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Review Article

Some highlights of virus research in 1991

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Introduction

This review presents accounts of a number of advances in virology research which were published mainly in 1991. As with our similar publications for previous years, it is highly selective and to a degree reflects the authors' own interests and outlooks. The review starts with an account of a single poliovirus paper on virus production in a cell-free system, and then treats the fast moving field of genomic characterization of newer hepatitis viruses. The next three sections concentrate on virion structures, for parvovirus, adenovirus and herpesvirus. There follow accounts of viral involvement in programmed cell death, of current understanding regarding antigen processing for recognition by T lymphocytes and of viral aspects of superantigens, and finally advances in the technology of monoclonal antibodies (MAbs) are outlined.

Synthesis of poliovirus in a cell-free system

This brief section registers a single paper on poliovirus, which described the synthesis in a cell-free system of infectious poliovirus by translation and replication of input poliovirus RNA (Molla *et al.*, 1991).

Molla *et al.* (1991) prepared cytoplasmic extracts of uninfected HeLa cells, and used these to translate poliovirus RNA (either from virions or transcribed *in vitro* from cDNA clones) to give an authentic and apparently complete range of polypeptides. It was found that this procedure also yielded plaque-forming virus, and that the yield was increased by addition of NTPs. Over 4×10^4 plaques per ml of reaction mixture could be obtained after incubation for up to 18 h, although this yield is small compared with the input of around 10^{11} RNA molecules. Various controls demonstrated that the active virus obtained resulted from translation and processing of the virus polyprotein, replication of virus RNA via a negative-strand intermediate by the newly synthesized polymerase and assembly of authentic poliovirus particles. The phenomenon did not represent just packaging of input RNA, and any contribution from intact HeLa cells was excluded.

In itself, this paper presents an elegant result but does not give immediate new information on mechanisms of virus replication. However, this is the first such *in vitro* synthesis of an infectious animal virus starting from its genome as the only viral component that has been achieved, so far as we are aware. As such it is a significant and impressive milestone, and this capability should transform further analyses of picornavirus replication and protein functions.

Genomes of viruses responsible for non-A, non-B hepatitis

Two viruses have been identified recently which are responsible for the majority of non-A, non-B hepatitis (NANBH): hepatitis C virus (HCV; Choo *et al.*, 1989), a flavivirus- or pestivirus-related agent associated with post-transfusion NANBH, and hepatitis E virus (HEV; Reyes *et al.*, 1990), also a positive-strand RNA virus (possibly calicivirus-like) which is associated with enterically transmitted NANBH. Neither virus has been grown *in vitro* and hence research on these agents relies on the application of recombinant DNA techniques. Here we describe an analysis of the genome sequence of HEV and summarize some of the data from the avalanche of papers on HCV.

The genome sequence of a Burmese HEV isolate, HEV(B), was determined by Tam *et al.* (1991) from cDNA prepared to RNA extracted from an experimentally infected macaque. The RNA is 7194 nucleotides in

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length, excluding the 3′ poly(A) tail, and has a base composition of 58% G+C. Two major open reading frames (ORFs), separated by 37 nucleotides, were predicted in the sequence: ORF1 (bases 28 to 5109) and ORF2 (bases 5147 to 7129). The ORF1-encoded polypeptide sequence does not show obvious similarity to other positive-strand RNA virus proteins, but contains amino acid motifs characteristic of RNA-dependent RNA polymerases and of NTP-binding sites like those found in RNA helicases. ORF1 therefore probably encodes a polyprotein precursor for non-structural proteins. ORF2 has a possible signal peptide sequence at its N terminus, though the significance of this is unclear since HEV is non-enveloped and any interaction of its proteins with cellular membranes is unknown. There follows a region relatively rich in basic amino acid residues, suggesting that ORF2 perhaps encodes a capsid protein which interacts with the viral RNA. Analysis of the immunoreactivity of a cDNA expression library prepared to a human isolate from Mexico, HEV(M), showed that ORF2 contains epitopes recognized by acute- and convalescent-phase sera (Yarborough *et al.*, 1991). In addition an epitope was identified in the HEV(M) library to be encoded by a third ORF, ORF3, which contains only 369 bases. ORF3 spans the sequence between ORFs 1 and 2, overlapping ORF1 by four bases and ORF2 by 328 bases. Northern blot hybridization of RNA from infected macaques revealed three polyadenylated 3′ coterminal transcripts: a full-length 7·5 kb RNA and two subgenomic RNAs of 3·7 and 2·0 kb (Tam *et al.*, 1991; Yarborough *et al.*, 1991). The genesis of these subgenomic transcripts and their translation properties remain to be established.

Recently the sequence of a Pakistani isolate of HEV, SAR-55, has been reported (Tsarev *et al.*, 1992). This has the same genome organization as HEV(B) and an overall 99% amino acid identity when the three ORFs are compared. However, a region of 100 amino acids from ORF1 showed 14% difference, and this region was confirmed as being particularly variable by comparison with an HEV isolate from Russia, OSH-1852. This region will provide a useful marker for molecular epidemiological studies on HEV variation.

The available data do not allow the firm assignment of HEV to a particular virus family. HEV has a particle morphology and genome layout (5′ encoding non-structural, and 3′ structural proteins) similar to those of caliciviruses. However, sequence analysis of the presumptive non-structural proteins of a feline calicivirus showed similarity to those of picornaviruses, including the presence of a cysteine protease (Neill, 1990); this activity is not predicted for HEV. Hence, HEV could represent a distinct genus in the *Caliciviridae* or the prototype of a new family.

