Chimpanzee Research: An Examination of Its Contribution to Biomedical Knowledge and Efficacy in Combating Human Diseases

SUPPLEMENT

Detailed reviews of each of the 27 papers citing the 14 chimpanzee papers from our random sample that had described human prophylactic, diagnostic or therapeutic methods were carried out, along with reviews of the cited chimpanzee studies, to assess the contribution of the cited chimpanzee studies to the methods described. The papers are summarized in **Table 1** (main document); full detailed reviews are described below:

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Human prophylactic, diagnostic or therapeutic methods

1. Cancer (non-specific): a therapeutic method

An extensive review by Tong and Stone (Tong and Stone 2003) summarized progress leading to clinical trials of the safety and efficacy of potential novel anticancer agents that modulate the activity of the CD40 cell surface receptor. CD40-related treatment approaches have been considered for the experimental therapy of human leukemias, lymphomas, and multiple myeloma, based on findings that binding of CD40 by its natural ligand CD154 (also known as CD40L) leads to growth modulation of malignant B cells. Furthermore, there is selective expression of the CD40 receptor on human epithelial and mesenchymal tumors but not on most normal, non-proliferating epithelial tissues, and ligation of CD40 to human breast, ovarian, cervical, bladder, non small-cell lung, and squamous epithelial carcinoma cells has been found to inhibit their growth via cell cycle blockage and/or induction of apoptosis. CD154 treatment also heightens tumor rejection immune responses via the activation of dendritic cells, and by increasing tumor immunogenicity by way of up-regulation of co-stimulatory molecule expression and cytokine production.

The review cited 155 publications, many of which have contributed to the development of these approaches to treatments involving CD154 recombinant protein and gene-therapy, and our understanding of the multifaceted molecular mechanisms of CD154–CD40 interactions. Overwhelmingly (over 100 of the 155 cited references), this information was generated by *in vitro* studies using cultures of human lymphoma cells, B lymphocytes, multiple myeloma cells, lymphocytic leukemia cells, Burkitt's lymphoma cells and various epithelial cells, as well as human studies. There were also 27 *in vitro* or *in vivo* studies in which rodents or their tissues and cells were used, both to study cellular and molecular processes, and also during preclinical drug testing.

In contrast to the citing Tong and Stone review in question, which examined the role of CD154 and the consequences of CD40-CD154 binding to the growth inhibition of malignant cells, the cited chimpanzee study from our random sample (Brams *et al.*, 2001) looked at the activity of a humanized antibody to CD154 in blocking B-cell activation via inhibition of CD40-CD154 binding, not for cancer treatment, but rather with a view to the treatment of autoimmune diseases and prevention of allograft rejection. The stated objective was essentially to determine if humanization of the anti-CD154 antibody had resulted in loss of affinity, specificity and functional activity of the molecule. The bulk of the cited paper reported the *in vitro* cloning, production and purification of humanized antibodies, *in vitro* binding and binding-inhibition studies, *in vitro* tests for antibody-mediated inhibition of B-cell proliferation and differentiation, *in vitro* assays of antigen-induced B-cell activation using human spleen cells, and *in vitro* assays of effector functions (such as induction of depletion and/or lysis of $CD154^{+}$ cells). The *in vivo* chimpanzee research in this paper comprised a 'safety study' in the form of an assay of the antibody's effects on lymphocyte populations, and pharmacokinetic measurements. Notably, these studies had been done previously, using cynomolgus monkeys.

The chimpanzee element of the cited Brams *et al*. paper cannot be considered to have contributed significantly to the citing Tong and Stone paper for a number of reasons.

It appeared in a paragraph summarizing the functions of CD40 in human cells, more specifically in a discussion of CD40-activated proinflammatory responses in cells of the reticuloendothelial system. Specifically, it is cited to support the observation of increased CD154 expression in T cells of human patients with systemic lupus erythematosus, which could be culpable for the disease pathogenesis by virtue of associated proinflammatory activities on interaction with CD40. The experiments in chimpanzees *per se* had nothing to do with this observation. The cited Brams *et al*. paper was not a chimpanzee-based study, but simply reported the use of chimpanzees to test safety and efficacy of therapeutic humanized antibodies developed and tested using *in vitro* technologies. The types of therapies were very different: Brams *et al.* dealt with the use of antibodies to block CD40-CD154 interaction in the treatment of autoimmune disease, whereas Tong and Stone discussed the use of CD154 itself in the therapy of various cancers.

Even if chimpanzee use had been central to the development of CD40-directed cancer therapy so far, there is no evidence of its use in current clinical practice. However, there are ongoing clinical trials in this area, for example using *ex vivo* transgenesis of patients' cells to express CD154 to treat chronic lymphocytic leukemia, malignant melanoma and mantle cell lymphoma, and use of adenoviral transfer of the CD154 gene to esophageal tumors to stimulate anti-tumor immune response.

2. Chronic obstructive pulmonary disease (COPD): a prophylactic method

A research paper by Suzuki *et al.* (Suzuki *et al.*, 2001) investigated the efficacy of prophylactic erythromycin therapy in patients with lower respiratory tract infections exacerbated by COPD. COPD predisposes sufferers to acute lower respiratory infections, often caused by human rhinoviruses (HRVs), RSVs, or influenza and parainfluenza viruses (Stenhouse 1967; McNamara *et al*., 1969; Monto 1995).

Long-term low-dose erythromycin therapy has been shown to be beneficial to patients with respiratory diseases such as bronchiolitis and bronchiectasis, for reasons other than its antibiotic properties (Kudoh 1998; Kudoh *et al*., 1998), which prompted this prospective, randomized, controlled trial of erythromycin therapy in lowering the frequency of the common cold and subsequent exacerbation of symptoms in patients with COPD.

After observing the 104 participants for 12 months and noting the frequency and severity of common colds and COPD exacerbations, Suzuki *et al.* found that prophylactic erythromycin therapy offered significant benefits to COPD patients, with considerably reduced rates of colds, repeat colds and exacerbations.

Most sources cited by Suzuki *et al.* as contributing to the development of this potentially therapeutic method were clinical studies of human patients. Observation of COPD exacerbations following bacterial airway infections and common colds was made in human patients (Smith *et al*., 1980), and the important implication of immune involvement and associated inflammation was achieved via epidemiological analysis of asthma sufferers (Gern and Busse 1999).

Prior evidence of successful erythromycin therapy against respiratory disease was obtained from human studies of erythromycin therapy in patients with diffuse panbronchiolitis (Kudoh 1998; Kudoh *et al*., 1998), and also from a study of influenza virus-induced lung injury in mice (Sato *et al*., 1998). Evidence of an anti-inflammatory as opposed to an anti-infective mechanism being responsible was obtained from studies of cultured human bronchial epithelial cells in which inflammatory cytokines were down-regulated (Khair *et al*., 1995).

The cited chimpanzee study (Huguenel *et al.*, 1997) involved an investigation into cold prevention by means of inhibiting HRV-binding to airway epithelia via binding to soluble intercellular adhesion molecule (ICAM)-1. This is not relevant to the citing Suzuki *et al.* study of erythromycin-based protection of COPD patients. It was cited in part of the Discussion devoted to a brief history of attempts to prevent and cure the common cold. Nevertheless, this chimpanzee study might still have been of benefit for COPD patients had it provided reliable information regarding the efficacy of soluble ICAM-1 in preventing human infection by cold-causing rhinoviruses. However, although a preliminary human study was cited (Turner *et al*., 1999), there is no evidence to suggest that any subsequent human trial of this type of agent has been successful.

3. Epstein-Barr virus: prophylactic methods

This extensive review of 112 papers by Khanna *et al.* (Khanna *et al.*, 1999*a*) detailed the phenotypes connected with a number of EBV-associated human diseases, the molecular bases of immune responses to them, and vaccine development.

Since the discovery of EBV 40 years ago, an immense volume of research has contributed to the understanding of its life cycle. Most of this knowledge has come from the study of EBV-infected lymphoblastoid cell lines (LCLs), revealing profound differences in gene expression in latently infected B-cells associated with Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, infectious mononucleosis, and post-transplant and X-linked lymphoproliferative diseases. The existence of strong memory T-cell responses to EBV infection in the peripheral blood of healthy virus carriers was demonstrated via *in vitro* examination of T-cells from seropositive donors (Moss *et al*., 1978; 1979). Analysis of cytotoxic T lymphocyte (CTL) responses from more than 50 human volunteers revealed that certain EBV latent antigens were more reactive than others (Khanna *et al*., 1992; Murray *et al*., 1992), and lytic antigens were characterized using synthetic peptides and recombinant vaccinia virus (Bogedain *et al*., 1995; Elliott *et al*., 1997; Steven *et al*., 1997; Pepperl *et al*., 1998; Khanna *et al*., 1999*b*; Tan *et al*., 1999). A role for class II-restricted $CD4^+$ CTLs in addition to class-I restricted $CD8^+$ CTLs in controlling EBV infection was suggested by *in vitro* studies of cultured cells (Misko *et al*., 1981; 1984).

Based on the considerable data provided by these studies, the authors concluded that the time was ripe to proceed towards vaccine development. They suggested two broad approaches: a vaccine against an EBV structural antigen, and use of CTL epitopes from EBV latent antigens, restricted through common human leukocyte antigen (the major histocompatibility complex of humans; HLA) alleles, as immunogens. This latter approach is of particular interest due to its link with the cited chimpanzee study from our random sample (Bertoni *et al.*, 1998). The strategy involves the generation

of an antiviral $CD8^+$ T-cell repertoire using synthetic immunodominant epitopes known to be recognized by the CTL response. Because Bertoni *et al.* had observed an overlap between the CTL repertoire in humans and chimpanzees, the authors of this review suggested that chimpanzees could '*provide an excellent platform for vaccine development.*'

It is clear, however, that this citation is speculative, linking an observation about CTL similarity between species to the hope of an eventual successful vaccine for EBV. The authors admit this, and other problems endemic in non-human primate models by stating, "*...the effect of minor HLA allelic variation on the presentation of CTL epitopes will need to be considered while formulating an EBV vaccine based on CTL epitopes."* (page 54), and, "*It would be a mistake to assume that experimental results obtained in these ... primate models had direct relevance to vaccine formulations that might offer protection against infectious mononucleosis [one of the main targets for a vaccine]"* (page 54).

4. Hepatitis A and B: prophylactic methods

Immunization with HAV and HBV vaccines is the predominant strategy for control of these infections. Because of significant overlap between individuals at risk of HAV and HBV, combination vaccines have been developed to improve protection rates. A review of 85 references by Koff (Koff 2002) described progress towards the development of HAV, HBV, and, in particular, combination vaccines.

Human clinical studies were the primary sources reported by Koff as contributing to the development of combination HAV and HBV vaccines; of a total of 85 references, 83 comprised clinical investigations, reviews, guidelines, and epidemiological and *in silico* studies. For example, an early study of an unidentified combination vaccine developed by Merck and Co. Inc., showed adequate immunogenicity in healthy human volunteers to the HAV but not the HBV component (Frey *et al*, 1999). A later study demonstrated high immunogenicity for both components, and high tolerance when given in a three-dose series at 0, 6 and 12 months (Knoll *et al*., 2000). A study of 843 healthy adults aged 17 to 60 years demonstrated 100% and 95% durability of vaccine-induced antibodies at four-years post vaccine, for anti-HAs and anti-HBs, respectively (Thoelen *et al*., 1999). Other cited studies investigated children aged 1-11 years, and 10-19 years, and young adult healthcare workers (Lee *et al*., 1999; Czeschinski *et al*., 2000; Kallinowski *et al*., 2000; Van Der Wielen *et al*., 2000). Overall, these studies showed improved immunogenicity and tolerance in adults and children, and pediatric use has been approved in Europe. Additionally, in 2001 the FDA approved the combination hepatitis A and B vaccine Twinrix (GlaxoSmithKline), which was already licensed in 70 countries outside the U.S..

