are poorly influenced by parenteral administration of inactivated vaccine and require a vaccination strategy designed to stimulate local secretory antibody (IgA) with living vaccine applied to the potential site of infection. Also, in the instance of influenza A, vaccine antigens tailored to resemble those of current, prevalent strains are required.

TABLE 3. POTENTIAL VACCINES FOR HUMAN VIRUS DISEASES

influenza (living, attenuated)	herpes viruses (varicella zoster: cytomegalovirus)
hepatitis A	rotavirus
hepatitis B	hepatitis non A/non B
respiratory syncytial virus	

What are the remaining human viral infectious diseases against which a vaccine might be developed, and what are the technical options available? Table 3 lists some significant diseases or viruses, in terms of morbidity or mortality, for which a vaccine is not yet available (hepatitis A, hepatitis non A/non B, rotavirus) or is in the process of development (influenza, varicella-zoster virus, cytomegalovirus, respiratory syncytial virus, hepatitis B).

The recent cultivation of the CR326 hepatitis A virus, by Provost & Hilleman (1979), in explant cultures of *Saguinus labiatus* (marmoset) liver and in foetal rhesus kidney cell cultures presumably foreshadows the cell culture production of inactivated, or living attenuated viral vaccine from this hepatotropic enterovirus. It has already been shown that an inactivated vaccine from virus-infected marmoset liver will protect marmosets against experimental infection (Provost & Hilleman 1978).

Japanese workers (Takahashi *et al.* 1974) have developed a live varicella vaccine attenuated by passage from human lung cells, through guinea pig embryo cells for 12 passages, and then back to WI-38 human fibroblasts. This was used to control hospital cross infection with varicella virus and to immunize 'high risk', immunosuppressed groups of patients on corticosteroids or suffering from malignant disease. This vaccine appears to be effective in stimulating antibody without producing serious side effects, provided that cancer or other cytotoxic chemotherapy is suspended when the vaccine is given. In particular, the vaccine strains do not appear, so far, to lead to latent infection and recrudescent episodes of zoster. It is suggested that clinical trials should be initiated in the U.S.A. and elsewhere (for a review see Gershon 1978). It should be noted, however, that the development of acycloguanosine, a nucleoside analogue with high specific activity against the herpes virus group (Editorial 1979), may substantially alter the requirement for a vaccine.

Rotaviruses, an important cause of gastroenteritis in human neonates, have not been propagated serially and to high yield in cell or organ cultures, although related viruses causing diarrhoea in calves can be grown in cell culture.

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Respiratory syncytial virus (RSV), an important respiratory pathogen of the newborn, grows readily in cell culture, but inactivated vaccines given parenterally have produced more severe disease, rather than protection, when the vaccinated child encountered 'wild' virus (Kapikian *et al.* 1969). Living attenuated vaccines are being developed from temperature sensitive (*ts*) mutants (see below, influenza) and are attenuated for animals and, to some extent, for children (Wright *et al.* 1971; Wright *et al.* 1976). There is, in addition, the complicating factor of the less vigorous immune response in the very young.

Hepatitis B virus and hepatitis non A/non B virus(es) have not been grown in cell culture, although both have been propagated in chimpanzees.

Various options for formulating a killed, or living, attenuated vaccine are set out in table 4.

TABLE 4. OPTIONS FOR FORMULATION OF VIRAL VACCINES

(*a*) inactivated viral vaccines
- inactivated whole virions
- disrupted virions
- virion components without nucleic acid
- selected, purified capsid or envelope proteins
- 'synthetic' viral polypeptides, with adjuvant

(*b*) living viral vaccines
- wild, non-virulent strains from same host species
- strains attenuated by passage in a different species
- (host range (*hr*) mutants)
- temperature sensitive (*ts*) mutants and cold adapted variants
- genetic 'recombinants' or reassortants

The use of inactivated whole virions, or disrupted virions, is a well established approach. For example, the inactivated influenza vaccine made by the Commonwealth Serum Laboratory, Melbourne, contains virions disrupted with deoxycholate. Immunogenicity is retained, but adverse reactions are less than with whole virus. The component antigens can be separated from the virion and viral nucleic acid, purified, and used as a vaccine in the hope of reducing hazard from residual uninactivated virus, or of further reducing vaccine reactions. Unfortunately, increasing purity seems to be accompanied by decreasing immunogenicity. Thus, Webster *et al.* 1977 found that purified influenza glycoproteins (haemagglutinin or neuraminidase) will boost pre-existing immunity but are poor immunogens in an immunologically unprimed individual. The presence of a few inactivated viral particles mixed with the polypeptides greatly improves the serological response. This point is of potential significance in procedures designed to produce viral polypeptides by DNA cloning techniques.