Turning to HCV, good progress has been made on genome sequence analysis, with complete sequences of seven isolates now published (Kato *et al.*, 1990; Choo *et al.*, 1991; Inchauspe *et al.*, 1991; Okamoto *et al.*, 1991, 1992; Takamizawa *et al.*, 1991; Chen *et al.*, 1992). The HCV genome is a positive-stranded RNA molecule about 9·4 kb long with a base composition of 59% G+C, which contains a single ORF encoding a polyprotein of 3010 to 3033 amino acids. Flaviviruses and pestiviruses have a similar organization and, although there is little primary sequence similarity overall (either at the nucleotide or amino acid level) among these three virus groups, the hydropathy profiles of their polyproteins are similar (Choo *et al.*, 1991; Takamizawa *et al.*, 1991). Hence the organization of the HCV genome is predicted as: 5′ untranslated region (5′ UTR; about 341 bases) followed by proteins designated C, E1, E2/NS1, NS2, NS3, NS4, NS5, and finally a short 3′ UTR. The actual sequence at the 3′ end is uncertain; both poly(A) (Choo *et al.*, 1989; Han *et al.*, 1991) and poly(U) (Kato *et al.*, 1990; Okamoto *et al.*, 1991; Takamizawa *et al.*, 1991; Chen *et al.*, 1992) have been reported. The 5′ UTR is highly conserved among different HCV isolates and contains blocks of sequences which show significant homology with pestivirus 5′ UTRs (Choo *et al.*, 1991; Han *et al.*, 1991). The HCV 5′ UTR also exhibits three or four AUG-initiated short ORFs upstream of the AUG thought to initiate the polyprotein. The significance of these ORFs is unknown, but cell-free translation of *in vitro* synthesized HCV RNA has shown that ribosomes bind directly to an internal sequence (between bases 101 and 332) in the 5′ UTR rather than scanning from the 5′ terminus as is the case with most eukaryotic mRNAs (Tsukiyama-Kohara *et al.*, 1992).

Processing of the HCV putative structural proteins has been investigated by cell-free translation of an RNA transcript expressing the 980 N-terminal residues of the polyprotein (Hijikata *et al.*, 1991); C (19K core or capsid protein), E1 (35K glycoprotein), E2/NS1 (70K glycoprotein) and part of NS2 were produced only in the presence of microsomal membranes, indicating that cotranslational processing was mediated by signal peptidases. Recombinant C protein or synthetic C peptides have been used to develop sensitive immunoassays for HCV infection, which are particularly useful as antibodies to C appear early after infection (Chiba *et al.*, 1991; Harada *et al.*, 1991; Hosein *et al.*, 1991; Nasoff *et al.*, 1991). The E1 and E2/NS1 proteins are heavily glycosylated and both are candidate envelope proteins of the virus. Antibodies to E1 (expressed by recombinant baculoviruses) were found in a relatively small proportion of patients designated HCV-positive by detection of anti-core or anti-C100 (a recombinant yeast-expressed antigen from the NS3–NS4 junction) antibodies (Matsuura *et al.*,

1992). Expression of E2/NS1 from cloned DNA indicated that the mature product was cell-associated (Spaete *et al.*, 1992); it therefore seems probable that it acts as a virion glycoprotein like the corresponding pestivirus protein, and unlike the non-structural, secreted flavivirus equivalent.

Turning to the HCV non-structural proteins, NS3 contains amino acid motifs characteristic of a serine protease and an RNA helicase, and similar motifs are found in the analogous proteins of flaviviruses and pestiviruses. Protease activity has been demonstrated experimentally for flaviviruses and pestiviruses (Falgout *et al.*, 1991; Preugschat & Strauss, 1991; Preugschat *et al.*, 1991; Wengler *et al.*, 1991; Wiskerchen & Collett, 1991), and the C-terminal region of West Nile flavivirus NS3 has been purified and shown to have RNA-stimulated NTPase activity, a characteristic of an RNA helicase (Wengler & Wengler, 1991). NS5 contains amino acid motifs diagnostic of an RNA-dependent RNA polymerase. The roles of NS2 and NS4 are unknown.

From the wealth of complete and partial sequence data on HCV genomes it has become obvious that considerable heterogeneity exists among different isolates (e.g. Nakao *et al.*, 1991), even isolates from the same patient (e.g. Oshima *et al.*, 1991). The mutation rate of an American strain of HCV has been estimated at $1{\cdot}92 \times 10^{-3}$ base substitutions per genome site per year, though the nucleotide changes were not distributed evenly throughout the genome (Ogata *et al.*, 1991). Hypervariable domains have been reported in the putative envelope proteins and in the NS4 and NS5 proteins (Kremsdorf *et al.*, 1991; Weiner *et al.*, 1991) which may hamper serological identification of HCV-infected individuals.

At least three major types of HCV have now been defined on the basis of sequence data (Chan *et al.*, 1991, 1992): type 1, which includes the American prototype strain HCV-1 and Japanese isolates such as HCV-BK and HC-J4; type 2, which includes other Japanese isolates like HC-J6 and HC-J8; and type 3, for which only partial sequences are available. The polyproteins of HCV types 1 and 2 differ in about 30% of their amino acids (Okamoto *et al.*, 1991), and similar differences have been reported for the limited regions sequenced for type 3 isolates (Chan *et al.*, 1991). Types 1 and 2 could be further subdivided into regional variants (Chan *et al.*, 1992; Okamoto *et al.*, 1992). Chan *et al.* (1991) reported that the three types co-exist in the same geographical area, at about the same prevalence. These observations raise the possibility of multiple infections with different virus types, which may be involved in the observed extreme chronicity of HCV infection. In addition, Pozzato *et al.* (1991) have suggested that severity of disease and response to interferon treatment may correlate with different HCV types.

Research on parvoviruses

The *Parvoviridae* comprise a family of small icosahedral viruses, which include a number of animal pathogens but only one recognized human pathogenic virus (B19 virus). The group includes autonomous viruses and others which are dependent on helper virus for completion of a productive replication cycle. Parvovirus genomes are linear ssDNA molecules of around 5000 bases, with terminal complex hairpin structures. There are basically two reading frames in a parvovirus genome, but variation of splicing for the transcripts allows in each case expression of a family of related proteins. One family specifies structural proteins and the other supplies functions associated with DNA replication. Most autonomous parvoviruses package in their virions DNA which is complementary to the mRNAs, i.e. it is negative-stranded; some autonomous parvoviruses (including B19) and helper-dependent viruses package both strands, in separate particles.

This section deals with X-ray crystallographic analysis of canine parvovirus 2 (CPV-2), followed by other current topics in parvovirus research. CPV-2 is a pathogenic virus of dogs which was first reported in 1978 and rapidly became established world-wide. The first high resolution analysis of a parvovirus virion structure by X-ray crystallography was reported in 1991 by Tsao *et al.* (1991) for CPV-2. A preliminary low-resolution analysis of the human parvovirus B19, using empty capsids obtained from a baculovirus expression system, was also published (Agbandje *et al.*, 1991).

The CPV-2 capsid has icosahedral geometry and is composed of 60 molecules of structural protein: most of these are the 584 residue species termed VP2; some are VP1, which is equivalent to VP2 with an extra N-terminal domain; and in DNA-containing particles some VP2 molecules have an N-terminal segment removed to give VP3.