Two chimpanzee studies were also cited by Koff. Prince *et al.* (Prince *et al.*, 1997) examined the prophylactic efficacy of DNA based immunization against hepatitis B virus in neonatal chimpanzees, following on from similar studies in mice. Two chimpanzees vaccinated at birth and boosted at 6 and 24 weeks with a plasmid coding for hepatitis B surface antigen produced transient antibody to the hepatitis B surface antigen, and proved resistant to HBV challenge at 33 weeks; in contrast, an unimmunized control chimpanzee developed detectable hepatitis B surface antigen and antibody to the core protein, the conventional markers of hepatitis B infection.

Koff also described a human study (Roy *et al*., 2000) published three years after the infant chimpanzee study, in which a novel DNA-based HBV vaccine gave promising results in human volunteers. The chimpanzee study appears to have been a trial run of this approach, with the aim of indicating safety and potential efficacy. Koff stated that additional studies of dose-response curve, ideal frequency of administration, duration of immunity, and evidence of safety would be necessary before this vaccine should be approved.

The cited chimpanzee study from our random sample (Ogata *et al.*, 1999) investigated the prophylactic efficacy of a licensed recombinant hepatitis B vaccine against HBV strain AS, in which an arginine for glycine substitution at surface gene codon 145 resulted in a variation in the antigenic surface protein. Such variation is considered to represent a possible antibody neutralization escape mutant. Four vaccinated chimpanzees developed antibodies to hepatitis B surface antigen (anti-HBs), and thereafter also proved resistant to intravenous challenge with mutant HBV strain AS, in contrast to two unvaccinated controls who succumbed. This result was also demonstrated by enzyme-linked immunosorbent assay (ELISA): serum anti-HBs in the vaccinated chimpanzees reacted not only with wild-type surface antigen, but also with mutant surface antigen. Consequently, Ogata *et al.* concluded that immunization of chimpanzees with licensed recombinant hepatitis B vaccines stimulates broadly reactive anti-HBs, affording protection against surface gene mutants of HBV.

Interestingly, however, Koff cited this chimpanzee study as potentially *complicating* our understanding of escape viruses (page 1190). He then acknowledged the inability of chimpanzee studies to accurately predict human outcomes, cautioning that "*continuous surveillance of breakthrough infections"* will be needed, despite the previously published result in chimpanzees.

Finally, in contrast to the seven human studies cited (Frey *et al*, 1999; Knoll *et al*., 2000; Thoelen *et al*., 1999; Lee *et al*., 1999; Czeschinski *et al*., 2000; Kallinowski *et al*., 2000; Van Der Wielen *et al*., 2000) and the stated goal of the author, neither of the cited chimpanzee studies examined the cross-protective effects of combined HAV and HBV vaccines – the major potential prophylactic method explored in this review by Koff.

5. Hepatitis viruses A through G: prophylactic and therapeutic methods

Around 100 million people worldwide and nearly 4 million in the United States are infected with HCV, probably the most common cause of chronic liver disease (Regev and Schiff 1999), with approximately 600,000 deaths resulting from hepatitis B in 2002 (WHO 2005). Regev and Schiff's review of 71 papers (Regev and Schiff 1999) describes the epidemiology, natural history and prophylactic options available for the prevention of HAV, HBV, HCV, HEV, HGV and the related transfusion-transmitted virus (TTV), and recent advances in the field.

Three human studies are cited examining the risks of HAV and efficacy of vaccines in patients with chronic liver disease, who are at particular risk of HAV. Patients with chronic HCV have a particularly substantial risk of fulminant hepatitis and death associated with HAV superinfection, supporting recommendations that patients with

chronic liver disease, and especially with chronic HCV infection, should be vaccinated against HAV (Vento *et al*., 1998).

Regev and Schiff also cited 12 papers that (with the exception of one molecular study) were human based, examining the efficacy of Interferon-α (IFN-α) and alternative treatments including liver transplantation for HBV (e.g. (Lau *et al*., 1997; Heijtink *et al*., 1997; Sokal *et al*., 1998)). Four cited papers predominantly relied on human clinical trials, and discussed the efficacy of alternative therapeutic regimes using the nucleoside analogues lamivudine and famciclovir (e.g. (Lai *et al*., 1998; Tassopoulos 1998)). Two cited papers described the development of escape mutants in vaccinated infants (Hsu *et al*., 1997) and of the incorporation of pre-S1 and pre-S2 antigens into the HBV vaccine to improve its immunogenicity in nonresponders (Waters *et al*., 1998). Implications of new strategies for improved outcomes in liver transplantation in HBV patients were described by three human studies (Dickson 1998; Ghany *et al*., 1998; Protzer-Knolle *et al*., 1998), and the epidemiology, natural history, and treatment of HCV by 48 almost exclusively human studies (one used a mathematical model, another relied on molecular techniques).

Two papers were cited investigating the epidemiology of HEV, with Wu *et al.* (Wu *et al.*, 1998) reporting human epidemiologic and molecular studies, and Mast *et al.* (Mast *et al.*, 1998) (the cited chimpanzee study from our random sample), using sera from both humans and chimpanzees to investigate the reliability of 12 anti-HEV assays.

Chimpanzees in the latter study were used as both positive and negative controls, though both were redundant, for more-relevant human controls existed in the form of undiluted sera obtained 2 months to 13 years after acute hepatitis E infection, and sera obtained from previously tested U.S. blood donors with no history of hepatitis. Their inclusion may have been to incorporate more varied strains of the virus into the investigation, but the authors acknowledge that the assays were designed to detect *human* antibodies, and that differences may exist in their ability to detect those from chimpanzees. The results also indicated profound between-assay discordance, and consequently Regev and Schiff recommended that anti-HEV seroprevalence data in non-HEV-endemic countries derived from these assays be interpreted with caution.

The cited Mast study was not cited elsewhere by Regev and Schiff, and so cannot be considered to have contributed to the development of anti-viral-hepatitis therapeutics as described. At best, Mast *et al.* may have contributed to our understanding of the epidemiology of HEV, but the chimpanzee sera used were redundant to human sera, as discussed above.

6. Hepatitis B virus: a prophylactic method

A research paper by McMahon *et al.* (McMahon *et al.*, 2005) described a prospective cohort study of almost 1,000 people vaccinated against HBV, and aimed to assess long-term vaccine efficacy.

Most cited references referred to epidemiological HBV research and *in vitro* methods (such as radioimmunoassay) of testing vaccinated groups of people, largely in studies

of immunization efficacy and duration: from a total of 38 references, 35 met these criteria, and another two pertained to computer analysis programs.

The cited chimpanzee study from our random sample (Ogata *et al.* 1999) and four of the citing paper's 37 additional references (Hsu *et al*., 1997; Carman *et al*., 1990; Fortuin *et al*., 1994; Hino *et al*., 1999) are mentioned in a section of the Discussion on variants of HBsAg in immunized people who had experienced breakthrough infections. Although mutant surface antigens were present in four of the six people with confirmed HBV infections – co-existing with the wild-type (natural) virus in three of these four cases – the clinical and epidemiological relevance of this is questionable, given that all were asymptomatic.

Ogata *et al.* was included in the discussion because it demonstrated that a commercial HBV vaccine had protected four chimpanzees against infection by a variant HBV strain named "AS." This suggested that the HBV vaccine used might be broadly protective against strains of the virus with mutations in its surface-antigen gene. Consequently, McMahon *et al.* consequently inferred that their detection of variant surface antigen in four people was most likely simply due to co-existing alternative strains of HBV in the population, rather than evidence of selection by anti-HBs (i.e. escape mutations), as has been observed in some neonates given post-exposure HBV vaccine and immune globulin (Carman *et al*., 1990; Hino *et al*., 1999). The cited chimpanzee study referenced eleven reports to support this theory, all of which are based on the study of infected humans (Ghany *et al*., 1998; Protzer-Knolle *et al*., 1998 Fortuin *et al*., 1994; Hino *et al*., 1999; Okamoto *et al*., 1992; Karthigesu *et al*., 1994; Kato *et al*., 1996; Brind *et al*., 1997; Nainan *et al*., 1997; Ngui *et al*., 1997; Ogata *et al*., 1997).

The cited chimpanzee study by Ogata *et al.* was clearly incidental to the main findings of McMahon *et al.*, which showed that the HBV vaccine can elicit protection against virus-induced hepatitis B for at least 15 years, and that the vaccine is more effective long-term when administered to people of a greater age. Furthermore, Ogata *et al*. described only one very specific variant of the HBsAg, with a glycine for arginine substitution at codon 145 (Gly-145-Arg), which is different from the variants observed in the citing McMahon *et al.* study of humans. Finally, Ogata *et al*. used an ELISA to demonstrate that serum anti-HBs antibodies from the vaccinated chimpanzees were immunoreactive with the AS-mutant surface. This might as easily have been employed using the human serum samples from McMahon *et al.*, which would have removed the need to extrapolate from an experiment that used a different HBsAg variant in a non-human species. Consequently the chimpanzee study cannot be considered to have made an essential contribution towards the duration of immunization of humans vaccinated against HBV.

7. Hepatitis B virus: prophylactic and therapeutic methods

In an extensive review with 300 references, Karayiannis (Karayiannis 2003) described past, present and potential future approaches to prophylaxis and antiviral treatment of HBV, a virus that (despite the availability of preventative vaccinations) chronically afflicts over 350 million people worldwide and also predisposes them to increased risks of cirrhosis, hepatic decompensation and hepatocellular carcinoma.

This review provided a brief summary of the genetics, life cycle and epidemiology of the virus, followed by a more in-depth discussion of treatment options. Current therapies comprised two main categories: *immunomodulators* such as IFN-α, thymosin and interleukin-12 (IL-12), and *nucleoside analogues* such as lamivudine and adefovir dipivoxil. Combination therapies involving more than one nucleoside analogue with or without an immunomodulator were also discussed. Proposed future treatments included *therapeutic vaccination* with recombinant viral antigens, new adjuvants to the traditional vaccine such as synthetic oligonucleotides that can stimulate T-helper cells and cytokine production, and peptide-based molecules containing T-cell epitopes; plasmid-based *DNA vaccines* encoding viral antigens, T-cell epitopes and cytokines; *antisense oligonucleotides* to inhibit viral gene expression, and *ribozymes* designed to interfere with viral gene activity by cleaving virus-specific RNA molecules.

Largely because current therapeutic methods are successful in only 20-30% of people, and even then their effects are transient, much research effort is being focused on these latter approaches.

The citations pertaining to current therapies were almost exclusively of reports describing *in vitro* experiments and human studies/clinical trials, which have clearly provided the majority of our knowledge of their efficacy, adverse reactions associated with them, mechanisms of action, long-term outcomes and viral mutations leading to drug resistance. A small proportion (approximately 2-3%) of the ~250 references referred to animal studies of these therapies used against duck and woodchuck hepatitis viruses, which note differences in efficacy with their use against human HBV.