With live attenuated vaccines, the use of wild, non-virulent strains from the same host species (obtained by screening large numbers of isolates for virulence) and attenuation by adaptation of a wild strain to grow in some other or unusual host (host range mutants) are classical approaches to vaccine construction.

Two examples of current vaccine technology may be considered in an attempt to illustrate these approaches and their problems in greater detail.

The first involves attempts to produce an inactivated vaccine against hepatitis B virus (HBV). This virus has not been grown in cell culture, and, so far, has only been transmitted to chimpanzees and a few other non-human primates.

The second involves genetic reassortment techniques that are of value with RNA viruses that have a segmented genome (e.g., influenza, rotavirus, orbivirus).

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Hepatitis B is a widely prevalent infection, particularly in underdeveloped countries in Africa, Asia and Oceania. As shown in table 5, a substantial proportion of acute cases of hepatitis is caused by HBV; in some outbreaks the mortality has been high. In addition, some cases of chronic hepatitis, cirrhosis and hepatocellular cancer are caused by, or associated with HBV infection; these conditions are particularly common in areas of high HBV prevalence There is a particular hazard for health care staff working in haemodialysis and oncology units, in institutions for the mentally retarded, in clinical pathology laboratories and, to a lesser extent, in the general wards of hospitals. Infection results from skin penetration accidents or mucosal/conjunctival contamination with infected blood or tissue fluids from HBV-infected cases or carriers.

TABLE 5. PREVALENCE AND MORBIDITY OF HEPATITIS B (HBV) INFECTION

HBV causes 20 to 50 % of cases of acute hepatitis
chronic carrier rates range from 0.1 to 20 % in various populations
antibody to hepatitis B surface antigen ranges from 5 to $\geqslant$ 80 % in different populations
probably about 5 % of world's population infected
there is an association with chronic liver disease and hepatocellular cancer

TABLE 6. POTENTIAL USES OF HEPATITIS B VACCINE

prevention of acute or chronic hepatitis in:
patients and staff in haemodialysis units, oncology units, institutions with Mongol children
seronegative promiscuous male homosexuals, and prostitutes
seronegative health care or relief agency staff working in highly endemic areas
prevention of vertical transmission of infection in African and Asian populations

Another group at risk from a different mode of infection, perhaps via infected saliva or semen, are the sexually promiscuous, particularly male homosexuals. In the high prevalence countries of the underdeveloped world, vertical transmission of the virus from a sick or carrier mother to her child is another important mode of infection, associated in particular with the establishment of chronic carriage. It appears that the moment of infection is at or after birth, rather than a transplacental infection, so that there is some hope that a vaccine given just before, or very early, in the incubation period might be able to stimulate immunity that would sever this particular chain of infection.

Overall, there is little doubt that there is a disease problem for which a preventive vaccine is required, one obvious prerequisite for development. (Summary of populations for vaccination in table 6.)

Several forms of HBV and antigens are found in the blood of an infected individual: the 42 nm particle (Dane particle) and 22 nm spheres or 22 nm wide filaments. The outer coats of the 42 nm particle and of the smaller filaments and spheres are antigenically the same and are made up of protein, lipid and carbohydrate (hepatitis B surface antigen, HBsAg). Inside the 42 nm particle there is another antigen (core antigen, HBcAg), DNA polymerase and a circular, double stranded DNA with a single stranded gap. There are also other antigen–antibody systems associated with the 42 nm particle: HBeAg (three antigens) and anti-HBe. The significance of these, for protection and vaccine composition, is under investigation (Prince & Vnek 1978). HBsAg has a common antigen '*a*', combined with subtype antigens, *d*, *y*, *w*, and *r*, in various allelic forms, *ayw*, *adw*, *ayr* and *adr*. There are various other subtype specificities of *a* and *w*, but these are not of significance in relation to vaccine production, as a serological response to the common antigen '*a*' is protective.

The polypeptide composition of HBsAg has been studied by numerous workers. The relative molecular masses of the polypeptides vary in different reports; a common pattern is shown in table 7.

Work in Edinburgh (Mackay & Burrell 1976), with use of chromatographic methods in addition to SDS–polyacrylamide gel electrophoresis (p.a.g.e), suggests that there may, in fact, be one large polypeptide of 60000–70000 relative molecular mass and distinct from serum albumin; it is possible that the seven or so lower mass polypeptides reported may represent fragments of the larger one. Work some years ago in Edinburgh also demonstrated that carbohydrate is present in HBsAg and is connected with its ability to react serologically (Burrell *et al.* 1973; Burrell *et al.* 1976). Workers elsewhere (Chairez *et al.* 1973) have found that two or more of the seven polypeptides found on p.a.g.e. are glycosylated; the removal of the carbohydrate appears not to impair the immunogenicity of the common antigen '*a*', which apparently depends on polypeptide rather than on carbohydrate determinants. The immunogenic similarity of the polypeptides is in line with the view that they may be fragments of a larger precursor.