The crystallographic analysis showed that VP2 possesses a β-barrel structure which is very similar to that seen in the capsid proteins of picornaviruses and small icosahedral plant RNA viruses. In CPV-2 VP2 there are large loops between the β-strands, so that the β-barrel forms only about one third of the whole protein. The β-barrel lies internal to the virion surface, which is composed of the loops. Some regions of polypeptide chain are disordered, mostly on the virion surface.

The greatest radius of the CPV-2 particle is 14 nm or 140 Å, achieved with a 22 Å protrusion or spike at the threefold axes. A cylindrical structure of β-sheets is

present at each fivefold axis, and is surrounded by a 15 Å deep depression or 'canyon', which by analogy with some picornaviruses might contain attachment sites for cellular molecules used as viral receptors.

In the interior of the capsid, a region of icosahedrally symmetric electron density was present in full but not empty capsids. This could be convincingly modelled as an 11 nucleotide ssDNA segment, associated with a hollow in the inner surface of the protein shell. Certain positions in the visible nucleotide chain were preferentially occupied by either purine or pyrimidine residues. In the whole particle there are 60 such ordered sections of DNA, accounting in all for some 13% of the genome. The only other icosahedral virus in which ordered elements of the genome have been observed is the RNA-containing bean pod mosaic virus.

The VP1 variant of the capsid protein (of which there are only a few molecules per virion) contains a basic N-terminal domain, which is probably internal and associated with DNA (but was not visible in the crystallographic structure). Separately, association of capsid proteins of several parvoviruses with the 3'-terminal hairpins of genomic DNA has been reported (Metcalf *et al.*, 1990; Willwand & Kaaden, 1990; Willwand & Hirt, 1991): this probably represents a phenomenon distinct from protein–DNA interactions in the whole capsid, and has been proposed as part of a genome encapsidation mechanism (Willwand & Kaaden, 1990).

Although the CPV-2 capsid protein is now seen to be structurally similar to the proteins of small icosahedral RNA viruses, the way in which CPV-2 VP2 molecules are arranged to build the capsid differs significantly from that seen in satellite tobacco necrosis virus, the only other structurally determined example of a virion composed of 60 protein molecules. The similarity of the parvovirus VP2 geometry to that of structural proteins of icosahedral RNA viruses is suggestive of a remote common evolutionary origin.

Turning to other aspects of CPV-2, this parvovirus is thought to have developed as a host range mutant from a virus of a group whose members infect cats, raccoons and mink. DNA sequence comparisons have been carried out, but did not allow identification of an immediate precursor of CPV-2 (Parrish *et al.*, 1988). Since its emergence, CPV-2 has been observed to be evolving rapidly. An antigenically distinct strain (CPV-2a) displaced the original strain by the early 1980s (Parrish *et al.*, 1985), and in a 1991 paper it was reported that after 1986 a new antigenic type (CPV-2b) displaced CPV-2a (Parrish *et al.*, 1991). Epitopes defining recent changes occupy exposed positions on the threefold axis spikes of the virion. Parrish *et al.* (1991) also analysed nucleotide changes in CPV-2 isolates and concluded that CPV-2 is undergoing a process of progressive evolution and antigenic drift comparable to that seen with influenza A virus. The primary selecting force on CPV-2 is likely to be escape from immunity in the canine population; another aspect could be that the virus is still in the process of optimizing adaptation to the canine host species.

Retained nucleotide substitutions were estimated to be accumulating in the CPV-2 genome at the rate of $1{\cdot}7 \times 10^{-4}$ per site per year. This rate is 10- to 100-fold lower than reported for regions of influenza virus or human immunodeficiency virus (HIV). No direct data are available on nucleotide substitution frequency during CPV-2 DNA replication, but since parvoviruses are replicated by cellular DNA polymerases the error rate is expected to be much lower than that for viral RNA-dependent RNA replicases. Rapid antigenic change thus appears not to be necessarily dependent on a high error rate in genome replication.

In another paper from the same laboratory, Parrish (1991) described the construction of recombinants between CPV-2 and feline panleukopenia virus (FPV) and their use to map virus properties to specific parts of the capsid. FPV is one of the group of viruses from which CPV-2 is thought to have originated. In the infectious molecular clones of the two viruses employed in this study there were 50 nucleotide differences, 16 of which specified coding differences. Eight recombinant DNAs were constructed, of which six yielded infectious virus. These viruses were tested for CPV-2- and FPV-specific antigenic sites, for dependence of haemagglutination on pH and for growth in canine and feline cells in culture. Epitopes for antibodies were mapped to the threefold axis spike. Haemagglutination by CPV-2 is much less pH-dependent than the FPV activity, and the recombinant analysis showed that this effect correlated with two VP2 residues located in a surface depression on the capsid surface. Host range effects were assigned to a section of VP2 containing four amino acid differences between the parental viruses and corresponding to a portion of the capsid surface close to that affecting haemagglutination.

We finish this section with an outline of aspects of site-specific integration into the host genome described for adeno-associated virus type 2 (AAV-2). Adeno-associated viruses are a group of parvoviruses which in normal tissue culture cells are dependent for productive infection on a helper virus, such as adenovirus or herpes simplex virus (HSV). In the absence of helper, AAV-2 infection results in efficient integration of tandem copies of the viral genome into the cellular genome to establish a latent condition. Cells carrying AAV-2 genomes do not show altered behaviour and AAV-2 can be rescued from such cells by superinfection with helper virus.

Recent papers have reported that AAV-2 integration into human genomic DNA occurs at a single locus; this specificity is so far unique among eukaryotic viruses. Kotin *et al.* (1990) reported that cellular DNA sequences flanking integrated AAV-2 DNA in a particular latently infected cell line were common to other AAV-carrying lines, and came from chromosome 19. The integration site was subsequently narrowed to a particular locality in the q arm of chromosome 19 (Kotin *et al.*, 1991; Samulski *et al.*, 1991). Samulski *et al.* (1991) used an AAV-2 construct carrying bacteriophage λ repressor-binding sequences to allow ready isolation of integrated genomes plus flanking sequences. The DNA sequences for a number of independent integrants showed that the breakpoints between host and viral DNA fell at various positions within a 100 bp region of host DNA, with variable loss of AAV DNA terminal sequences. Very short sequence identities between host and viral sequences (two or three nucleotides) were observed at the breakpoints. The 100 bp host region involved was flanked by 21 bp direct repeats.