As one would expect, citations describing proposed future therapeutic techniques were largely animal-based. Cited research into therapeutic vaccines (recombinant viral antigens, adjuvants, oligonucleotides and peptides) were a mixture of human studies, and experiments involving transgenic mice, woodchucks and even orangutans (with the caveat, "It remains to be seen whether similar responses are observed in human trials.") Cited research into the use of antisense nucleic acids and ribozymes came mostly from *in vitro* experiments, though some reports of research involving duck hepatitis B virus (DHBV) were included.

The cited chimpanzee study (Pancholi *et al.*, 2001) occurred in a description of research into DNA-based vaccines, in which pieces of DNA containing genes encoding viral proteins, T-cell epitopes and/or cytokines are injected intramuscularly, allowing the expression of these genes in a native environment and the production of relevant proteins that, it is hoped, might exert a therapeutic or protective effect. Several references (11 from a total of 24) were made to *in vivo* non-human studies, mostly using mice (transgenic and non-transgenic), but also ducks and, notably, NHPs. The NHP experiments were mainly for the assessment of safety and the induction of immune response, though results were variable; unlike chimpanzees and rhesus macaques, *Aotus* monkeys did not show a humoral immune response to DNA vaccination (Gramzinski *et al*., 1998), and in another cited chimpanzee study responses to DNA vaccination were variable: some individuals seroconverted while others didn't (Sallberg *et al*., 1998).

Pancholi *et al.* described the immunization of a rare chronic HBV-carrier chimpanzee with a DNA vaccine encoding the HBV viral surface antigen HBsAg, followed by a "boost" with a recombinant canarypox virus carrying HBV genes. The result was a dramatic reduction of HBV DNA, a temporary decline of the HBsAg levels, and a down-regulation of HBV covalently-closed circular DNA (cccDNA) – an important viral transcriptional template, the reduction of which indicates viral clearance in the liver.

Overall, the contribution of the chimpanzee HBV model to our knowledge of the virus, its life cycle and mechanisms of infection, and to the development of agents designed to combat it, could be considered more limited than proponents of chimpanzee research claim, given the lack of citations involving chimpanzee studies in the earlier part of the citing review. The authors are conscious of an ongoing argument about the contribution and necessity of chimpanzees to the historical progress of HBV research and vaccine development, but it is not within the scope of this review to address it.

It is difficult to ascertain the contribution of the Pancholi *et al*. chimpanzee study to the development of DNA-based vaccines safe and efficacious in humans, due to the paucity of human data. Existing studies suggesting that this form of vaccine is safe and well tolerated by human subjects are very few and involve small samples (10 patients (Mancini-Bourgine *et al*., 2004); 16 patients (Rottinghaus *et al*., 2003)).

Clearly this potential prophylactic method is some way from being sufficiently developed for routine human use. However, even if DNA vaccines were ultimately shown to be safe and effective in human beings, the contribution of chimpanzee research would seem scant. Differences in response to DNA vaccines between NHP species as described in the citing review by Karayiannis suggest that extrapolation from chimpanzee to human would be neither reliable nor predictive. As Pancholi *et al.* stated: "*Because chronically HBV-infected chimpanzees are rare, it may be more feasible [than performing further chimpanzee-based research] to contemplate a clinical trial*" (page 453).

8. Hepatitis C virus: a diagnostic method

Since 1989, HCV enzyme immunoassays (EIAs) using glycosylated viral envelope proteins E1 and E2 have been used for routine screening of donated blood (Donohue *et al*., 1992; Kleinman *et al*., 1992). However, E1 and E2 are difficult to express and purify, which hampers the detection of envelope antibodies in blood samples.

Consequently, in this research paper Hüssy *et al.* (Hüssy *et al.*, 1997) investigated the expression of recombinant E1 and E2 within *E. coli* and *Sf9* insect cells via baculoviral vectors. Ni^{2+}–NTA chromatography was used to purify the envelope proteins, which were subsequently tested using serum samples in an HCV EIA. Sources of serum were normal blood donors, chronically HCV-infected patients, a mixed titer panel, and several seroconversion panels.

Sera from infected patients (confirmed by 100% positive screening results with the (traditional) Cobas-Core anti-HCV EIA (Roche Diagnostic Systems)) revealed 10-40% anti-E1 positive sera using *Sf9*, and 93% using *E. coli* proteins. E2 screenings

yielded 70-73% and 70-80% anti-envelope activity for *E. coli* and *Sf9* proteins, respectively. Consequently Hüssy *et al.* concluded that both the recombinant E1 and E2 proteins expressed in *E. coli* and *Sf9* cells offered utility for the detection of envelope antibodies in the serum of HCV infected patients. In particular, they recommended the use of recombinant E1 expressed in *E. coli,* and co-expressed E1 and E2 proteins from *Sf9* cells.

Hüssy *et al.* described several previous studies that contributed substantially to the development of this diagnostic method. Hsu *et al.* (Hsu *et al.*, 1993) determined that the whole structural region of HCV can be expressed in insect cells, and that sera reacted with both E1 and E2, although the former, is more reactive suggesting that E1 recombinant antigens in particular could be useful diagnostic markers of infection. Nishihara *et al.* (Nishihara *et al.*, 1993) demonstrated the reactivity of E2 in an ELISA. Successful expression of E1 and E2 proteins by infection of mammalian cells with recombinant vaccinia viruses was achieved by Ralston *et al.* (Ralston *et al.* 1993), and in Chinese hamster ovary cells by Spaete *et al.* (Spaete *et al.*, 1992) and Lesniewski *et al*. (Lesniewski *et al*., 1995). Expression and purification of glycosylated HCV E2 envelope protein was achieved in *Sf9* insect cells by Hüssy *et al.* (Hüssy *et al* ., 1996).

The cited chimpanzee study from our random sample by Wang *et al.* (Wang *et al.*, 1996) described an investigation into the reactivity of humans and chimpanzees to various epitopes of HCV H strain structural proteins. They found that in 10 HCV-infected humans, epitopes were mostly mapped to the capsid and E1 proteins but not to E2. This human outcome is twice cited by Hüssy *et al.*, and in contrast the chimpanzee data within the cited Wang *et al.* paper made no contribution to the citing Hüssy *et al* paper.

Additionally, Wang *et al.* identified key differences between the immune response of humans and chimpanzees to HCV infection. Humans reacted chiefly to epitopes of the capsid and E1 proteins, but not to the E2 protein, and just over half reacted to the host-coded GOR antigen induced by the viral infection. Although HCV-rechallenged chimpanzees reacted to capsid epitopes, chronically infected chimpanzees did not, and neither group reacted to GOR. Although these findings are interesting, particularly to scientists who propose that knowledge of *differences* between species is important in the bigger picture, they are not relevant to the subject of the citing Hüssy paper.

In conclusion, the chimpanzee research described by Wang *et al.* did not contribute to the development of this new method for the expression of E1 and E2-recombinant proteins via recombinant baculoviruses within *E. coli* and in *Sf9* insect cells, as described by Hüssy *et al.* Additionally, although the sensitivity of the HCV EIA using the E1 and E2 thus derived offered good sensitivity in most cases, it did not equal that of a traditional Cobas-Core anti-HCV EIA.

9. Hepatitis C virus: a therapeutic method

A review of 91 references by Feld and Hoofnagle (Feld and Hoofnagle, 2005) described the evolution of interferon (IFN)-based therapy for patients with chronic hepatitis C, from the early days of short-term and ineffective monotherapy in 1986 to

contemporary practice with pegylated interferon/ribavirin (a broad-spectrum antiviral agent) combination treatments.

Over this time, despite a demonstrable improvement in sustained response rates from an average of 9% of patients to 55%, it remains the case that around 45-50% of hepatitis C patients do not mount a sustained response to these drugs. In addition, this form of combination therapy is expensive and associated with numerous and significant side-effects. It therefore follows that more efficacious and better-tolerated treatments are sorely needed, and Feld and Hoofnagle suggested that, by elucidating the mechanisms of action of interferon and ribavirin, these can be realized. Central to this endeavor will be to elucidate further why one third of patients completely fail to respond to treatment, and why viral kinetics differ among patients of all response types.

The authors showed that the major contributors to this therapeutic protocol, by far, have been *in vitro* and clinical research (76 out of 91 references cited). Clinical investigations of patients who do not respond to IFN-based therapy have focused on various issues: viral strain and quasispecies diversity; pharmacokinetic profiles (IFN and ribavirin dose and drug levels); disease characteristics (severity and activity of hepatitis); the effects of co-morbidities (obesity, diabetes, renal disease and immunodeficiency); problems in IFN cell signaling and actions; and potentially modifiable environmental factors (alcohol, smoking and adjunctive medications). *In vitro* and human clinical research have shown that IFN doesn't act directly on the virus, but affects the expression of interferon-specific genes (ISGs) to induce a general "antiviral state" within the infected cell by affecting viral mRNA stability and translation.

The cited chimpanzee study from our random sample (Bigger *et al*., 2001) and two additional cited chimpanzee studies (Su *et al*., 2002; Bigger *et al*., 2004) examined gene expression at various stages of HCV infection, and showed that IFN genes were not induced, though there was an increase in expression of many ISGs. However, not only did these studies simply "confirm" previous findings ("*Molecular and microarray data from chimpanzees acutely or chronically infected with HCV confirm the importance of IFN pathways and the innate immune system in the host response to infection"*) (page 969), but concurrent similar studies using human peripheral blood mononuclear cells and liver biopsies prior to treatment with IFN/Ribavirin were revealing human-specific results, in particular that non-responding patients have high baseline levels of ISG expression (Ji *et al*., 2003; Chen *et al*., 2005). In the cited chimpanzee study, Bigger *et al*. described liver-specific gene expression changes in "*an acute-resolving HCV infection in a [single] chimpanzee."* Clearly, little can be concluded from a sample size of one.

One important difference between the human and chimpanzee studies is that the latter have examined acute, rather than chronic, infection. However, there is absolutely no impediment to a similar gene expression study of acute hepatitis C in humans; human-based studies of acute hepatitis C are numerous and reveal important data (Aberle *et al*., 2006; Delic *et al*., 2005; Kamal *et al*., 2006; Micallef *et al*., 2006).

Finally, although this chemotherapeutic protocol offers some degree of hope to HCV sufferers, unfortunately half of chronically infected patients treated with this combination did not achieve sustained viral clearance.

10. Hepatitis C virus: a therapeutic method

Although IFN has been the most successful therapeutic agent for the treatment of chronic hepatitis C, it is effective in eradicating the virus in less than half of patients overall (Tsubota *et al*., 1994). The HCV-1b viral strain is widely distributed worldwide and is unfortunately more resistant to IFN therapy than many other subtypes, although IFN sensitivity of the strain varies with geographical location. In Japan, an area of the viral genome known as the interferon sensitivity-determining region (ISDR) is crucial to the degree of sensitivity of a patient to IFN therapy, but this is not the case in other geographical regions.