TABLE 7. HEPATITIS B SURFACE ANTIGEN: COMPOSITION AND ANTIGENS

$10^3 \times$ relative molecular mass polypeptides	percentage of total	antigens†
22	33	*adw*
25 CHO	22	*adr*
31 CHO	3	*ayw*
35 CHO	1.5	*ayr*
40	4	
44	4	
52 CHO	8	
68	24.5	
lipid		

† Similar amino acid content/sequence; CHO, carbohydrate present.

The fine structure of the 22 nm spheres has also been investigated in Edinburgh (Mackay *et al.* 1978). Small circular 4–6 nm 'washer'-like subunits are built up into a spherical particle.

HBsAg is stable at 100 °C and resists proteolytic degradation until the polypeptides are exposed by detergent treatment. Perhaps because of these properties, the blood of chronic carriers of HBV often contains impressive amounts of HBsAg, e.g. 10^{13} 22 nm particles/ml or 100 μg/ml of antigen. This provides a rich source of material for a vaccine, which might not, in fact, be bettered by successful attempts either to grow the virus in a cell culture system in the future, or to produce its polypeptides by cloning the DNA in *E. coli* (see below).

Finally, various workers (Millman *et al.* 1971; Neurath *et al.* 1974), including those in Edinburgh (Burrell 1975), have shown that HBsAg purified from plasma is difficult to free from traces of serum proteins, particularly albumin. The potential problems of contamination of a vaccine with traces of serum protein, or with liver specific protein (LSP), are well dissected by Purcell & Gerin (1978*a*), who conclude that a hypothetical risk of induced 'auto-immune' disease with materials containing these contaminants has not been realized in practice during the use of whole blood transfusion, or injection of plasma or plasma fractions that were (unknowingly) contaminated with HBsAg.

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The first steps in the evolution of a hepatitis B vaccine were taken by Krugman *et al.* (1971). A group of mentally retarded children entering the Willowbrook State School, where HBV was highly prevalent, were immunized with heated (100 °C × 1 min) serum from a chronic HBsAg carrier and exhibited some resistance when subsequently challenged with live virus. In extensions of these preliminary observations, Purcell & Gerin (1978*b*) and Hilleman *et al.* (1978), in the U.S.A., and Maupas *et al.* (1978), in France, among others, have, by various techniques, purified 22 nm particles of HBsAg from the serum of carriers, have inactivated the material with heat or formalin, have safety tested it in chimpanzees, and, finally, have challenged the vaccinated chimpanzees with live virus. (Results obtained by Hilleman's group are given, by way of illustration, in table 8.)

TABLE 8. TESTS OF INACTIVATED HEPATITIS B SURFACE ANTIGEN, 22 nm PARTICLES, IN CHIMPANZEES

(After Hilleman *et al.* (1978).)

	fraction of animals showing:			
		response to viral challenge†		
group	antibody (anti-HBs) response to vaccine	HBsAg	anti-HBc	l.f.t.↑‡
vaccinated§	5/6	0/6	0/6	0/6
unvaccinated	0/5	5/5	4/5	5/5

† Challenge: 10^3 chimpanzee 50 % infective doses, intravenously.
‡ L.f.t.↑: abnormal liver function tests.
§ Vaccine: ad, ay, 3 doses, 20 μg, at monthly intervals.

TABLE 9. USE OF INACTIVATED 22 nm HEPATITIS B SURFACE ANTIGEN VACCINE IN DIALYSIS UNITS

(After Maupas *et al.* (1978).)

category	subclinical infection	overt hepatitis†	total
staff			
vaccinated‡	11	6 (3)	162 (100)
refused vaccination	2	8 (33)	24 (100)
patients			
vaccinated	5	5 (9)	55 (100)
refused vaccination	2	25 (80)	31 (100)

† Acute or chronic hepatitis, percentage in parentheses.
‡ Vaccine: ad + ay, 22 nm purified particles, 2–10 μg protein/ml, formalin, alum.

Although such purified 22 nm particle vaccines have proved to be less immunogenic, as measured by antibody response, in man than in chimpanzees, the results are nevertheless encouraging, and such vaccines are now under test in man in double blind trials. The 22 nm particle vaccine prepared by Maupas *et al.* (1978) has been tested in staff and patients exposed to HBV in several haemodialysis units in the Loire Valley. Although the control group was selected by a decision not to be vaccinated, rather than chosen at random and given a *placebo* inoculation, the results suggest an encouraging diminution of overt and chronic hepatitis in staff and patients (table 9).