The mechanism of this very interesting phenomenon remains to be examined. In principle, both cell and virus could contribute specific cis-acting DNA sequences and trans-acting gene products. Earlier work had shown that AAV-2 integration occurs efficiently in the absence of functional AAV-2 genes, but Samulski *et al.* hint that AAV-2 sequences in addition to the terminal repeats may be needed for site-specificity. Outside parvovirus virology *per se*, the system is also of interest as an experimentally approachable example of non-homologous recombination, and has potential for use in controlled introduction of DNA into human cells, as for gene therapy.

A separate, curious observation relating to AAV-2 integration was made in 1991. Thomson *et al.* (1991), engaged on sequence analyses of the genome of human herpesvirus 6, discovered an ORF encoding a protein sequence convincingly homologous to the sequences of parvoviral replication (rep) proteins and to that of AAV-2 in particular. These authors speculated that the known capacity of AAV-2 for non-homologous insertion could have introduced the gene from AAV-2 into the herpesviral genome. The functional significance remains unclear but tantalizing. Parvoviral rep proteins are probably broadly similar in function to the T antigens of polyomaviruses, and have been shown to exert various effects on non-parvoviral gene expression: Heilbronn *et al.* (1990) reported that AAV-2 rep could effectively suppress HSV-induced DNA synthesis.

Adenovirus structure

The virion structure of adenovirus 2 as determined by cryoelectron microscopy and image reconstruction was reported by Stewart *et al.* (1991). The previously determined X-ray crystallographic structure for the major capsomer, from the same group, allowed validation of the whole-virion work, and overall the structure and its interpretation are notably refined and informative.

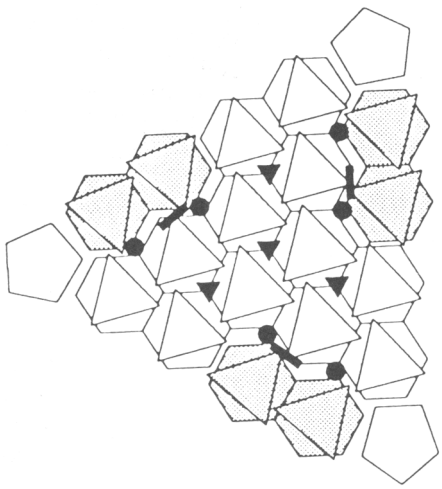

Fig. 1. A group of 12 hexons making up one face of the adenovirus capsid is shown, together with three groups of two hexons each from the adjacent faces; the latter are shaded. Pentons are shown at the three corners. Each hexon (consisting of a homotrimer) is shown as a hexagon with its outer 'tower' part as a superimposed triangle. Note that the hexons in the group of 12 are closely packed whereas packing between hexons from adjacent groups of 12 is more open, and that the orientation of the towers differs between adjacent groups. The small filled bars, triangles and circles represent inferred locations of minor proteins of the capsid with specific 'cementing' roles. Reproduced from Stewart *et al.* (1991) with permission. © Cell Press.

The virion consists of an icosahedral protein shell containing 240 hexon and 12 penton capsomers. Excluding the fibres which protrude from the fivefold vertices, the greatest diameter is 91 nm. Each hexon capsomer is a trimer of a 967 residue polypeptide, and in plan view (with respect to the virion surface) has a hexagonal base topped by a triangular 'tower' structure. The electron microscopic reconstruction is in good agreement with the X-ray crystallographic structure for the hexon capsomer and shows the triangular structure of the towers clearly, at a resolution of about 3 nm.

The virion can be regarded as being formed by 20 groups each of 12 hexons with their hexagonal bases tightly packed and all their towers in the same orientation, as indicated in Fig. 1. This unit corresponds to a face of the icosahedron; adjacent units have their towers in opposing orientations and two of the four hexons along each edge of the icosahedron are contributed from each adjoining group of 12 hexons (Fig. 1). The capsid is significantly distorted from a flat-faceted icosahedron into a rounded structure, with more exten-

sive inter-hexon contacts along the edges than a true icosahedron would allow. This outcome implies that the well known electron microscopic images of negatively stained adenovirus particles with flat faces and sharply defined edges are artefactual.

The penton fibre of adenovirus 2 is a structure of length 37 nm, longer than previously supposed (Ruigrok *et al.*, 1990). Only one third of the fibre is visible in the reconstruction. It appears that after the innermost third there is a bend in the fibre which renders the outer part invisible in the enforced icosahedral reconstruction. It is known that four peptide species in addition to the hexon and penton base proteins are included in the capsid shell. By a combination of close examination of the shell reconstruction and use of existing data on these extra proteins, Stewart *et al.* (1991) accounted convincingly for the locations of three, namely polypeptides VI, VIII and IX (see Fig. 1). All are involved in distinct subclasses of binding between adjacent hexons, two on the surface and one internally; they probably all have roles in stabilizing the capsid structure, and the authors emphasized that this is likely to prove a general if previously underappreciated aspect of such assemblies.

Adenoviral DNA is thought to be packaged in a complex with virus-encoded histone-like protein. Stewart *et al.* (1991) did not observe general icosahedrally ordered structure in the capsid interior, although they registered that some partial ordering might exist adjacent to the inner surface of the shell.

Structure and assembly of herpesvirus capsids

Herpesvirus particles are large and complex objects by general standards of virus architecture. The virion consists of an icosahedral capsid containing the genomic DNA; the capsid is surrounded by an amorphous protein layer, the tegument; this in turn is enclosed in a lipid membrane carrying surface glycoproteins. The herpesvirus icosahedral capsid is some 125 nm in diameter, five times greater than the parvovirus capsid and with a 100-fold greater internal volume.

Herpesvirus capsids have been studied in recent years by cryo-electron microscopy of unstained particles followed by computerized image processing. The first such analysis, for HSV-1, was by Schrag *et al.* (1989). This gave a view of the capsid structure to 4 nm resolution and proposed the existence of an additional shell of protein inside the well recognized outer shell. In the past 3 years this work has been extended and corrected in a number of papers by W. W. Newcomb, J. C. Brown and their colleagues to give information on various aspects of capsid structure – geometry, structure of capsomers, location of proteins, internal proteins in immature capsids and configuration of encapsidated DNA. This section describes these topics, combining data for HSV-1 and the structurally closely similar equine herpesvirus type 1, and goes on to describe an aspect of capsid formation involving a recently characterized virus-encoded proteinase.

