CELL MEDIATED IMMUNITY IN VIRUS INFECTIONS

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by

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INTRODUCTION

Many key concepts concerning the nature of immunity have originated from the very practical need to control virus infections. This year, 1996, has been designated the "Year of the Vaccine" commemorating the 200th anniversary of Edward Jenner's vaccination of James Phipps with cowpox virus, and subsequent challenge with smallpox virus. Insight into the nature of viruses, and how viruses interact with mammalian cells, has evolved since the turn of the century. Our concepts of immunity developed concurrently, beginning with Pasteur's treatment of Joseph Meissner with "aged" rabies virus. Antibody-mediated protection conferred by attenuated, live yellow fever virus won the Nobel Prize for Max Theiler in 1951. Perhaps the most exciting area of immunology when I graduated from the University of Queensland Veterinary School in 1962 was the nature of virus neutralization (1), a topic that is still being resolved with the monoclonal antibody (mAb) technology originated by Georges Köhler and César Milstein (Nobel Prize 1985). Crystallographic analysis of influenza virus neuraminidase-mAb complexes, and variants of neuraminidase selected by mAbs, led to the clear demonstration that Ig molecules normally bind to tertiary structure on proteins (2).

The present award is for our discovery (3–6) from experiments with lymphocytic choriomeningitis virus (LCMV) that the nature of T cell-mediated immunity (CMI) is essentially different, focusing on the recognition of cell-surface major histocompatibility complex (MHC) glycoproteins that have been modified as a consequence of infection. My intention here is to place these findings in historical context, and to develop some aspects to the present day in the context of viral immunity and pathology.

THE AUSTRALIAN SCHOOL OF VIRAL PATHOGENESIS AND IMMUNITY

The strengths in both virology and immunology at the John Curtin School of Medical Research (JCSMR) where we did the LCMV experiments were a direct consequence of themes developed in Australia by F.M. (Sir Mac) Burnet. Over a period of more than 20 years, Sir Mac built the Walter and Eliza Hall Institute (WEHI) in Melbourne into a major international center for virus

(particularly influenza) research. In the late 1940's the only person working there on another virus disease was Frank Fenner, who at Burnet's suggestion studied the epidemiology and pathogenesis of ectromelia (mousepox). Following Burnet's demonstration that ectromelia could be titrated by pock assay on chick chorioallantoic membrane, Fenner did careful quantitative studies of virus distribution in a range of tissues (7). His conclusion was that the virus replicated initially at the site of inoculation in the dermis, then the regional lymph nodes, then generalized via the liver and spleen, and again to the skin, where it produced a smallpox-like rash. He also noted that delayed type "allergy" appeared earlier than serum antibody. In 1949 Fenner became head of the Department of Microbiology in the JCSMR at the Australian National University, which had just been formed largely as a consequence of initiatives taken by Sir Howard (later Lord) Florey (Nobel Prize 1945). Fenner built a very strong center for virology research.

In 1957, having just enunciated the clonal selection theory (8), Burnet abandoned virology and re-directed the work of the WEHI to basic immunology, a focus continued with great distinction by his protégé G.J.V. Nossal. Nossal recruited J.F.A.P. Miller, who established a strong program in T-cell immunology that was to have major influence both locally and on the international scene. Some of the WEHI virologists joined Fenner's department in Canberra. One of the few remaining in Melbourne was G.L. Ada, who switched to immunology. Gordon Ada then moved to Canberra in 1968, replacing Fenner who had become Director of the JCSMR. By the time that I arrived, the microbiology department in Canberra had a strong research effort in immunology, interfacing with the virologists remaining from the Fenner era.

Fenner's ectromelia model was subsequently pursued at the JCSMR in two ways that were to influence our discovery. The first was that C.A. Mims made extensive use of fluorescence microscopy to define patterns of virus growth in different organ sites (9), helping to keep the general area of whole-animal viral pathogenesis alive through the 1 960's, when most virologists (following Ender's, Weller and Robbins, Nobel Prize 1 954) turned their attention to *in vitro* tissue culture systems. Fenner's final contribution to experimental viral immunology was to recruit R.V. Blanden back to Australia from the Trudeau Institute, where he had been working with George Mackaness (a Florey trainee and former JCSMR scientist) on CMI in bacterial infections (10). Bob Blanden applied the approaches that he had developed with Mackaness to the ectromelia model, using both adoptive transfer experiments and depletion with anti-thymocyte serum to show the crucial role of T lymphocytes in controlling the infection (11–13).

I had followed Mim's papers for many years, and returned to Australia from Edinburgh at the end of 1971 to work with him. He had warned me that he might be moving to a Chair in Microbiology in London, and left before the end of 1972. I inherited his laboratory, his technician (Gail Essery) and the LCMV model that had been brought to Canberra some years before by Fritz Lehmann-Grube. Rolf Zinkernagel arrived in 1973, to work with Blan-

den on CMI in Salmonellosis. Due to space requirements, Gordon Ada moved Rolf into the laboratory with me, which is how our collaboration started. Rolf was later to use our experimental data for his Ph.D. thesis but, though I helped him with the writing, the relationship was always as equal colleagues rather than supervisor and student.