In efforts to improve the safety of the hepatitis B surface antigen vaccines, and, in particular, to rid them of traces of human proteins, other workers (Hollinger *et al.* 1978) are investigating

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the use of purified polypeptides from HBsAg. This approach has been complicated by the loss of material during purification, which, in turn, leads to a requirement for larger starting volumes of plasma from HBsAg carriers, and, also, by the lower immunogenicity of the purified viral polypeptides in terms of antibody response in experimental animals, a situation perhaps analogous to that observed with purified haemagglutinin and neuraminidase from influenza

TABLE 10. IMMUNOGENICITY OF PURIFIED HBsAg POLYPEPTIDES IN CHIMPANZEES, AND RESULTS OF CHALLENGE OF IMMUNIZED ANIMALS WITH HEPATITIS B VIRUS

(After Hollinger *et al.* (1978).)

	fraction of animals showing:			
		response to viral challenge†		
group	antibody (anti-HBs) response to vaccine	HBsAg	anti-HBc	l.f.t.↑
vaccinated‡	4/4	0/4	0/4	0/4
controls		inoculum positive in other experiments		

† 3×10^5 Chimpanzee 50 % infective doses of HBV, intravenously.
‡ Doses (4) of 1.0 ml of mixture of alum and 40 μg protein, P22/25 polypeptides, intramuscularly.

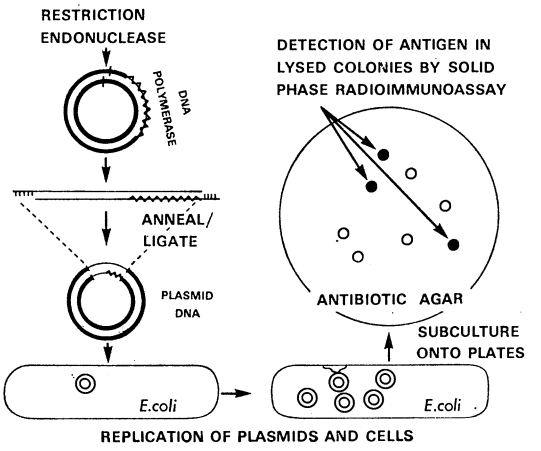

FIGURE 1. Steps in the cloning of hepatitis B virus DNA in *E. coli*. The viral DNA is extracted from Dane particles and the single stranded gap is closed with the endogenous DNA polymerase. The HBV and plasmid DNAs are treated with a restriction endonuclease giving a staggered cut and the complementary sequences are annealed so that the HBV DNA is inserted into the plasmid. The hybrid DNA is then introduced into *E. coli* and replicated in it. A restriction endonuclease site chosen for insertion of the HBV DNA is in a plasmid gene specifying an antibiotic resistance, so that the altered antibiotic resistance of the transfected *E. coli* can be used in colony selection and identification.

virus. Extraction and purification of the 22000 and the 25000 relative molecular mass polypeptides involves treatment with 0.5 M urea, 10 g/l SDS and 1 % (by volume) 2-mercaptoethanol before p.a.g.e. separation. There was some evidence that this altered the conformation of antigenic sites, an effect that might produce less avid antibody than that formed against whole viral antigens. Thus, these purified polypeptides had to be administered in several doses and with an adjuvant. Small numbers of chimpanzees were immunized with an alum-P22/25 vaccine and subsequently resisted challenge with living HBV (table 10).

This preliminary demonstration of the immunogenicity of isolated HBsAg polypeptides raises the possibility that the segment of the HBV DNA coding for HBsAg polypeptides might be cloned in *E. coli* by the techniques described by Professor Murray in this symposium, and that the polypeptide(s) of the sequence containing the epitopes for the '*a*' antigen, could be

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produced in large quantity for vaccine purposes. The first steps to this approach to vaccine production have been taken in several centres. Burrell *et al.* (1979), working in Edinburgh and at the Microbiological Research Establishment, Porton Down, have cloned a portion of the HBV genome in *E. coli* HB101 by means of plasmid pBR322 (figure 1). Some colonies of the transfected *E. coli* express HBV gene functions, namely synthesis of HBcAg. Some, but not substantial, synthesis of HBsAg has been detected. Slightly more recently, Sninsky *et al.* (1979), at Stanford, have cloned HBV DNA with plasmid pACYR 184 and *E. coli* K12. Finally, Fritsch *et al.* (1978), at the Pasteur Institute, also report the cloning of HBV genome in *E. coli*, but with bacteriophage λ gt WES λ B as a vector. In the latter studies, the whole genome was cloned, but no mention is made of gene expression or tests for HBV antigens.

These advances are very much within the spirit of this meeting on 'New horizons in industrial microbiology'. Clearly, however, there are a number of questions that remain to be resolved before the implications for polypeptide vaccine production are clear.