The most refined picture so far achieved for the structure of the HSV-1 capsid was reported by Booy *et al.* (1991). The capsid is 125 nm in diameter with 162 capsomers, which project some 11 nm from the 4 nm thick 'floor' of the capsid, and have hollow cores extending into the particle's interior. The 12 capsomers at the icosahedral vertices (pentons) show a definite pentagonal cross-section while the other 150 (hexons) are hexagonal in cross-section. Cumulative indirect evidence indicates that both types are composed of the same protein species, VP5 (five molecules in each penton, six in each hexon) (Schrag *et al.*, 1989; Newcomb & Brown, 1991). Distinct 'triplex' structures are seen to join the bases of groups of three adjacent capsomers. This description of external features is consistent with those in previous accounts. However, Booy *et al.* (1991) in their analyses of both empty and DNA-filled capsids found no sign of the inner shell reported by Schrag *et al.* (1989) and it now seems essentially certain that the earlier result was artefactual.

The truly novel feature in the report of Booy *et al.* (1991) was their visualization of the genomic DNA in filled capsids. At high magnification in cryo-electron micrographs the DNA was visible as, to use the authors' words, 'punctate arrays or finely spaced, curvilinear striations'. Computer procedures allowed separation of DNA images from those of the surrounding capsid proteins. The DNA appeared densely and uniformly packed to fill the entire interior of the capsid, with an inter-strand spacing of 2·6 nm. It was concluded that the DNA was packed in a locally ordered manner, with bundles of parallel duplex strands. Notably, this packing is closely similar to that already observed for the bacteriophages T4 and λ. The previous model for HSV DNA packing, dating from the early 1970s, was that the DNA was wound on a 'fibrillar spool'. It now seems probable that this result was based on artefacts of sample preparation. Booy *et al.* (1991) observed various patterns of capsid DNA strands, which certainly suggest a long range winding scheme, but their data do not support the presence of a spool structure.

Herpesvirus capsids are apparently first assembled as protein shells which lack DNA but do contain substantial amounts of internal protein, which is lost when DNA is packaged. Three distinct forms of capsids, differing in density, can be recovered from infected cells. The

heaviest (C capsids) contain DNA. The intermediate B capsids are the precursors of C capsids and contain internal protein but no DNA. The lightest, A capsids, are empty and are thought to be dead-end aberrant processing products of B capsids.

The major internal protein constituent is a species termed ICP35 in HSV-1 (and various other names, e.g. p40, Vmw40, Vp22a, NCP-4). This undergoes extensive processing so that there is a family of related forms. Newcomb & Brown (1991) were able to show that treatment of HSV-1 capsids with 2 M-guanidine hydrochloride released pentons plus the ICP35 core and also several proteins. On renaturation, ICP35 assembled into toroidal structures. The function of the temporary internal structural element is not well defined; it could have roles in assembly of the nascent capsid shell and in subsequent DNA packaging. Recent work has identified a virus-encoded proteinase involved in processing the internal protein; comparable results have been obtained for HSV-1 and for the widely diverged betaherpesvirus simian cytomegalovirus (SCMV) (Liu & Roizman, 1991*a*, *b*; Preston *et al.*, 1992; Welch *et al.*, 1991*b*).

The coding arrangement for the structural protein and the proteinase is curious: the proteinase functional sequences are encoded by the 5′-terminal half of the HSV-1 UL26 ORF and ICP35 is encoded by the 3′ part of the same ORF. The proteinase is expressed by an mRNA which contains the whole ORF and is translated into a 635 residue product which represents the entire ORF and in effect comprises proteinase fused to ICP35. The mRNA for ICP35 initiates within the UL26 ORF, traverses only the 3′ portion of the ORF and is translated into a 329 residue product. Proteinase action on ICP35 consists of trimming the C terminus. The enzyme is also active in cutting between the two domains of the whole ORF product to yield the N-terminal proteinase domain and a form equivalent to ICP35 with an N-terminal extension. In SCMV two further transcript species translate into smaller portions of the 3′ region of the ORF (Welch *et al.*, 1991*a*).

The detail of biochemical logic underlying this maturation process remains not wholly clear: one could imagine that fusion of enzyme to substrate allows targeting of the enzyme to the capsid interior. It is evident that the process is an essential step in capsid formation, and as such it provides a new possible target for antiviral intervention. The herpesvirus proteinase has not been unambiguously assigned to one of the recognized groups of mechanistically related proteinases. Welch *et al.* (1991*b*) proposed on the basis of conserved elements in the sequences of six herpesviral proteinases that they could be related to the maturational proteinase of bacteriophage T4.

Viruses and programmed cell death

The cell biology term 'apoptosis' has recently been appearing in virological contexts. This is the name given to a particular process of cell death, characterized by loss of cell volume, degradation of genomic DNA into fragments representing a few nucleosomes and cytoplasmic blebbing. Apoptosis is a controlled, active process and is a normal physiological event, for instance, in organism development and maturation of populations of lymphocytes (see review by Williams, 1991). One gene which apparently plays a role in control of apoptosis is the oncogene *bcl-2*, whose product is thought to act to inhibit initiation of the apoptotic process. In follicular B cell lymphomas *bcl-2* is aberrantly expressed at a high level consequent on a characteristic chromosomal translocation, and thus cells accumulate to form a lymphoma because of a reduction in cell loss rather than an increase in cell proliferation. The mechanism of action of the *bcl-2* protein is not known. Uniquely for an oncogene product, it is apparently localized to the inner mitochondrial membrane (Hockenbery *et al.*, 1990). This section deals with the effects of Epstein–Barr virus (EBV) infection on apoptosis and also mentions involvement of apoptosis in infection by a baculovirus.

The human lymphotropic herpesvirus EBV readily immortalizes B lymphocytes, and the resulting lymphoblastoid cell lines (LBLs) express eight 'latent cycle' genes, including six whose products are localized to the nucleus (EBNA 1, EBNA 2, EBNA 3a, EBNA 3b, EBNA 3c and LP) and two membrane proteins (LMP 1 and LMP 2). EBV is associated with the disease Burkitt's lymphoma (BL). Cells from BL biopsies display a different version of the viral latent state from LBLs: the only viral gene expressed is EBNA 1 and the cells possess a distinct set of surface proteins. However, BL cells maintained in culture drift toward the LBL phenotype, with expression of more EBV latent genes and change in surface characteristics. Gregory *et al.* (1990) described subcloning of passaged BL cells to give BL-like clones (termed group I) and LBL-like clones (termed group III). In a subsequent paper, Gregory *et al.* (1991) then reported that the group I cells were highly sensitive in suboptimal culture conditions (such as low serum) to death by apoptosis, whereas the group III cells were resistant: the increased expression of EBV genes in group III cells apparently conferred resistance. This observation correlates with the somewhat paradoxical susceptibility of original BL cells to apoptosis.