To elucidate the molecular basis of IFN resistance and the differences in IFN sensitivity for isolates of the same HCV-1b strain between Japan and other regions, Nakano *et al.* (Nakano *et al.*, 1999) described an investigation of the nucleotide sequences of HCV-1b isolates from around the world, concluding that a distinct group of HCV-1b isolates exists in Japan. Despite the phylogenetic proximity between HCV-1b isolates and the probable lack of statistical difference between them, they found differences at specific nucleotide residues that enabled their characterization. Nakano *et al.* concluded that differences in isolates of the same viral strain, and in their relative geographic distributions, account for the variable interferon sensitivities of human populations.

As described by Nakano *et al.*, epidemiological differences between HCV-1b isolates had been previously suggested by studies of hepatitis C patients (Khorsi *et al*., 1997; Zeuzem *et al*., 1997). A decade ago, a human-based Japanese study (Enomoto *et al*., 1995) showed that the presence of a specific viral amino-acid sequence ($NS5A_{2209-2248}$) known as the ISDR correlated with IFN responsiveness, and could therefore be used as a predictive marker: patients with a wild-type (natural) ISDR rarely respond to IFN therapy, whereas patients with an intermediate or mutant ISDR are much more responsive (Enomoto *et al*., 1996). These conclusions were reached by genomic comparison of HCV-1b viral isolates from non-responding patients before and after interferon treatment, and a subsequent cohort study.

The cited chimpanzee study from our random sample by Wang *et al.* (Wang *et al.*, 1996) described an investigation into the reactivity of humans and chimpanzees to various epitopes of HCV H strain structural proteins, concluding that there are distinct differences between the two species. HCV-infected humans reacted chiefly to epitopes of the capsid and E1 proteins, but not to the E2 protein, and over half reacted to the host-coded GOR antigen induced by the viral infection. Although HCV-rechallenged chimpanzees reacted to capsid epitopes, chronically infected chimpanzees did not, and neither group reacted to GOR.

This study was cited by Nakano *et al.* because it showed that E1 peptides were reactive to sera from human patients infected with HCV, suggesting the existence of B-cell and/or T-cell epitopes in this region. Nakano *et al.* had found that two of the three specific amino-acid substitutions identified between the Japanese (J) and Non-

Japanese (NJ) HCV-1b isolates were in the E1 region. Because mutations here appear to change the immunogenicity of the antigen, this supports the hypothesis that immune responses to the NJ and J isolates are different.

However, the inclusion of chimpanzees in the cited Wang *et al.* study serves only to highlight differences in the immune response of humans and chimpanzees to the hepatitis C virus. In fact, this study was cited by Nakano *et al.* only to demonstrate that peptides corresponding to putative E1 were reactive to sera from human patients infected with HCV, and the component of chimpanzee research contained within this paper must therefore be considered incidental to the citing paper by Nakano *et al.* Finally, while the citing Nakano *et al.* paper clearly contributed to our understanding of the variation in HCV responses to vaccination, no improved vaccines were described.

11. Hepatitis E virus: prophylactic methods

HEV accounts for the majority of enterally transmitted non-A, non-B hepatitis worldwide. In developing countries with poor sanitary conditions and high population density, it is responsible for more than 50% of cases of sporadic acute hepatitis and acute hepatic failure. Worm and Wirnsberger's review of 133 references (Worm and Wirnsberger, 2004) described populations and groups at risk of infection by HEV, its molecular biology, genetic diversity, appropriate vaccination strategies and, most importantly, progress towards the development of a vaccine.

Various aspects of the molecular biology and transmissibility of the virus were ascertained using methods such as immune electron microscopy, PCR, ELISA and genetic studies (Balayan *et al*., 1983; Reyes *et al*., 1990; Tam *et al*., 1991; Bradley 1992; Koonin *et al*., 1992; Jothikumar *et al*., 1993). Genomic studies revealed that despite substantial genetic diversity between viral genotypes and strains, all share at least one epitope encoded within open reading frame 2 (ORF2; a region of the viral genome), which elicits neutralizing antibodies (Yarbrough *et al*., 1991) - a prerequisite for the development of a broadly protective vaccine.

Although natural infection is best mimicked by vaccination with inactivated or attenuated whole-virus particles, and therefore most likely to provide broad immunity, this was considered unfeasible because HEV does not yet grow adequately in cell cultures. Most HEV vaccines under development (as described by Worm and Wirnsberger) were therefore based on recombinant proteins derived from immunogenic parts of the HEV capsid gene. DNA based vaccines and approaches using transgenic tomatoes had also been developed (Ma *et al*. 2003).

Strategies for the expression of recombinant ORF2 encoded proteins or peptides containing HEV epitopes were developed using *Escherichia coli* (Purdy *et al*., 1993; Im *et al*., 2001), and also by using a baculovirus vector in insect cells such as *Spodoptera frugiperda* (*Sf9*) cells (McAtee *et al*., 1996; Robinson *et al*., 1998; Li *et al*., 2000). The superiority of insect cell expression (specifically, superior detection of convalescent-phase anti-HEV antibodies) was demonstrated by ELISA (McAtee *et al*., 1996; Robinson *et al*., 1998; Li *et al*., 2000), therefore it was hoped that baculovirus-expressed proteins might prove better at inducing neutralizing antibodies.

The development of DNA vaccines was also described, in which purified plasmid DNA containing coding sequences with immunogenic activity is administered intramuscularly. Although antigen is subsequently expressed by liver cells *in vivo*, as in a natural infection, tests in mice and cynomolgus monkeys yielded inconsistent results. While some anti-HEV activity was induced in all test animals, only two of the four cynomolgus monkeys were protected against HEV infection with a heterologous strain (Kamili *et al*, 2002; 2004). An ORF2 partial gene ("HEV-E2" representing amino acid residues 394–604) was transferred into tomato plants to produce a plant-derived oral vaccine for HEV (Ma *et al*., 2003), and ELISAs have demonstrated the subsequent low-level expression of HEV-E2 derived proteins.

At the time the citing paper (Worm and Wirnsberger) was written in 2004, one vaccine candidate had passed a phase I clinical trial and was being tested in a field trial in Nepal (Safary 2001). This was based on ORF2 from a Pakistani strain expressed using a recombinant baculovirus (Robinson *et al*., 1998), and was initially tested using cynomolgus monkeys and rhesus macaques. However, further tests were considered necessary to determine its long-term efficacy.

Worm and Wirnsberger cited techniques including serodiagnostic assays such as Western blots and ELISAs as making important contributions to the development of this vaccine, which were developed using recombinant proteins and synthetic peptides derived from antigenic domains within ORF2 and ORF3 (Dawson *et al*., 1992; Goldsmith *et al*., 1992; Paul *et al*., 1994). Other methods that, in their opinion, had contributed to HEV vaccine development in general are listed below.

As stated previously, the cited chimpanzee study from our random sample (Mast *et al.*), used sera from both humans and chimpanzees to investigate the reliability of 12 anti-HEV assays, and there is profound discordance between assay results. Chimpanzees were used to provide positive and negative controls, even though more relevant human controls existed in the form of undiluted sera obtained 2 months to 13 years after acute hepatitis E infection, and sera obtained from previously tested U.S. blood donors without a history of hepatitis.

The Mast *et al*. paper was not cited elsewhere by Worm and Wirnsberger and therefore, while it highlights a difficulty in assessing the efficacy of putative HEV vaccines, it cannot be considered to have contributed to their development. Recently, a WHO anti-HEV IgG antibodies standard was defined, which may assist appropriate assessment of immune response in vaccine programs (Ferguson *et al*., 2002).

Other NHP studies cited by Worm and Wirnsberger yielded conflicting results. Some reports showed that passive transfer of convalescent sera from humans and experimentally infected monkeys protected cross-challenged monkeys against hepatitis E disease but not against infection (Tsarev *et al*., 1994; Pillot *et al*., 1995), whereas others reported no efficacy with passive immunization of rhesus monkeys with human convalescent-phase serum (Chauhan *et al*., 1998).

In spite of considerable genetic diversity, a common antigenic epitope exists across HEV isolates. Confirmation of cross-reactivity of acute and convalescent-phase sera from geographically-distinct regions was achieved using sera from human patients and a fluorescent antibody test (Krawczynski and Bradley 1989), and later

demonstrated in cynomolgus monkeys (Bradley 1990) and rhesus macaques (Arankalle *et al*., 1995). The relevance of these results to humans is clearly less than the results obtained using human sera, and the necessity of obtaining them at all appears unclear, given that human results had already been published.

Epidemiological and clinical studies of naturally infected humans were cited by Worm and Wirnsberger as contributing to HEV vaccine development by revealing the longevity of IgG antibodies (12-14 years in most adults) (Goldsmith *et al*., 1992; Chauhan *et al*., 1998; Khuroo *et al*., 1993; Koshy *et al*, 1996; Aggarwal *et al*., 1997; Worm *et al*., 1998) compared to IgM (up to 6 months) (Bryan *et al*., 1994). Molecular studies also revealed important aspects of the structure of recombinant proteins used for vaccination (Riddell *et al*., 2000; Schofield *et al*., 2003).

12. Hepatitis E virus: a diagnostic method

HEV is a common etiologic agent of enterally transmitted non-A, non-B hepatitis, with outbreaks usually occurring in developing countries of Asia and Africa, the central Asian republics of the former Soviet Union, and the Middle East (Obriadina *et al*., 2002). Efficient diagnosis and control of such outbreaks requires HEV assays sensitive to different geographic HEV variants, but a previous survey of 12 of these assays demonstrated poor concordance and in some cases poor sensitivity between them, particularly against different HEV strains (Mast *et al*., 1998). In this research paper citing a chimpanzee study from our sample, Obriadina *et al.* reported the development and evaluation of a new, potentially improved EIA.

The new EIA utilized two new recombinant proteins as antigenic targets: one (fragment pB166), against which antibodies have been shown to cross-neutralize different geographic HEV strains (Obriadina *et al*, 2002; Meng *et al*., 2001), and another (MPII), composed of strongly and broadly immunoreactive epitopes comprised of short regions derived from the HEV Burma pORF2, and from the HEV Burma and Mexico strain pORF3s (Obriadina *et al*, 2002; Khudyakov *et al*., 1993; 1994).

This EIA was evaluated using serum specimens from 322 acutely-infected HEV patients from all over the world, 552 normal blood donors (NBD) from the non-endemic countries U.S. and Russia; and four experimentally infected chimpanzees. Specimens from 21 healthy Russian infants and 11 naive chimpanzees provided anti-HEV-negative controls.

The new assay achieved 100% sensitivity, detecting anti-HEV activity in all acutely infected patients. Around 16% of NBD specimens were found to be anti-HEV IgG positive, though over 90% of these immunoreacted with overlapping synthetic peptides spanning the entire HEV pORF2, indicating a specificity of at least 90%. The combination of these two antigens in this new assay was concluded to offer an efficient diagnostic target for the reliable detection of HEV-specific antibodies to different geographic HEV variants.

Obriadina *et al.* cited 11 papers (from a total of 32 references) as contributing to the development of this assay, which outlined the cloning of the HEV genome and the expression of recombinant antigens (Purdy *et al*., 1992; Tsarev *et al*., 1993; Li *et al*.,

1994; Favorov *et al*., 1996; Fields *et al*., 1996; McAtee *et al*., 1996; Li *et al*., 1997; Anderson *et al*., 1999; Lin *et al*., 2000). The first recombinant antigens expressed in *Escherichia coli* strongly immunoreacted with acute phase sera, but showed diminished immunoreactivity with convalescent sera (Mast *et al*., 1998; Dawson *et al*., 1992; Goldsmith *et al*., 1992; Li *et al*., 1994; Favorov *et al*., 1996): later recombinant antigens, however, expressed in both *E. coli* and insect cells, demonstrated strong and broad immunoreactivity with both acute and convalescent serum specimens (Tsarev *et al*., 1993; Li *et al*., 1994; McAtee *et al*., 1996; Li *et al*., 1997; Anderson *et al*., 1999).