These are:

(1) the degree to which the HBV gene for HBsAg is expressed in *E. coli*;

(2) if completely expressed, whether *E. coli* protein synthesis can be manipulated so that the HBsAg gene product becomes a predominant part of the net protein synthesis of the cell;

(3) whether unglycosylated HBsAg polypeptide will have the antigenic specificity and immunogenicity of the polypeptide(s) extracted from the native HBsAg 22 nm particle; in particular, whether the quality of antibody produced will be avid and will neutralize infectivity;

(4) whether the HBsAg polypeptide synthesized will be linked to polypeptides synthesized by plasmid or phage genes on either side of the integrated sequences of HBV DNA, for example, as a fused polypeptide with β lactamase coded by the adjacent plasmid gene. These additional polypeptide sequences could confer unwanted antigenic specificities that might be difficult to remove by enzyme treatment and further fractionation.

Recombinant DNA techniques may also have a place in the production of vaccine polypeptides from RNA viruses such as influenza or rotaviruses. However, before discussing that aspect, brief mention may be made of some other genetic recombinant or, more correctly, reassortment techniques that are of value in the construction of inactivated, and also of living, attenuated influenza A vaccines. These serve as a model for similar manipulations with other RNA viruses with segmented genomes.

It has been known since the observations of Burnet & Lind in the early 1950s at the Walter and Eliza Hall Institute, Melbourne, that strains of influenza virus A with different 'host range' properties (e.g. ability to grow in mouse brain, to produce pocks on chorioallantive membrane, etc.) and with different antigens would, when inoculated into the allantoic cavity of chick embryos, 'recombine' at high frequency to produce hybrids with the antigens of one parent and the host range properties of the other. More recently, the genetic basis for the phenomenon became clear when it was found that the genes of influenza A viruses consist of eight segments of RNA with different nucleotide compositions and different oligonucleotide maps. These segments can be separated by electrophoresis on gels, by analysis of the differing migration patterns from reassorted strains and by complementation techniques their functions have been determined (Palese 1977; figure 2).

When two different strains of influenza virus A infect the same cell, the replicated RNA genes from each parent may be reassorted and packaged into different virions yielding hybrids that can be selected by various techniques (figure 3). In this way it is possible to recombine a

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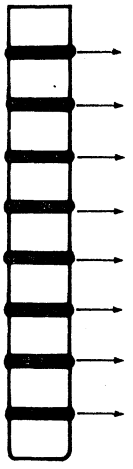
'master' strain with vigorous growth characteristics, valuable in vaccine production, with an antigenically different, poorly growing strain isolated from a current prevalence of influenza. This approach is now widely used for the production of new strains for inactivated vaccines. A similar approach is now being used to produce attenuated influenza A strains for use as a living vaccine (for a review see Beare 1975). Mackenzie (1969) found that temperature sensitive (*ts*) mutants of the mouse virulent WSN strain of influenza virus tended to be less virulent for mice, and suggested the use of 'master' strains with a '*ts*' lesion for the preparation of reassortants to be used as living attenuated vaccine strains. Mills *et al.* (1969), noting the lower temperature (about 33 °C) of the nasopharynx, compared with that of the lower parts of the lung (about 37 °C), decided that *ts* mutants might produce an immunizing infection limited to the upper respiratory tract, and have conducted numerous volunteer vaccine trials utilizing this principle.



RNA gel	gene product	relative molecular mass	molecules per virion
	P3	85000	15
	P1 (RNA polymerases)	95000	15
	P2 (RNA polymerases)	87000	15
	haemagglutinin	75000	1000
	nucleoprotein	53000	1100
	neuraminidase	45000	200
	matrix protein	25000	2300
	non-struct. protein	23000	0

After Palese (1977).

FIGURE 2. Diagrammatic representation of the separation of the segments of RNA making up the influenza (PR8) virus genome, and of the properties and function of the gene products from these segments.

The techniques of genetic reassortment and the use of *ts* mutants can be combined to produce living attenuated vaccine strains of defined and highly selected characteristics (for a review see Chanock & Murphy 1979).

The same approach may be of value with other RNA viruses with segmented genomes. One interesting example is the rotavirus, responsible for severe outbreaks of gastroenteritis in the newborn. Human rotavirus do not grow serially or to high titre in cell culture, although bovine and porcine strains are able to do so. Work in Melbourne (Rodger *et al.* 1975) has shown that these viruses have a genome of double stranded RNA with eleven segments and that the gel patterns of human and bovine rotaviruses are different (Kalica *et al.* 1976; Rodger & Holmes 1979).

Efforts are therefore in hand (Chanock *et al.* 1978) to generate reassortants, between human and bovine viruses, that will have the cell culture tropisms (host range properties) of the bovine strain with the surface antigens of the human.