In further work from the same group, Henderson *et al.* (1991) showed that group III cell lines exhibited a pronounced increase in expression of *bcl-2* relative to group I. They also introduced a set of vectors each

expressing an EBV latent protein into EBV-negative, apoptosis-sensitive lymphoma cells and examined apoptosis susceptibility and *bcl-2* expression. Only cells expressing LMP 1 became refractory to apoptosis, and only cells expressing LMP 1 showed increased expression of *bcl-2*. LMP 1 was previously known to have pleiotropic effects on cellular gene expression and phenotype, and Henderson *et al.* carried out experiments with BL cells expressing transfected *bcl-2* to build up a case that the effects of LMP 1 on *bcl-2* and on apoptosis were indeed causally related. They also noted briefly that *bcl-2* regulation by EBV proteins may involve more than LMP 1, since cells expressing both LMP 1 and EBNA 2 showed increased *bcl-2* expression.

From this work it was concluded that, in addition to EBV's action in transforming B cells to indefinite proliferation, the virus also acts to render these cells resistant to apoptotic death. This makes sense in the context of B lymphocyte biology: most B cells are programmed to have a short life span and to die by apoptosis. This work also increases perception of LMP as an effector of multiple aspects of EBV-induced change in cell phenotype. There is one additional fact, intriguing but of unknown significance, to register here: EBV possesses a gene, BHRF1, which is a clear homologue of *bcl-2* (Cleary *et al.*, 1986).

We finish this section with a brief mention of an insect viral system. During infection of cultured insect cells with the baculovirus *Autographa californica* nuclear polyhedrosis virus, cell lysis normally does not take place until at least 72 h post-infection, by which time the progeny virus particles are protected inside protein inclusion bodies. Clem *et al.* (1991) found that mutants in the p35 gene of the virus caused premature death of cells from some insects but not others, giving much lower titres of progeny, and that cell death was by characteristically apoptotic mechanisms. The p35 protein of wild-type virus thus may well act to prevent apoptosis, although its mode of action is unknown. Clem *et al.* speculated that apoptosis may be employed as a viral defence system in insects and that the p35 gene accordingly may act to increase viral host range and effective virulence.

Processing of antigens for presentation to class I- and class II-restricted T cells

Very notable advances are currently being made in analysis of the cellular mechanisms employed to present antigens to T lymphocytes, with implications for understanding viral interactions both at the cellular level and in terms of the whole organism.

T cells recognize on the surfaces of virus-infected cells or on specialized antigen-presenting cells complexes that consist of short peptides bound to highly polymorphic membrane proteins encoded by either class I or class II major histocompatibility complex (MHC) genes. T cell subtypes can be distinguished by the cell surface proteins CD4 and CD8. T cells recognizing peptide antigens associated with class I MHC molecules have a $CD8^+$, $CD4^-$ phenotype and those recognizing peptides associated with class II MHC molecules a $CD4^+$, $CD8^-$ phenotype. These phenotypes correlate to some extent with T cell functions. Helper T cells have a $CD4^+$, $CD8^-$ phenotype and recognize peptides in association with class II MHC molecules. In general, cytotoxic T lymphocytes (CTLs) are primarily class I-restricted, although in certain viral infections class II-restricted CTLs may also be important (for reviews see Long, 1989; Yewdell & Bennink, 1990; Randall & Souberbielle, 1990). In the past year, impressive progress has been made in analysing natural peptides bound to both class I and class II MHC molecules and in elucidating further the mechanisms by which such peptides are generated and how they become associated with MHC molecules.

To analyse natural peptides bound to MHC molecules, class I and class II molecules from appropriate cell lines have been purified by binding to MAbs, and the bound peptides recovered and sequenced (for review see Germain, 1991). The peptides eluted from class I MHC molecules contained eight or nine residues (Schumacher *et al.*, 1991; Madden *et al.*, 1991; Jardetzky *et al.*, 1991; Falk *et al.*, 1991). When multiple peptides associated with a single class I species were sequenced the different peptides were found to contain consensus amino acids at their N and C termini. Residues at other positions were variable, although at certain positions there appeared to be some bias towards particular amino acids (Jardetzky *et al.*, 1991; Falk *et al.*, 1991). These findings give rise to the expectation that more effective methods may be developed for predicting from protein sequences peptides which will be active with individual MHC molecules (Janeway, 1991*b*). The hope is that a combination of direct peptide sequencing and improved predictive programs for CTL sites may advance the use of suitable peptide cocktails for vaccines. When considering the possible composition of peptide vaccines it may also be of significance that synthetic octa- or nonapeptides corresponding to the eluted peptides proved to be significantly more potent for presentation to T cells than the longer synthetic oligopeptides previously employed.

In contrast to the situation observed with MHC class I peptide associations, natural peptides bound to class II MHC molecules were found to range between 13 and 17 residues (Rudensky *et al.* 1991). Here it appears that the peptides bound may have precisely defined N termini and that the C-terminal positions may vary. Further-

more, analysis of multiple peptides from a single class II species detected no conservation of particular amino acids at given residues and it may, therefore, not be feasible to predict dominant class II-restricted T cell sites.

These differences in the nature of sets of peptides associated with the two classes of molecules are considered to result from the different processing mechanisms utilized for class I and class II presentation (Peters *et al.*, 1991; Germain & Hendrix, 1991; for reviews see Hackett, 1991; Yewdell & Bennink, 1990). In turn, differences in the processing pathways reflect the different functions of the two classes. In general, the primary function for class I presentation is to sample and present peptides derived from intracellular proteins (endogenous antigens), so facilitating recognition of virus-infected cells. Class II molecules, however, present to T helper cells peptides derived from exogenous antigens captured from extracellular fluid by specialized antigen-presenting cells such as macrophages, dendritic cells and antigen-specific B cells.