The discovery that the minimal size fragment that can efficiently model the HEV neutralization epitope is 166 aa in length, its location at 452-617 aa of the pORF2 (pB166), and its ability to cross-neutralize different HEV genotypes, all derived from *in vitro* assays of immunized mouse sera (Meng *et al*., 2001). The discovery that the pB166/MPII antigenic combination resulted in excellent sensitivity relied upon acute phase serum specimens from human patients infected with geographically distinct HEV strains (Obriadina *et al*, 2002).

The chimpanzee study from our random sample (Mast *et al.*) was primarily cited in the section of the discussion describing the poor concordance, sensitivity and variable efficiency of previously available HEV assays, particularly against different HEV strains. Hence, while this study helped establish the need for a new HEV assay, it contributed very little to the actual development and evaluation of this new assay as described in the citing paper by Obriadina *et al.* Furthermore, the studies of chimpanzee sera were conducted alongside studies of sera from acute and convalescent human patients and normal human blood donors with no history of hepatitis. It is difficult to ascertain what additional benefit any attempt to extrapolate from chimpanzee to human outcomes would have yielded, given that human outcomes were already directly available.

Finally, one of the key outcomes of the cited paper were the highly discrepant existing HEV assay results from U.S. blood donors that were presumed HEV negative, necessitating great caution when interpreting positive assay results - an outcome that relied exclusively on humans.

For these reasons, the cited chimpanzee study must be concluded to have contributed minimally to the development of this new recombinant HEV assay which, while providing a promising prototype diagnostic assay, was insufficiently developed for routine use.

13. Hepatocellular carcinoma: a diagnostic method

A review of 81 papers by Kim and Wang (Kim and Wang, 2003) described an emerging human diagnostic technique using of various methods of global gene expression profiling to detect liver cancer, with a view to concurrently elucidating the molecular mechanisms underlying the development of HCC and preneoplastic liver disease, and to improve identification of metastatic potential/likelihood of reoccurrence and to discover new therapeutic targets.

Liver cancer is the third most deadly and the fifth most common type of cancer worldwide, and it was anticipated that data from these investigations would enable the identification of individuals at high risk of developing HCC, thereby facilitating early diagnosis, as well as the provision of adjuvant therapy to patients liable to suffer from a recurrence of HCC and/or related metastatic neoplasms. These investigations should also identify novel diagnostic markers and candidate therapeutic targets.

This review indicated that human genetic studies have been almost exclusively responsible for implicating the involvement of certain genes and mutations in hepatocarcinogenesis. Gene expression profiling is augmenting this information significantly, and genomic techniques such as differential display, suppression subtractive hybridization, representational difference analysis, serial analysis of gene expression (SAGE) and microarray analysis are enabling the identification of genes associated with neoplastic and preneoplastic liver diseases. It is encouraging to note that since the publication of this review, a number of reports have built on this technology and its application to this field: gene expression profiling has been and is being used to derive diagnostic and prognostic markers for hepatocellular carcinoma, to predict clinical outcome and survival in patients, and to classify types of carcinoma (Lee *et al*., 2004; Lee and Thorgeirsson 2005; Nishida *et al*., 2003; Ye *et al*., 2003).

The review summarized the contribution of these methods to identifying candidate genes associated with HCC using human and animal model systems. From a total of 30 references profiling gene expression, 27 were human-based, and three animal-based. Of the three animal-based references, one involved chimpanzees.

The cited chimpanzee study from our random sample (Bigger *et al.*) described the cDNA microarray-based analysis of liver gene expression in a chimpanzee infected with HCV. It must be concluded that this study is incidental to Kim and Wang's review for the following reasons: 1) The chimpanzee study was included only in a table of references that profiled liver disease gene expression, and was not mentioned in the text of Kim and Wang's review at all; 2) although investigation of preneoplastic liver diseases is clearly important in HCC research, the main focus of Kim and Wang's review was HCC itself, rather than HCV infection or other diseases associated with HCC; 3) 27 of the 30 references in the table of papers profiling gene expression in liver diseases were human-specific. These also included references to microarray and SAGE studies of human HCV infection (Honda *et al*., 2001; Yamashita *et al*., 2001), which the chimpanzee study replicated.

14. Human immunodeficiency virus: a prophylactic and therapeutic method

Armbruster *et al.* (Armbruster *et al.,* 2002) described a three-week phase I clinical trial of the anti-HIV-1 human monoclonal antibody (hMAb) 4E10, both alone and in combination with two other hMAbs, 2F5 and 2G12, for the treatment of AIDS. All were safe and well tolerated. Previously, AIDS treatment had relied upon highly active antiretroviral therapy (HAART) to prevent integration of the viral genome at the reverse transcription step, or to block viral assembly. However, drug resistance, incomplete suppression of viral replication, and adverse side effects have long indicated the need for therapies acting on additional targets – leading to the

development of fully human antibodies directed towards HIV proteins, in an attempt to block the viral life-cycle.

The development of this approach stems from experiments involving the immortalization of human peripheral blood lymphocytes from the blood of HIV-1-positive volunteers, in which cell lines and stable hybridomas are established via cell fusion or EBV transformation, to produce hMAbs against HIV-1. Investigations of the therapeutic potential of combinations of hMAbs (such as Armbruster *et al*.) were inspired by clinical research showing that HIV quickly develops resistance to all current drugs used against it; combination therapy is therefore much more effective than monotherapy in preventing viral escape mutants.

The vast majority of references in the Armbruster paper, therefore, are clinical studies of anti-HIV therapies and *in vitro* methods of MAb production and characterization (a total of 20 out of 25 references). Several studies involved the assessment of MAbs in monkeys. The cited chimpanzee study from our random sample (Conley *et al*. 1996) sought to determine whether hMAb 2F5, an antibody to gp41, had any prophylactic antiviral activity *in vivo* and whether the presence of the antibody would alter the course of virus replication if an infection were established. While single doses of 2F5 administered to chimpanzees challenged with high doses of a primary virus significantly delayed seroconversion, this cannot be considered to have contributed to the outcome of the citing paper.

Firstly, the 2F5 and 2G12 hMAbs being assessed in combination with 4E10 in the Armbruster *et al*. clinical trial had already gone through human clinical trials to assess their safety and efficacy (Armbruster *et al.* 2002). Secondly, the citation of Conley *et al*. was in a section of the paper speculating that the highest probability of success with hMAbs could be passive immunization with 4E10, 2F5 and 2G12 in preventive settings. Though based on the chimpanzee results, this approach is some way from being suitable for widespread human use. Since the publication of the citing paper, a clinical trial was completed last year (2005) although its results are not yet available. One paper reports a proof-of-principle study (Trkola *et al*., 2005) showing that a cocktail of 4E10, 2F5 and 2G12 hMAbs can delay viral rebound in HIV-infected patients whose antiretroviral therapy has been interrupted, but there were major caveats: the trial was not randomized or blinded, only two of eight chronically infected patients showed rebound delay, the antibodies used must be highly potent and used at a high dose, and there was evidence of rapid escape.

15. Human immunodeficiency virus: a prophylactic and therapeutic method

Similar to their 2002 study, Armbruster *et al.* (Armbruster *et al.*, 2004) described a three-week phase I clinical trial of anti-HIV-1 hMAbs 2F5, 2G12 and 4E10, employing a combination protocol to reduce the possibility of viral escape due through HIV evolving drug resistance. This study assessed the safety, immunogenicity and pharmacokinetics of the hMAb 4E10 alone and in combination with 2F5 and 2G12 in eight clinically healthy HIV-1-infected human volunteers. Previously, 4E10 had been shown to be safe in animals, but its safety in humans was unknown. Details of the development of this protocol are provided in Review 14, above.

The authors concluded that 4E10 singly and in combination with 2F5 and 2G12 was safe and well tolerated by HIV-1-infected subjects: plasma samples showed no detectable levels of 2G12 immune complexes, and lack of proteinuria supported the hypothesis that immune complex disease (ICD), was not present.
$CD4^+$ cell counts showed no evidence of accelerated decline and HIV-1 RNA copy numbers did not vary significantly in almost all patients. Additional studies were therefore recommended to establish the utility of the hMAb combination in therapeutic and prophylactic applications, such as use in combination with or during strategic treatment interruption of HAART regimens, and in prophylactive immunization. Only with respect to the latter was the cited chimpanzee study from our random sample by Conley *et al.* mentioned, describing seroconversion delays in chimpanzees infused with 2F5 and challenged with high doses of a primary HIV-1 isolate. While Conley *et al*. suggested a potential field of further development with respect to prophylactic applications, it did not play an integral role to this particular study, which examined the safety, immunogenicity and pharmacokinetics of the hMAb protocol in healthy HIV-1-infected human volunteers. Furthermore, while the potentials of this protocol are exciting it remains some way from being sufficiently developed for routine human use.

16. Human immunodeficiency virus: a prophylactic and therapeutic method

An extensive review of 130 references by Bardsley-Elliot and Perry (Bardsley-Elliot and Perry, 2000) provided a comprehensive and detailed overview of the use of the drug Nevirapine, an HIV-1 specific non-nucleoside reverse-transcriptase inhibitor, against pediatric HIV infection. The authors described its pharmacodynamic and pharmacokinetic properties, clinical efficacy, tolerability, and assessed the economic considerations of its use in preventing perinatal HIV transmission and in the management of pediatric HIV infection.

The review concluded that Nevirapine is a medically effective and cost-effective drug in the fight against pediatric HIV infection, when administered either prophylactically as a single intrapartum dose to the mother and postpartum dose to the neonate, or as a combination therapy treatment with other anti-retrovirals to HIV-infected infants and children.

The authors cited numerous published clinical papers that demonstrate the safety and efficacy of the drug: human studies and randomized controlled clinical trials were responsible for the discoveries that monotherapy with Nevirapine gave rise to drug-resistant strains of HIV, of the increased efficacy of combination therapy, and the elucidation of adverse reactions and the tolerability of Nevirapine. The pharmacokinetic properties of the drug were discovered from human clinical trials and radiolabeling experiments involving human volunteers. In the pre-clinical development phase, the antiviral activity, prophylactic properties and low toxicity of Nevirapine had been demonstrated in various cultured cell lines including human T-cells (evidenced by 13 studies) (Merluzzi *et al*., 1990; Koup *et al*., 1991; Richman *et al*., 1991; Balzarini *et al*., 1993; Mazulli *et al*., 1994; Rusconi *et al*., 1994; Merrill *et al*., 1996; Patel and Benfield 1996; Zhang *et al*., 1996*a*, *b*; Anonymous 1998*e*; de Bethune *et al*., 1998; Zhang *et al*., 1998; Grob *et al*., 1997) and had also been shown

to inhibit HIV-1 activity and infectivity in cell-free *in vitro* tests (Zhang *et al*., 1996*a*; de Bethune *et al*., 1998).