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Finally, in line with the comments already made on the use of recombinant DNA techniques with hepatitis B, it might be possible to produce selected envelope proteins of the influenza virus, or capsid proteins of a rotavirus. This would involve isolation of the RNA gene concerned, production of a complementary DNA copy with the enzyme reverse transcriptase (RVT) and insertion of this copy, in its double stranded DNA form, into plasmid or phage DNA (figure 4).

A part from the problems of viral gene expression in *E. coli* and the immunogenicity of any polypeptides synthesized (as already discussed in relation to hepatitis B), a crucial aspect is the completeness and fidelity of copying of the RNA segment by RVT. Dr Air at the Australian

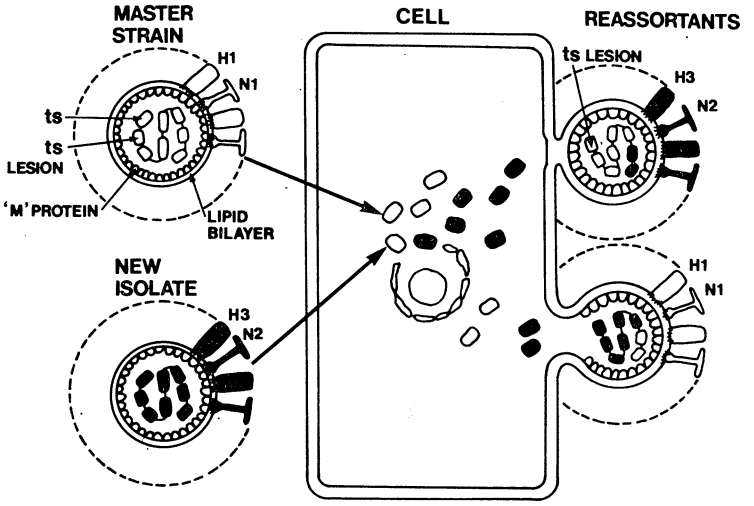

FIGURE 3. Example of reassortment of genes between a master strain influenza virus with 'ts' lesion and H1N1 envelope proteins and a new isolate with H3N2 envelope proteins. Doubly infected cells will yield some virions with the envelope proteins of the new isolate and the ts lesions (and, probably, the favourable replicative properties) of the master strain. These hybrids may be selected with the aid of an antiserum against H1N1 antigens and the presence of the ts lesions verified by growth at the permissive and nonpermissive temperatures.

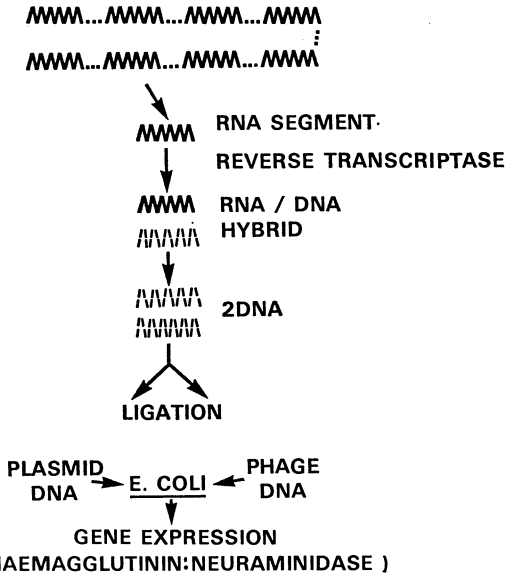

FIGURE 4. Possible scheme for cloning a complementary DNA copy of that part of the genome of a segmented RNA virus coding for envelope glycoproteins important in stimulating protective antibody.

National University, Canberra (personal communication), using influenza virus genes and tumour virus (AMV) reverse transcriptase has obtained full transcripts of the RNA, as have Emtage *et al.* (1979*a*) at Searle Research Laboratories, Bucks., U.K. Within recent months Sleigh *et al.* (1979) at the C.S.I.R.O. Molecular and Cellular Biology Unit, North Ryde, N.S.W., Australia, and the M.R.C. Molecular Biology Laboratory, Cambridge, U.K., have cloned ds DNA copies of the RNA haemagglutinin gene of various influenza A strains in *E. coli* with plasmid BR322 as a vector; expression of the haemagglutinin was not studied. However, Emtage *et al.* (1979*b*) have obtained expression of antigenic determinants of an avian influenza A virus previously cloned in *E. coli* and discuss techniques of maximizing expression of antigens.

References (Marmion)

Beare, A. S. 1975 Live viruses for immunisation against influenza. *Prog. med. Virol.* **20**, 49–83.

Burrell, C. J. 1975 Host components in hepatitis B antigen. *J. gen. Virol.* **27**, 117–126.

Burrell, C. J., Leadbetter, G., Mackay, P. & Marmion, B. P. 1976 Tryptic cleavage of antibody binding sites from hepatitis B surface antigen particles. *J. gen. Virol.* **33**, 41–50.