Much has been learned of these two quite distinct processing pathways over the last few years. For endogenous antigens, proteinase complexes in the cytoplasm ('proteasomes') continually break down proteins to short peptides which then become transferred to the lumen of the endoplasmic reticulum (ER). There has been much recent debate as to whether transfer from the cytoplasm to the ER is an active process, using ATP-driven peptide transporters, although the bulk of evidence suggests that this is indeed the case (Powis *et al.*, 1991; Spies & DeMars, 1991; Kelly *et al.*, 1991; Spies *et al.*, 1991; Attaya *et al.*, 1991; Levy *et al.*, 1991; for reviews and comments see Dobberstein, 1991; Parham, 1991). It is considered that in the ER peptides with the appropriate affinity for newly synthesized class I heavy chains induce a conformational change in the heavy chain which then results in its stable association with β_2 microglobulin, and enables transport of the complex through the Golgi apparatus to the cell surface (Elliot *et al.*, 1991).

Until recently it was assumed that the polymorphism of class I molecules was the sole variable in selecting peptides to be bound. However, as a further twist it has now been reported that polymorphism of peptide transporters may also influence the peptides loaded by class I MHC molecules (Powis *et al.*, 1992; Parham, 1992). Interestingly, the genes encoding the peptide transporters are located within the class II MHC gene cluster (Deverson *et al.*, 1990; Trowsdale *et al.*, 1990; Spies *et al.*, 1990; Monaco *et al.*, 1990). Since genes for proteasome components are also located in the class II gene cluster (Brown *et al.*, 1991; Glynne *et al.*, 1991; Ortiz-Navarrete *et al.*, 1991; Martinez & Monaco, 1991; Kelly *et al.*, 1991; Robertson, 1991) it appears that there may exist a cassette of linked genes involved with antigen processing and presentation.

The processing pathway which results in the association of class II MHC molecules with peptides is quite distinct from the class I mechanism. Newly synthesized α and β chains of class II molecules do not normally become loaded with peptides in the ER (Germain & Hendrix, 1991). Rather, they become associated with a protein termed the invariant (Ii) chain, which appears to have two functions, namely to prevent peptides in the ER from binding to the class II molecule (Roche & Cresswell, 1990; Teyton *et al.*, 1990; Germain & Hendrix, 1991) and to chaperone the class II molecule specifically to endosomal acidic vesicles (Lotteau *et al.*, 1990; Bakke & Dobberstein, 1990). It is in these acidic vesicles in antigen-presenting cells that exogenous antigens are proteolysed to peptides. In these peptide-rich compartments, the Ii chain dissociates from the class II molecule which then becomes loaded with a peptide. Recent evidence suggests that this association of peptides with class II molecules is irreversible (Lanzavecchia *et al.*, 1992).

In terms of vaccine design, the separate processing pathways for class I and class II antigen presentation present a potential problem. Given that class I-restricted CTLs play a critical role in response to viral infections and that peptides which become associated with class I MHC molecules are derived from endogenous antigens, the problem is how to induce class I-restricted CTLs by immunization with inactivated virus or purified virus antigens (Randall & Souberbielle, 1990). However, it is possible for exogenous antigen to be processed for class I presentation if, rather than being confined to endosomal vesicles, some antigen also enters the cytoplasm. This can be achieved relatively easily in tissue culture cells using a variety of methods. Recent work has also demonstrated that class I-restricted CTLs are induced *in vivo* if virus antigens are presented in certain structures, including immune-stimulating complexes (Iscoms) and solid matrix–antibody–antigen complexes (e.g. Takahashi *et al.*, 1990; Randall & Young, 1991). It has even been reported that oral immunization with Iscoms can induce good CTL responses (Mowat *et al.*, 1991). However, the efficiency of induction of class I-restricted T cells with exogenous antigens may be substantially lower than with infectious virus (Randall & Young, 1991). Nevertheless, it may be possible to develop more efficient ways of inducing class I-restricted T cells with virus antigens by investigating how exogenous antigen can enter the class I presentation pathway *in vivo*. For example, it may be that there are specialized antigen-presenting cells *in vivo* capable of processing antigen for class I presentation. If these could be identified it may be possible to 'tag'

antigens such that they specifically bind to these cells. Alternatively, it may be possible to incorporate antigens into structures which fuse with plasma membranes and release their contents into the cytoplasm.

Superantigens and viruses

The term 'superantigen' is currently applied to two classes of molecules which act on class II-restricted T cells. The characteristic feature of superantigens is that they exert effects on large subsets of such T cells (representing perhaps 10% of the repertoire of T cell $\alpha\beta$ receptors) with a specificity determined almost exclusively by the Vβ portion of the receptor. This is in distinction to standard antigens presented by class II MHC molecules, which interact with only a small subset of T cells whose specificity is contributed to by all the variable portions of the receptor (Vα, Jα, Vβ, Dβ, Jβ).

The first class of superantigens comprises microbial proteins, for instance staphylococcal toxins. It is considered that these act to crosslink a part of the Vβ domain of the T cell receptor not employed in normal antigen recognition with a class II molecule on a presenting cell. The second class, of direct relevance to this review, contains endogenous antigens found in mice. These are recognized in two ways. First, as alloantigens in mixed cultures of spleen cells with cells from a distinct mouse strain (but with the same MHC genotype), they may cause extensive proliferation of CD4$^+$ T cells carrying a particular Vβ sequence; genomic loci controlling such activation have been mapped and are referred to as minor lymphocyte-stimulating (*mls*) elements. The second phenotype of *mls* elements is that they cause in their carriers deletion of the subset of mature CD4$^+$ T cells carrying the appropriate Vβ sequence; this presumably results from the action of systems responsible during T cell maturation in the thymus for elimination of cells reactive with self antigens (for review see Janeway, 1991*a*).

This aspect of murine immunology has recently acquired a virological dimension. Genetic mapping indicated that one *mls* locus lay close to the positions of an integrated mammary tumour virus (MTV) genome (Woodland *et al.*, 1990) and more extensive analyses demonstrated complete concordance between the positions of a number of integrated MTVs and *mls* loci (Dyson *et al.*, 1991; Frankel *et al.*, 1991; Woodland *et al.*, 1991; see review by Janeway, 1991*a*). In closely related work, Marrack *et al.* (1991) described a maternally inherited superantigen of C3H mice. This was transmitted to progeny after birth apparently by suckling, and so behaved like a non-defective, milk-transmitted, exogenous MTV. In subclasses of C3H mice, activity of the superantigen correlated with exposure to exogenous MTV.