The cited chimpanzee study from our random sample by Grob *et al.* (Grob *et al.*, 1997), published almost one year after Nevirapine was approved by the FDA, showed that in three chimpanzees compared with one untreated control, administration of Nevirapine just prior to HIV exposure prevented latent or chronic HIV infection: HIV DNA was present in some samples of blood cells from the Nevirapine treated chimpanzees, though infectious virus could not be recovered from them.

This study correlates with the prior *in vitro* findings revealing the efficacy and tolerability of the treatment. However, it cannot be considered to have been a crucial element in its successful clinical application, because it simply confirmed the findings of previous *in vitro* studies, it is just one of 130 references in this review, and it simply correlates with the plethora of prior discoveries from the human studies and clinical trials – many of which demonstrated its clinical antiviral efficacy (Carr *et al*., 1996; Cheeseman *et al*., 1995; D'Aquila *et al*., 1996; Havlir *et al*., 1995*a*, *b*; Luzuriaga *et al*., 1996).

17. Human immunodeficiency virus: a prophylactic method

A review of 108 papers (Hone *et al.*, 2002) described advances toward the development of an HIV-1 vaccine to provide both systemic and mucosal immunity. The authors cited the chimpanzee study by Conley *et al.* (Conley *et al.*, 1996) from our random sample as just one of nine NHP studies demonstrating that HIV-l or simian immunodeficiency virus neutralizing antibodies provide protection against lentiviruses. This principle was by no means established conclusively however, as six other NHP investigations were cited where no such association was observed.

Importantly, this principle was also relatively basic in comparison to other important components of HIV-1 vaccine development described in the review, namely: 1) seventeen papers justifying a focus on the development of oral, rather than parenteral, vaccines (based on the negligible mucosal humoral responses of parentally administered vaccines and the widespread acceptance and reliability of the oral route in large-scale vaccination programs); 2) fifteen papers indicating that sIgA (secretory IgA) could be more effective than IgG at affording cross-protective immunity against different envelope antigen variants of HIV-1 and is more adaptive to external mucous environments; 3) ten papers justifying the development of cheaper bacterial vectors for the delivery of an HIV-1 vaccine, based on evidence that a *Salmonella* DNA vaccine vector system produced $CD8^+$ T cell responses in both mucosal and systemic lymphocyte populations; 4) Seven papers describing conformationally constrained HIV-l Env and gp120 immunogens that have allowed the formulation of HIV-1 vaccines inducing humoral responses; 5) six papers further describing the limitations of conventional HIV-l Env and gp140 subunit vaccines that have resulted in poor immunogenicity, largely because the relevant epitopes are inaccessible - and hence the need to engineer immunogens exposing these epitopes to the surface and/or adjuvants that prolong immunogenicity; and finally, 6) five papers describing the neutralization of primary HIV-l isolates for up to a year or more post-vaccination, via the cholera toxin catalytic domain CtxA1 adjuvant.

The elements described by these 60 papers clearly contributed considerably more to the authors' development of an HIV-1 vaccine than did the cited chimpanzee study from our random sample.

18. Human immunodeficiency virus: prophylactic and therapeutic methods

A wide-ranging review of 93 references by Sleasman *et al.* (Sleasman *et al.*, 2003) described the HIV-1 virus and subsequent epidemic, the origin of the virus, its molecular biology and life-cycle, the infectious process *in vivo*, including immune response and physiological consequences, and finishes with a detailed summary of the treatment and prevention of HIV-1 infection.

One of the 93 references in this review relates to an experiment involving chimpanzees (Conley *et al.*, 1996), with another two papers referring to the possible origin of HIV in chimpanzees (Korber *et al*., 1998; Gao *et al*., 1999). The Conley *et al.* citation is in a section dealing with HIV-specific immunity, which describes the emergence of cytotoxic T lymphocytes and anti-HIV antibodies following infection, resulting in the inhibition of viral replication and subsequent decreases in plasma virus levels. However, other cited studies demonstrated contrary results in humans *in vivo* (Halsey *et al*., 1992; Robertson *et al*., 1992). Consequently, Sleasman *et al.* stated that:

"*Most applications that have been based on adoptive transfer of antibody or immunization targeted toward enhancing mucosal immunity in animal models have not provided complete protection against viral challenge,*" and, "*...the likelihood that traditional protein-based vaccines will provide effective immunity against infection is uncertain*" (page S590).

This lack of certainty is further illustrated in the cited chimpanzee study by reference to an anti-gp120 antibody that protects against HIV infection in chimpanzees, but fails to do so in human PBMC cultures (Emini *et al*., 1992), and by reference to a study in which post-seroconversion antigen recognition patterns in chimpanzees infected with HIV (strain SF-2) differ from those observed in humans (el-Amada *et al*., 1995).

The contribution of the single cited chimpanzee experiment to these HIV therapies is substantially limited by its inconsistency with other cited human data.

19. Human immunodeficiency virus: a diagnostic method

The assessment of candidate HIV vaccines requires rapid and reliable assays, with discrete end points, to measure HIV-1 virus-neutralizing antibodies (VNAbs). Yang *et al.* (Yang *et al.*, 1998) reported their development of a new assay of this type based on PCR-based assays of viral DNA from infected cell cultures. Their protocol consistently revealed HIV DNA in H9 cells after 15 minutes, a substantially shorter time-frame than other DNA-based assays (1 hour postinfection (Stevenson *et al*., 1990); 1 to 2 hours post-infection (Hewlett *et al*., 1991); 24 hours after infection (Robb *et al*., 1992)).

To measure the performance of this assay relative to others, six monoclonal antibodies, a positive control (HIV Ig) and a negative control (human IgG1 paraprotein - HuIgG) were each exposed to five viral isolates, and cultured in PBMCs. Yang *et al*.'s method compared favorably with other assays in terms of specificity and reliability, with a well defined end point, and was unaffected by the presence of partial reverse transcripts in virions or the endogenous reverse transcription of HIV-1 that affects other assays (Zhang *et al*., 1996a; Trono 1992). Yang *et al.* therefore concluded that their novel assay offered a simple, economic, rapid, sensitive, and reproducible approach to evaluating to candidate HIV vaccines when compared with other VNAb assays.

Much of Yang *et al.*'*s* paper was dedicated to a comprehensive description of their methods of viral incubation, measurements of infectivity, and extraction and detection of viral nucleic acids. Their 27 citations of other sources are relatively few, which mainly comprise papers describing molecular methods. Ten papers were referenced with regard to previous attempts to incubate and test for HIV, along with several papers describing previous protocols used.

The chimpanzee study from our random sample (Conley *et al*., 1996) was cited to demonstrate that passive immunization with the anti-gp41 HIV neutralizing monoclonal antibody 2F5 delays or prevents subsequent infection in chimpanzees. This citation demonstrates only that effective HIV antibodies can indeed neutralize HIV in chimpanzees, to varying degrees. Two other cited papers also describe the neutralization of laboratory-adapted strains of HIV-1 or even primary HIV isolates in other species, through passive immunization with monoclonal or polyclonal VNAbs, or active immunization with certain candidate AIDS vaccines that elicited VNAbs (Gauduin *et al*., 1995; Zolla-Pazner *et al*., 1997).

Consequently, the cited chimpanzee paper by Conley *et al.* must be considered to be of peripheral relevance Yang *et al.*'*s* paper, which focused on the development of an assay to measure viral DNA levels, primarily for application to putative human HIV vaccines. Notably, PCR-based diagnostic methods may now be considered ancillary to other quicker and more robust techniques such as Enzyme-Linked Immunosorbent Assays (ELISAs), Enzyme Immunoassays (EIAs), and immunochromatographic assays: highly specific assays such as Western blots, indirect fluorescent antibody (IFA) assays or the radioimmunoprecipitation assay (RIPA) are sometimes used to verify reactive screening test results, with PCR-based methods used to help resolve indeterminate results (Constantine, 2006).

20. Human immunodeficiency virus: therapeutic methods

An extensive review of 258 papers by Gallo (Gallo 2002) described in depth the history and current state of knowledge about the transmission and pathogenesis of two human retroviruses HIV and human T-cell leukemia virus (HTLV). The search for the existence of human retroviruses was described, citing the confounding contribution of animal studies and the importance of tissue culture, electron microscopy and sensitive immunological, biochemical and molecular techniques. The association of these viruses with the diseases they cause was detailed, as were their life-cycles, pathological mechanisms and the potential for vaccine development in the future. The

latter consideration is especially significant for HIV, as 50% of patients are known to fail in therapy within two years (Gallo 2002).

An in-depth analysis of the major contributors to this review revealed an almost exclusive presence of human-based *in vivo*, *in vitro* and *ex vivo* research. This applies to: the discovery of HTLV and its identification as the causal agent of T-cell leukemia relied upon culture of many types of human cells and astute clinical observation; human and *in vitro* research showed the virus can cause neurological disease and was central to genetic analysis of the virus and its etiology; elucidation of the viral mechanisms and effects of viral genes - in other words how the virus causes disease - was all achieved *in vitro*. The discovery and isolation of HIV, its link to AIDS and its effects on T cells, characterization of its genes and structural proteins and development of a blood test for it, all relied on human-based and *in vitro* experimentation. Likewise, the characterization of HIV heterogeneity, discovery of CD4 as the viral receptor, presence of HIV in the nervous system and semen, identification of macrophages as target cells, and HIV's relation to simian immunodeficiency virus (SIV).

The review continued in this vein: HIV pathogenesis has also been tremendously reliant upon human and *in vitro* methods, including the association of latently infected cells to viral rebound after therapy; the identification of natural killer (NK) cells as targets for infection; the evidence that the outcome of HIV infection can be estimated by viral kinetics. Resistance to infection has also been characterized using these methods, and the suppression of viral replication by chemokines.

Sections of the review on cellular pathogenesis, the death of T cells and bystander cells, involvement of cell to cell contact and humoral factors, generative abnormalities in uninfected cells, HIV-1 and neoplasties, therapeutic considerations for the future and vaccine development: all almost exclusively cited human-based methods in support of their findings. Salient examples include the implication of dendritic cells and lymph nodes; the observation that many HIV-infected cells do not die while some non-infected bystanders do; mechanisms of $CD4^+$ cell death and the involvement of viral Tat, Nef and Vpr proteins; impaired hematopoiesis and lymphopoiesis; cell killing by the HIV envelope; the development of protease inhibitor resistant mutants; correlation of increased endogenous levels of chemokines with protection against infection and/or retarded development of AIDS; the development of inhibitors of viral fusion and coreceptor interaction; progress towards drugs targeting IFNα and Tat, and therapeutic antibodies.

There were a small number of citations that referred to studies involving non-human primates. The association of the activation state of the immune system to indicate disease progression included the citation of a study using sooty mangabeys, though this was completed subsequently to five co-cited human and *in vitro* studies. A discussion of the mechanisms of uninfected cell death cited a study that involved an analysis of lymph nodes from macaques; the same study, however, reported concurrent results from the lymph nodes of HIV-infected humans, and was co-cited with seven *in vitro* investigations.