Burrell, C. J., Mackay, P., Greenaway, P. J., Hofschneider, P. H. & Murray, K. 1979 Expression in *E. coli* of hepatitis B virus DNA sequences cloned in plasmid pBR322. *Nature, Lond.* **279**, 43–47.

Burrell, C. J., Proudfoot, E., Keen, G. A. & Marmion, B. P. 1973 Carbohydrates in hepatitis B antigen. *Nature, new Biol.* **243**, 260–262.

Buxton, A. & Fraser, G. 1977 Rickettsias and viruses. *Animal microbiology*, vol. 2. London: Blackwell.

Chairez, R., Steiner, S., Melnick, J. L. & Dreesman, G. R. 1973 Glycoproteins associated with hepatitis B antigens. *Intervirology* **1**, 224–228.

Chanock, R. M. & Murphy, B. R. 1979 Genetic approaches to control of influenza. *Perspect. Biol. Med.* **22**, 537–547.

Chanock, R. M., Wyatt, R. G. & Kapikian, A. Z. 1978 Immunization of infants and young children against rotaviral gastroenteritis – prospects and problems. *J. Am. med. Ass.* **173**, 570–572.

Dixon, C. W. 1962 *Smallpox*, ch. 12, pp. 249–281. London: Churchill.

Editorial 1979 A new antiviral drug for herpes. *Med. J. Aust.* **1**, 557–558.

Emtage, J. S., Catlin, G. H. & Carey N. H. 1979*a* Polyadenylation and reverse transcription of influenza viral DNA. *Nucl. Acids Res.* **6**, 1221–1239.

Emtage, J. S., Tacon, W. C. A., Catlin, G. H., Jenkins, B., Porter, A. G. & Carey, N. H. 1979*b* Influenza antigenic determinants are expressed from hemagglutinin genes cloned in *Escherichia coli*. *Nature, Lond.* (In the press.)

Fenner, F. 1977 The eradication of smallpox. *Prog. med. Virol.* **23**, 1–21.

Fritsch, A., Pourcel, C., Charnay, P. & Tiollais, P. 1978 Clonage due génome du virus d l'hépatite B dans *Escherichia coli*. *C.r. hebd. Séanc. Acad. Sci., Paris* D **287**, 1453–1456.

Gershon, A. A. 1978 Varicella–zoster virus. Prospects for active immunisation. *Am. J. clin. Path.* **70**, 170–174.

Hilleman, M. R., Bertland, A. U., Buynak, E. B., Lampson, G. P., McAlear, W. J., McLean, A. A., Roehm, R. R. & Tytell, A. A. 1978 Clinical and laboratory studies of HBsAg vaccine. In *Viral hepatitis* (ed. G. N. Vyas, S. N. Cohen & R. Schmid), pp. 525–537.

Hollinger, B. F., Dreesman, G. R., Sanchez, Y., Cabral, G. A. & Melnick, J. L. 1978 Experimental hepatitis B. polypeptide vaccine in chimpanzees. In *Viral hepatitis* (ed. G. N. Vyas, S. N. Cohen & R. Schmid), pp. 557–567. Philadelphia: Franklin Institute Press.

Jenner, E. 1798 *An inquiry into the causes and effects of the variolae vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of cowpox.* London: Sampson Low.

Kalica, A. R., Garon, G. F., Wyatt, R. G., Mebus, C. A., Van Kirk, D. H., Chanock, R. M. & Kapikian, A. Z. 1976 Differentiation of human and calf reovirus-like agents associated with diarrhoea using polyacrylamide gel electrophoresis of RNA. *Virology* **74**, 86–92.

Kapikian, A. Z., Mitchell, R. H., Chanock, R. M., Shwedoff, R. A. & Stewart, C. E. 1969 An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am. J. Epidemiol.* **89**, 405–421.

Krugman, S., Giles, J. P. & Hammond, J. 1971 Viral hepatitis, type B (MS-2 strain): studies on active immunisation. *J. Am. med. Ass.* **217**, 41–45.

Mackay, P. & Burrell, C. J. 1976 Examination of the polypeptides of hepatitis B surface antigen. *J. gen. Virol.* **33**, 181–191.