These papers thus suggested that for at least some *mls* loci endogenous MTV genomes might be directly involved in generating superantigens. Subsequent papers have pursued this idea and have demonstrated that the *mls* phenotype can be conferred by just one of the MTV genome's coding regions, namely a 320 codon ORF in the 3′ long terminal repeat. This ORF is common to MTV isolates but is not found in other retroviruses. Choi *et al.* (1991), using the exogenous C3H MTV mentioned above, cloned the 3′ ORF into an expression vector and introduced this into B lymphoma cells. The ORF-carrying cells were found to express superantigen specifically, as assayed by stimulation of interleukin-2 from a Vβ14$^+$ T cell line and by induction of proliferation of Vβ14$^+$ T cells. Another paper, by Acha-Orbea *et al.* (1991), described the behaviour of mice transgenic either for the whole genome of an MTV isolate or for the 3′ ORF; both groups of animals exhibited specific deletion of Vβ14$^+$ T cells.

Thus, there is now firm evidence that integrated murine MTV genomes constitute *mls* loci and that the product of the 3′ ORF unique to this group of retroviruses is involved in the *mls* phenotype, most likely directly as the superantigen molecule. The superantigen protein has not been identified, but is presumably expressed on the surface of B cells or is perhaps secreted. Known *mls* loci react with a range of Vβ sequences, and it is suggested that specificity determinants may reside in a variable, C-terminal part of the 3′ ORF proteins. In a separate murine retroviral system, Hügin *et al.* (1991) reported that a defective leukaemia virus which causes an immunodeficiency syndrome is also responsible for presentation on B cells of a superantigen causing proliferation of CD4$^+$ T cells, and that this effect was specifically inhibited by addition of a MAb against the *gag* p30 protein of the virus, suggesting a mechanism distinct from that employed by the endogenous MTVs.

Two classes of proposal have been made concerning possible roles of MTV-related superantigens. On the one hand, induced T cell proliferation might represent the creation of a cell population susceptible to infection by an exogenous superantigen-expressing virus, to the benefit of the virus. On the other hand, the endogenous *mls* elements may act in the host's interest by avoiding response and susceptibility to incoming viral or bacterial superantigens. There are probably 20 to 30 endogenous MTV loci in mice; these loci are of relatively recent evolutionary origin and may not have counterparts in other mammals. The general significance of these phenomena thus remains obscure, although they do contribute a possible extra dimension in considering the complexities of T cell dynamics in human AIDS.

Advances in MAb technology

MAbs have had an enormous impact in virology and biomedical sciences in general over the last decade and novel applications are still being developed (Harris, 1991). However, there are major drawbacks to conventional methods for both isolating and producing MAbs. For example, to obtain a source of B lymphocytes for hybridoma production animals normally have to be immunized. Commercial production of MAbs is also expensive since it requires either the use of animals (for production of ascitic fluid) or the large-scale culture of hybridoma cells. Furthermore, conventional technology for the production of human MAbs has proved largely unsuccessful.

To circumvent these problems, much effort is being put into producing functional human and rodent antibody fragments (Fab or single chain Fv fragments) in bacterial expression vectors (e.g. Buchner & Rudolph, 1991; Skerra *et al.*, 1991; for reviews see Winter & Milstein, 1991; Burton, 1991). This is achieved by manipulating the variable segments of immunoglobulin genes that encode the variable domains of both light and heavy chains. Using sets of 'universal' primers it is possible to amplify and rescue these VH and VL gene segments for direct cloning and expression. Thus combinatorial libraries can be generated in which there is a random association of VH and VL gene segments. A significant limitation of this approach is that in a random combinatorial library at least 10^8 VH and VL pairs would have to be constructed to have a reasonable chance of rescuing a rare original gene pairing that specified an antibody with high affinity for a particular antigen (Winter & Milstein, 1991; Gherardi & Milstein, 1992). However, the construction of libraries from mRNA from immune lymphocytes, rather than from DNA, may significantly improve the chances of obtaining high affinity antibody to specific antigens (Burton, 1991).

Since it is not ethically feasible to isolate lymphocytes from specifically immunized humans to generate combinatorial libraries, the prospect of obtaining high affinity human antibodies to immunologically uncommon antigens by these techniques seems at first sight remote. However, a number of ingenious solutions to this particular problem are being pursued. For example, to increase the chances of obtaining human antibodies with desired specificity, mice exhibiting severe combined immunodeficiency and populated with human peripheral blood lymphocytes were immunized in order to induce secondary immune responses to specific antigens. Monoclonal human Fab fragments to these antigens were then generated by rescuing the appropriate VH and VL pairs from lymphocytes taken from these animals through the generation of combinatorial libraries (Duchosal *et al.*, 1992). Alternatively, using a purely molecular approach, it has been shown that both single chain and Fab fragments can be expressed as surface components of recombinant filamentous bacteriophage. The genes encoding desirable VH and VL pairs can then be isolated by selection from a bacteriophage library using immobilized antigen (McCafferty *et al.*, 1990; Clackson *et al.*, 1991; Hoogenboom *et al.*, 1991; Persson *et al.*, 1991; Barbas *et al.*, 1991; Kang *et al.*, 1991). However, the approach predominantly gives only low affinity antibodies using combinatorial libraries generated from non-immune lymphocytes. Thus, systems are also being developed to enable affinity maturation of selected bacteriophage antibody *in vitro* (Gram *et al.*, 1992).

This area of work offers the prospect that MAbs may routinely be generated with no recourse to immunizing animals. Furthermore, the technology should be equally applicable to cloning and expressing human antibodies. Indeed, human Fab fragments have been generated against hepatitis B surface antigen (Zebedee *et al.*, 1992) and gp120 of HIV-1 (Burton *et al.*, 1991) by antigen selection from a random combinatorial library expressed on the surface of bacteriophage. The use of human MAbs in protecting high risk individuals from infection with HIV looks particularly promising given the observation that a human MAb with neutralizing activity has been reported to protect chimpanzees from infection with virus (Emini *et al.*, 1992).

An alternative molecular approach to making human MAbs is also becoming better established. Here the complementarity-determining regions (CDRs) of rodent MAbs of defined specificity are grafted onto human antibody frames by genetic manipulation (Riechmann *et al.*, 1988). For example, using this approach the CDRs from a murine MAb that neutralizes human respiratory syncytial virus (RSV) have been transferred to a human IgG frame. The resulting reshaped human antibody, after some minor adjustments, retained the ability to cross-react with clinical isolates of RSV, and was active prophylactically and therapeutically against RSV infection in mice (Tempest *et al.*, 1991). Although there remain many technical problems to be resolved in cloning and expressing antibodies, it may be that we are seeing the beginning of the end of conventional methods for making MAbs. These technical advances mean that the widespread clinical use of human MAbs in combating virus infections may become a realistic proposition.

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