The cited chimpanzee study from our random sample is another example: Goh *et al.* (Goh *et al.*, 1998) is among five studies cited by Gallo in reference to a viral strategy

to increase HIV expression in the infected cell by prolonging or arresting cells at the G2-M cell cycle checkpoint, prior to which viral genome activity is optimal. Notably, Goh *et al*. report the concurrent investigation of this topic in humans. Of the other four studies, two were human-based and published three years earlier in 1995 (Jowett *et al*., 1995; Rogel *et al*., 1995), and two were reviews also published in 1998 (Cullen 1998; Ememan and Malim 1998). The cited chimpanzee study simply confirmed the findings made by the prior human studies and the concurrent reviews. It therefore did not play an essential role in this review.

21. Malaria: prophylactic methods

In a review citing 104 references, Moore and Hill (Moore and Hill, 2004) examined preclinical and clinical progress towards the development of recombinant DNA/virus-vector prime-boost vaccines against malaria.

Studies in humans (28 of 104) and a variety of non-human species (51 of 104) were credited as assisting the development of this work and previous attempts to develop a cure for malaria. A study of West African children supported a role of major histocompatability complex (MHC) class I-restricted T-cell responses in protective immunity (Hill *et al*., 1991). Murine malaria models were credited as elucidating the role of IFN in mediating protection against pre-erythrocytic stage viral challenge (Good and Doolan 1999). Human volunteers challenged with non-irradiated sporozoites were protected by the administration of irradiated sporozoites (Nussenweig and Nussenweig 1989; Hoffman *et al*., 2002), but the approach was considered impractical because of the large number of irradiated sporozoites required for widespread vaccination. Mouse and human studies were cited to support the injection of naked DNA plasmid into muscle cells, resulting in gene expression and leading to the induction of protective immune responses to encoded proteins (Wolff *et al*., 1990; Ulmer *et al*., 1993; Hasan *et al*., 1999). However, although studies in a range of species determined that plasmid DNA induced both humoral and cellular immune responses, immunogenicity in humans proved insufficient to protect against malaria challenge (McConkey *et al*., 2003).

Moore and Hill also credited a murine malaria model with aiding preclinical design and development of efficacious vaccines. However, they cautioned that despite encouraging results with recombinant viral vectors as a vaccine platform, human applications need to be replication-incompetent.

The cited chimpanzee study from our random sample (Pancholi *et al.*, 2001) was credited with presenting "*promising data*" on prime-boost immunization for other diseases such as hepatitis B virus, but not against malaria.

Three other chimpanzee studies were also cited by Moore and Hill. A study of two chimpanzees immunized using DNA followed by recombinant modified vaccinia virus failed to show protection against a high-dose heterologous sporozoite (malaria) challenge (Schneider *et al*., 2001). The two remaining chimpanzee studies were cited in a section on alternative prime-boost vaccine strategies. They were described as ongoing collaborations with groups at the University of Pennsylvania, examining the efficacy of recombinant malaria vaccines based on alternative strains of adenovirus

(ADV) that can overcome the pre-existing anti-ADV response (Farina *et al*., 2001; Cohen *et al*., 2002).

Hence, although one can speculate that these other chimpanzee studies may possibly contribute in some important way to the development of a human malaria vaccine at some future time, this was not yet evident in the citing paper by Moore and Hill. And although the chimpanzee study from our random sample was credited assisting with the development of prime-boost immunization for other diseases, it was not credited with assisting with the development of a human malaria vaccine – the subject of Moore and Hill's paper.

22. Organ transplant rejection: therapeutic methods

A review of 87 papers by Matthews *et al.* (Matthews *et al.*, 2003) summarized the ongoing search for tolerance therapies that would thwart the alloimmune response following organ transplantation, without compromising the patient's protective immune response.

Three main groups of potential therapeutic methods were described. *Chimerism* involves the creation of a state in which large numbers of donor cells are maintained in the recipient. Matthews *et al.* mentioned three kinds of donor cells: bone marrow cells (e.g. Cosimi, 2002), $CD34^+$ (stem cell)-enriched peripheral blood mononuclear cells with total lymphoid irradiation (Millan *et al*., 2002*a*, *b*), and pancreatic Islets of Langerhans transplants with $CD34^+$-enriched stem cells (Ferreira *et al*., 2002). Most such therapies described were at the pre-clinical stage of investigation, although a few trials involving two to six human patients were underway, with mixed results.

Targeting of cell-surface receptors results in T-cell inactivation, thereby delaying allograft rejection, because T-cell activation is central to the inflammation and tissue damage that results in allograft rejection. For example, the binding of the B7 ligand - which is expressed by antigen-presenting cells – to the T-cell CD28 receptor stimulates T-cell proliferation; hence CD28/B7 blockade inhibits T-cell responses (Matthews *et al*., 2003). Although several such methods were under development, once again most were at the pre-clinical stage of investigation, and Matthews *et al.* stated that "*...results to date illustrate the profound difficulties in translating animal model success to the clinical arena.*"

Anti-CD2 antibodies developed in rats were also being tested in humans and monkeys for their capacity to deactivate T-cell CD2 receptors, but Matthews *et al.* were only guardedly optimistic about the outcomes, citing concerns about the limited efficacy and ensuing toxicity of anti-CD4 mAbs in mouse studies, and the failure of Genmab's HuMax CD4 (Zanolimumab) to demonstrate efficacy superior to that of the placebo in a phase II clinical trial of 155 patients with active rheumatoid arthritis (Genmab 2002).

Finally, *T-cell depleting therapies* are aimed at inducing a transient but profound depletion in T-cell levels in the presence of alloantigens. However, research in this area remained pre-clinical.

According to Matthews *et al.* these potential therapeutic methods have primarily been explored using mice, rats, and non-human primates, namely baboons, rhesus macaques and cynomolgus monkeys. One chimpanzee study from our random sample by Newman *et al.* (Newman *et al.*, 2001) was also cited. In this study chimpanzees were used to test an anti-CD4 mAb designed to target CD4 T-cell receptors. Keliximab, a primatized IgG1 CD4 mAb, was reconfigured from IgG1 to IgG4, and two amino acid substitutions were incorporated. However, although demonstrated by Newman *et al.* to be safe and efficacious in modulating T-cell receptor responsiveness in chimpanzees, as stated, a later Phase II clinical trial of another fully humanized anti-CD4 (Humax-CD4) mAb in 155 rheumatoid arthritis patients failed to demonstrate efficacy. It had been hoped this drug would prove efficacious, because T-cell activation is central to the inflammation and tissue damage that results in the clinical signs of rheumatoid arthritis. Following its failure in this clinical trial, however, development of this drug for rheumatoid arthritis sufferers was discontinued (Genmab 2002).

Given that the properties and use of CD4 monoclonal antibodies had already been investigated in seven earlier studies cited by Newman *et al.* at least six of which involved human clinical trials (Herzog *et al*., 1989; 1987; Hafler *et al*., 1988; Mathieson *et al*., 1990; Morel *et al*., 1990; Homeff *et al*., 1991; 1995), and the failure of the human trial for RA noted above, the Newman *et al.* chimpanzee study cannot be considered to have contributed significantly to the development of immunosuppressive chemotherapeutics for organ transplantation recipients that have proven to be efficacious in humans.

23. Respiratory syncytial virus: prophylactic methods

In a review of 150 papers, Kneyber *et al.* (Kneyber *et al.*, 2002) documented research carried out over a 40-year period with the aim of generating specific and effective human vaccines against RSV, and present recent advances within the field. RSV is the most common causative agent of viral respiratory tract infections in infants and young children, with 100,000 children hospitalized annually in the U.S. as a result.

The review summarized the structure and function of the virus, and the epidemiology of and immune response to infection. Lessons learned from past experiences with RSV vaccines were also described, with some being ineffective in humans, while another killed a number of children.

Attempts to develop vaccines against RSV have relied heavily upon data gained using animal models. Many of the citations within the review that describe experimental data refer to studies that have used rodents to determine the host immune response and also the safety and efficacy of potential vaccines. Some that proved promising in rodent models have progressed to human clinical trials, and through this process a number of chimpanzee studies (six) are also cited, including the study by Crowe *et al.,* 1999 from our random sample. In common with all of the other citations describing chimpanzee studies, this study utilized chimpanzees in the hope of determining the safety and efficacy of a potential new vaccine. In this case, although the vaccine appeared to provide chimpanzees subjected to the trials with protection against RSV infection, the attenuated virus from which it was derived did not behave as expected and the trial was not extended to humans. Consequently the cited Crowe

et al. study, in common with several others cited in the review, described a potential vaccine that was ultimately abandoned. In the absence of human clinical data, it is impossible to know whether this vaccine would have been effective and safe in human patients, and this chimpanzee study clearly has not contributed towards the development of an RSV vaccine.

Despite the fact that RSV vaccine development has been ongoing for many years and has been explored using a substantial number of animal studies, the only currently available prophylactic therapies for RSV are palivizumab (a humanized mouse-monoclonal antibody) and RespiGam (purified human IgG antibodies), which are used only for short-term protection from RSV-infection in high-risk individuals. Kneyber *et al*. therefore concluded that "*it will probably be at least another 5 to 10 years before any routine vaccination against RSV becomes daily practice.*"

24. Rheumatoid arthritis: a therapeutic method

Hepburn *et al.* (Hepburn *et al.*, 2003) described a randomized, double-blind, placebo-controlled human study of the pharmacokinetic and pharmacodynamic properties of the proposed RA drug Clenoliximab, a chimeric macaque/human monoclonal antibody specific for the human CD4 glycoprotein.

This protein, present chiefly on the surface of helper T-cells, is intimately involved in the immune response, binding to antigen-presenting cells bearing MHC II molecules. Because T-cell activation is central to the inflammation and tissue damage that results in the clinical signs of rheumatoid arthritis, neutralization of their CD4 surface molecules, and the subsequent down-regulation of their activities, has potential therapeutic value.

As described by Hepburn *et al.* such neutralization properties of anti-CD4 antibodies have been demonstrated *in vitro* and in human patients, where they have been shown to inhibit T-cell proliferation and cytokine/lymphokine production by dose-dependent coating of the $CD4^+$ cells (Takeuchi *et al*., 1987; Schrezenmeier and Fleischer 1988; Yocum *et al*., 1998; Reddy *et al*., 2000). However, the advent of Clenoliximab was prompted by the undesirable depletion of $CD4^+$ T-cells by its forerunner, Keliximab: thus, to circumvent this depletion activity, the Keliximab antibody was re-engineered to avoid binding complement and detection by cytotoxic cells.

Interestingly, the degree of $CD4^+$ cell depletion by Keliximab was much greater in humans compared to chimpanzees (Anderson *et al*., 1997; Jorgensen *et al*., 1998; Veale *et al*., 1999), which makes the decision to use chimpanzees as a model in which to characterize 'improved' derivatives of these drugs (as in the randomly-selected cited chimpanzee study by Newman *et al.*), somewhat puzzling.

In this research study, Hepburn *et al.* investigated the *mechanism of action* of anti-CD4 antibodies and showed that, in single and multiple dose human trials, Clenoliximab down-modulates the density of the CD4 protein by stripping it from the cell surface, without depleting $CD4^+$ cell numbers. They demonstrated this by analyzing sampled blood from patients using flow cytometry and immunoassays. The cited chimpanzee study from our random sample by Newman *et al.* describes the fact that the drug is known to inhibit T-cell proliferation and function, although the three

additional studies cited by Newman *et al.* that also show this were based on *in vitro* and human-based experiments (Reddy *et al*., 2000; Mould *et al*., 1999; Mason *et al*., 2002).