Mackay, P., Burrell, C. J., Gowans, E. J. & Marmion, B. P. 1978 A model for the ultrastructure of hepatitis B surface antigen. In *Viral hepatitis* (ed. G. N. Vyas, S. N. Cohen & R. Schmid), pp. 706–707. Philadelphia: Franklin Institute Press.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

MacKenzie, J. S. 1969 Virulence of temperature sensitive mutants of influenza virus. *Br. med. J.* **3**, 757–758.
Maupas, P. L., Goudeau, A., Coursaget, P., Drucker, J., Barin, F. & Andre, M. 1978 Immunisation against hepatitis B in man; a pilot study of two years duration. In *Viral hepatitis* (ed. G. N. Vyas, S. N. Cohen & R. Schmid), pp. 539–556. Philadelphia: Franklin Institute Press.
Melnick, J. L. 1977 Viral vaccines. *Prog. med. Virol.* **23**, 158–195.
Millman, I., Hutanen, H., Merino, F., Bayer, M. E. & Blumberg, B. S. 1971 Australia antigen: physical and chemical properties. *Res. Commun. chem. Pathol. Pharmacol.* **2**, 667.
Mills, J., Van Kirk, J., Hill, D. A. & Chanock, R. M. 1969 Evaluation of influenza virus mutants for possible use in a live virus vaccine. *Bull. Wld Hlth Org.* **41**, 599–606.
Neurath, A. R., Prince, A. M. & Lippin, A. 1974 Hepatitis B antigen: antigenic sites related to human serum proteins revealed by affinity chromatography. *Proc. natn. Acad. Sci. U.S.A.* **1**, 2663.
Palese, P. 1977 The genes of influenza virus. *Cell* **10**, 1–10.
Prince, A. M. & Vnek, J. 1978 A new candidate hepatitis B vaccine. In *Viral hepatitis* (ed. G. N. Vyas, S. N. Cohen & R. Schmid), p. 712. Philadelphia: Franklin Institute Press.
Provost, P. J. & Hilleman, M. R. 1978 An inactivated hepatitis A vaccine prepared from infected marmoset liver. *Proc. Soc. exp. Biol. Med.* **159**, 201.
Provost, P. J. & Hilleman, M. R. 1979 Propagation of human hepatitis A virus in cell culture *in vitro*. *Proc. Soc. exp. Biol. Med.* **160**, 213–221.
Purcell, R. H. & Gerin, J. L. 1978*a* Hepatitis B vaccines: on the threshold. *Am. J. clin. Path.* **70**, 159–169.
Purcell, R. H. & Gerin, J. L. 1978*b* Hepatitis B vaccines. A status report. *Viral hepatitis* (ed. G. N. Vyas, S. N. Cohen & R. Schmid), pp. 491–523. Philadelphia: Franklin Institute Press.
Rodger, S. M. & Holmes, I. H. 1979 Comparison of the genomes of bovine, human and simian rotavirus and detection of genome variation between bovine strains. *J. Virol.* (In the press.)
Rodger, S. M., Schnagl, R. D. & Holmes, I. H. 1975 Biochemical and biophysical characteristics of diarrhoea viruses of human and calf origin. *J. Virol.* **16**, 1229–1235.
Sabin, A. B. 1978 Overview and horizons in prevention of some human infectious diseases by vaccination. *Am. J. clin. Path.* **70**, 114–127.
Sleigh, M. J., Both, G. W. & Brownlee, G. C. 1979 Influenza virus haemagglutinin gene: cloning and characterisation of a double-stranded DNA copy. *Nucl. Acids Res.* **7**, 879–893.
Sninsky, J. J., Siddiqui, A., Robinson, W. S. & Cohen, S. N. 1979 Cloning and endonuclease mapping of the hepatitis B viral genome. *Nature, Lond.* **279**, 346–348.
Takahashi, M., Otsuka, T., Okuno, Y., Asano, Y., Yazaki, T. & Isomura, S. 1974 Live vaccine used to prevent the spread of varicella in children in hospital. *Lancet* ii, 1288–1290.
Webster, R. G., Glezen, P. W., Hannon, C. & Laver, W. G. 1977 Potentiation of the immune response to influenza virus subunit vaccines. *J. Immun.* **119**, 2073–2077.
Witte, J. J. & Axnick, N. W. 1975 The benefits from 10 years of measles immunisation in the United States. *Publ. Hlth Rep., Wash.* **90**, 205–207.
Wright, P. F., Tatsuhiko, S., Fleet, W., Sell, S. H., Thompson, J. & Karzon, D. T. 1976 Evaluation of a live attenuated respiratory syncytial virus vaccine in infants. *J. Pediat.* **88**, 931–936.
Wright, P. F., Woodend, W. G. & Chanock, R. M. 1971 Temperature sensitive mutants of respiratory syncytial virus: *in vivo* studies in hamsters. *J. infect. Dis* **122**, 501–512.

Discussion

ANON. I understand that in some of the populations in which hepatitis is endemic there are abnormal sex ratios. Why is this, and what effect would a hepatitis vaccine have on the ratios?

B. P. MARMION. I agree that certain populations exhibit this trait, but I have nothing useful to say about it. Perhaps the unusual sex ratios are due to differential mortalities.

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