As of March 2006, no anti-CD4 antibodies appeared to be in clinical trials for the development of chemotherapeutics for arthritis sufferers (according to ClinicalTrials.gov). As stated previously, one CD4 antibody, HuMax CD4 (Zanolimumab), developed by German pharmaceutical company Genmab A/S, failed to demonstrate therapeutic efficacy greater than that of the placebo in a phase II clinical trial of 155 patients with active rheumatoid arthritis. Further development was consequently discontinued (Genmab 2002). Lack of progression of chemotherapeutics through clinical trials towards the marketplace usually indicates safety or efficacy concerns, although Hepburn *et al.* provide no conclusions either way with respect to Clenoxilimab.

Even if anti-CD4 therapeutic antibodies had proved to be safe and efficacious for human sufferers of rheumatoid arthritis, the chimpanzee study cited by Newman *et al.* could not be considered to have made an important contribution for several reasons. Firstly, the properties and use of CD4 monoclonal antibodies had already been investigated in seven earlier studies cited by Newman *et al.*, at least six of which involved human clinical trials (Herzog *et al*., 1989; 1987; Hafler *et al*., 1988; Mathieson *et al*., 1990; Morel *et al*., 1990; Homeff *et al*., 1991; 1995). Furthermore, a variety of *in vitro* techniques had been used to demonstrate the stability and affinity of the Clenoliximab antibody, and rats were also used to assay its pharmacokinetic properties. *In vivo* coating/depletion properties were then assayed in chimpanzees - which had already been investigated *in vitro*.

25. Rhinoviral colds: a prophylactic method

Human rhinoviruses bind to upper respiratory ICAM-1 cellular receptors in order to invade the respiratory epithelium and elicit their pathological effects. Turner *et al.* (Turner *et al.*, 1999) described a phase I clinical trial of Tremacamra, a soluble recombinant ICAM-1 glycoprotein that was hoped to offer potential efficacy in the prevention of viral-source colds via blockade of the ICAM-1 receptor.

The study comprised four separate randomized, double-blind, placebo-controlled trials, using a total of 196 volunteers between the ages of 18 and 60. Subjects were isolated in a hotel room for the first 8 days, and symptoms were recorded through day 14.

Based on symptom scores, mucus weight, and onset of colds in experimentally infected subjects, it was concluded that Tremacamra reduces the severity of experimental rhinoviral colds. However, the authors noted that further study would be required to ascertain Tremacamra's clinical utility, and as of March 2006, no evidence of progression through further trials towards the marketplace could be located, which usually indicates that safety or efficacy concerns have arisen at some point.

As described by Turner *et al.* human *in vivo* and *in vitro* studies contributed most to the development of Tremacarma: Abraham and Colonno (Abraham and Colonno, 1984) used human cell cultures to establish that most rhinoviruses attach to the same

ICAM-1 cellular receptor; a soluble recombinant ICAM-1 glycoprotein (now Tremacamra) was developed in human cell cultures (human embryonic lung fibroblasts, HeLa cells and human adenoid explants) (Marlin *et al*., 1990; Crump *et al*., 1993); and the recent suggestion that host inflammatory response may play a role in the pathogenesis of rhinoviral common cold symptoms is attributed to several sources (Zhu *et al*., 1997; 1996; Grunberg *et al*., 1997; Teran *et al*., 1997; Turner *et al*., 1998), each of which also used human subjects and/or cell cultures.

The cited chimpanzee study from our random sample (Huguenel *et al.*, 1997) was one of two previous *in vivo* studies of ICAM-1 receptor blockade for the prevention of rhinovirus infection. Intranasal administration with soluble ICAM-1 alleviated rhinovirus type 16 infection in 16 chimpanzees challenged with rhinovirus then treated intranasally with ICAM-1.

The other *in vivo* study employed human volunteers, treated with intranasal monoclonal antibodies to ICAM-1, before being experimentally given colds (Hayden *et al*., 1988). The most beneficial outcomes were reduced symptoms and lower viral shedding during the medication period.

Turner *et al.* considered that "*both of these previous studies suggested the potential effectiveness of receptor blockade.*" The human study examined the prophylactic effects of ICAM-1 receptor blockade, which was also the subject of Turner *et al.*'*s* clinical investigation; and both were completed well prior to the chimpanzee study, which investigated *therapeutic* effects. Consequently, the chimpanzee study cannot be considered to have made an essential contribution to the development of this prophylactic method.

Finally, as stated, despite the promising results of the citing Turner *et al.* paper, Tremacamra has not progressed towards clinical application in the last seven years.

26. Systemic lupus erythematosus: therapeutic methods

A review of 77 papers by Gescuk and Davis (Gescuk and Davis, 2002) described the potential of new therapeutic agents for the autoimmune disease SLE, for which the drug of choice for 30 years, cyclophosphamide, is sometimes ineffective and has a significant number of known adverse effects.

The new therapies discussed by Gescuk and Davis were (i) new immunosuppressives such as mycophenolate mofetil (MMF), leflunomide and tacrolimus that act to inhibit one or more of purine or pyrimidine synthesis, lymphocyte proliferation, T-cell dependent antibody responses and inflammatory cytokine responses; (ii) biological therapies to block co-stimulatory molecules involved in T-cell activation, auto-antibody production and complement activation; (iii) T- and B-cell tolerizing agents; (iv) immunoablation techniques such as autologous hematopoietic stem cell transplantation, and (v) hormonal medications such as dehydroepiandrosterone.

Because the objective of this review was to describe emerging future therapies for SLE, many of the cited references are human-oriented pilot studies and clinical trials, (53 of 77). However, in the case of therapies that had not yet reached this stage many of the citations were for pre-clinical animal studies (19 of 77), chiefly in mice.

The sole cited study involving chimpanzees (Brams *et al*., 2001) was referenced in the section of the review dedicated to anti-CD40 ligand (CD40L; also known as CD154) based therapies. The basis for these is that CD40L is expressed on activated helper T-cells, which binds to CD40 on B cells stimulating antibody production; because CD40L is expressed on more T cells and for a longer period in lupus patients, it is hoped that blocking CD40L could inhibit autoantibody production and ameliorate symptoms in lupus patients.

There were several citations regarding studies in murine models of the efficacy and safety of this approach, for example decrease in incidence of renal disease resulting from autoantibody production and prolonged survival (Daikh and Wofsy 2001). However, this is confounded by a phase II clinical trial that showed the anti-CD40L MAb "IDEC131" produced no clinical benefit, and a finding in monkeys of thromboembolic events after treatment with an alternative anti-CD40L MAb.

As previously described (see review number 1), the cited Brams *et al*. paper was not a chimpanzee-based study, but simply reported the use of chimpanzees to test the safety and efficacy of the therapeutic humanized anti-CD40L (also known as anti-CD154) antibody IDEC-131, in blocking B-cell activation via inhibition of CD40-CD40L binding with a view to the treatment of autoimmune diseases and prevention of allograft rejection. The antibody had been developed and tested using *in vitro* technologies, and a description of this formed the core of the paper.

The chimpanzee element of this paper did not contribute to the conclusions of the citing Gescuk and Davis paper: not only was a study with an alternative monoclonal antibody to CD40L halted due to a high incidence of thromboembolic events in other treated primate species (Kawai *et al*., 2000), but more importantly the therapeutic protocol was not pursued because a phase II study of IDEC-131 failed to demonstrate clinical benefit (Gescuk and Davis 2002).

Since the publication of the citing paper in 2002, an open-label multiple-dose study to evaluate the safety, efficacy, and pharmacokinetics of a humanized anti-CD40L antibody in patients with proliferative lupus nephritis has been reported (Boumpas *et al*., 2003). The study was terminated prematurely because of thromboembolic events occurring in patients, including 2 myocardial infarctions. Also, a clinical trial is currently recruiting in order to "study the regulation of CD154 in patients with lupus in hopes of inhibiting its abnormal and deleterious expression" (ClinicalTrials.gov identifier NCT00008749).

27. Transmissible spongiform encephalopathy: diagnostic methods

While infected blood donors cannot be identified with certainty, infection with human forms of TSE such as variant Creutzfeldt-Jakob Disease (vCJD) will continue to pose a risk for recipients of transfusions. For example, ostensibly healthy individuals with preclinical vCJD can be infectious for at least three years prior to the onset of symptoms (Brown 2005). Two lines of defense are therefore desirable: 1) the development of effective screening assays for the diagnosis of TSE, and 2) elimination of the etiological agents of TSE (PrP^{TSE}) during blood processing via

inactivation or removal. In this review of 26 references, Brown described progress made towards these aims and the validation of proposed methods.

Much of the work to date concerning transmissible spongiform encephalopathy (TSE), dating back to the 1960s, has involved mice and hamsters with rodent-adapted strains of scrapie or human TSEs. Notably, Brown points out, '*…it is always problematic to what extent such models reflect the human situation.*' Nevertheless, experimental bovine spongiform encephalopathy (BSE), scrapie, mink encephalopathy, CJD, sporadic CJD (sCJD), vCJD, and Gerstmann-Sträussler-Scheinker syndromes have all been used to elucidate human TSEs in species as diverse as the goat, the sheep, the cow, the rat, the mink, the guinea pig, the microcebe, the chimpanzee, and various monkeys, for example. Until very recently, results suggested that although low levels of infectivity could often be detected in the blood of rodents with experimentally induced disease, no infectivity could be demonstrated in the blood of animals with *naturally* acquired disease.

Important recent human-based papers, however, have described the almost certain transfer of vCJD to two people from two blood donors in the preclinical phase of disease (Hunter *et al*., 2002; Llewelyn *et al*., 2004; Peden *et al*., 2004). Genetic studies of humans have given rise to the theory that genetic susceptibility may affect the outcome of transfusion exposure to vCJD, as it does to oral exposure to BSE (Brown *et al*., 2000).

Screening assays with high levels of sensitivity are essential, due to the low levels of PrP^{TSE} found in infected blood, even in the clinical phase of disease: Western blot assays and ELISAs are inadequate. A variety of assays have been investigated: 1) a combination of competitive antibody capture and capillary electrophoresis (CE); 2) the conformation-dependent immunoassay (CDI), based on conformational changes in the protein during denaturation; 3) screening for intensely fluorescent targets (SIFT) resulting from aggregations of misfolded PrP^{TSE}, which increases the number of sites available to bind to labeled antibodies; 4) assays using a ligand to bind the PrP^{TSE} to nucleic acid to facilitate PCR amplification (an immuno-PCR assay). TSEs investigated in these assays include scrapie and CJD, and species utilized include hamsters, sheep, chimpanzees and human donors (Safar *et al*., 1998; Schmerr *et al*., 1999; Barletta *et al*., 2005; Bieschke *et al*., 2000; Cervenakova *et al*., 2003; Yang *et al*., 2005).

The chimpanzee study from our random sample (Cervenakova *et al.* 2003) was cited to illustrate the failure of CE to discriminate between normal and CJD-infected chimpanzees or humans, or saline controls. As a result, Brown stated that this assay, along with the others, "remains in limbo with respect to a human screening test methodology." The remainder of Brown's citing paper was largely devoted to a discussion of the development of methods for filtering donor blood, and technical issues associated with the validation of filtered blood, as well as a description of two recently developed tissue culture bioassays with sensitivities comparable to animal-based bioassays, with the potential to greatly reduce the time, space and financial constraints of bioassay testing (Klöhn *et al*., 2003).