EARLY EXPERIMENTS WITH LCMV AND THE DISCOVERY OF MHC RESTRICTION

My initial focus with the LCMV model was to combine contemporary T-cell immunology approaches with the capacity to quantitate inflammatory pathology (14) by counting cells in mouse cerebrospinal fluid (CSF), using a CSF tap technique that I had learned from R.I. Carp (15). This proved to be a very powerful approach (Fig. 2), providing strong support for the theme developed initially by W.P. Rowe and J.E. Hotchin (and later by D. H. Gilden, G.A. Cole and N. Nathanson) that clinical LCM is an immunopathological disease (16–18). The most visually satisfying experiment done at this time was to use Evan's blue injection to show that the virus-immune T-cells were causing a total breakdown of the blood-brain barrier to protein, an experiment that reaches back to the dye studies of Paul Ehrlich (Nobel Prize 1908). Much of this work never appeared in the primary literature, and was only published (3) in an article that G. Möller requested for *Transplantation Reviews.* This also carried the first, tentative description of the MHC-restriction finding.

The formal collaboration with Rolf Zinkernagel started when I suggested that we might look to see if the CSF cells that I was obtaining (Fig. 2) from clinically affected mice could be shown to have cytotoxic T lymphocyte (CTL) activity. Having worked in Lausanne, he was very familiar with the ^{51}Cr release assay that was being used so effectively by J.-C. Cerottini and K.T. Brunner (19) to study alloreactivity and had tried (unsuccessfully) to apply the approach to the *Salmonella* model. The CTL assay had already been used with spleen cells from LCMV-infected mice by M.B.A. Oldstone, O. Marker, M. Volkert and colleagues (20,21). Fortuitously, we were using H-2^k- compatible CBA/H mice and L929 fibroblasts (L cells) as a source of T lymphocytes and virus-infected targets respectively. The first experiment worked beautifully, and we were able to show that the lytic activity was mediated by Thy-1 positive cells (22).

At about this time, the very active group of cellular immunologists that made up Gordon Ada's weekly "bible class" had been discussing recent publications on immune response (Ir) gene effects from B. Benacerraf (Nobel Prize 1980) and D.H. Katz at Harvard , which used a very complex *in vivo* mouse model of T-cell/B cell collaboration (23). We later read of the experiments of E. Shevach and A. Rosenthal at NIH, Bethesda, who were looking at the Ir gene question using *in vitro* stimulation of guinea pig T-cells (24). The paradigm (25) in this East Coast USA immunology axis was that the Ir genes, which had been mapped to the I-region (now MHC class II) between the loci for the "strong" transplantation antigens (H-2K and H-2D, now MHC class I),

encoded all (or part of) the "enigmatic" T-cell receptor (TCR). This interpretation would probably have quickly been revised when it became apparent that the Ir gene product (Ia antigen) was expressed on macrophages (26).

We then saw a report by H. McDevitt, G.R. Mitchell and M.B.A. Oldstone that there was an "Ir gene" effect in the LCM immunopathology model (27). This stimulated us to accumulate all the mouse strains that were available (CBA/H, BALB/c, and C57BL/6J) in Canberra to see if we could correlate the level of CTL activity with H-2 type. The big surprise was that only the H-2^k T-cells were lytic for the MHC compatible, LCMV-infected L cells (3,4). We had no appropriate H-2^b or H-2^k cell lines available, so used primary peritoneal macrophages (28) to provide a source of LCMV-infected targets to demonstrate reciprocal exclusion of T-cell recognition. The next important experiment was with the H-K^kD^d A/J mouse strain (29) , establishing that CTL recognition mapped to H-$2D^d$ and (perhaps) to H-$2K^k$. Later studies on immunodominance hierarchies (30,31) were to show us how lucky we had been to have a system available where both the H-2K and H-2D alleles were associated with a potent LCMV-specific CTL response. The basic findings with LCMV were rapidly replicated for ectromelia by Bob Blanden's student Ian Gardner (32). Bob also used his contacts as a more established immunologist to bring in a range of H-2 recombinant and mutant mouse strains over the next 12–18 months that were used (with both the LCMV and ectromelia models) to map virus-specific CTL effector function to the MHC class I alleles (33,34). The more detailed description of the actual experiments, the preceding history of the MHC and the way that the TCR repertoire develops during ontogeny are being covered in the accompanying article by Rolf Zinkernagel (35).

THE "SINGLE TCR ALTERED SELF" HYPOTHESIS

Right from the time that we made the discovery (3–6), we considered that there might be two possible explanations for our findings (Fig. 1). The first was based on the "TCR" type hypothesis of Katz and Benacerraf, which was formulated as the various dual recognition models proposed through the 1970's and early 1980's. The second was that the virus was in some way modifying the MHC molecule (4–6), either by complexing with it on the cell surface or by inducing some other (perhaps allosteric) change. This idea of an "altered self" molecule recognized by a single TCR seemed reasonable for virus-infected cells, though we were a bit perturbed when the recognition of "minor" histocompatibility antigens was later shown to obey the same rules (36,37). Evidence against the dual recognition model as originally stated (like-like interaction) came from adoptive transfer studies, where we showed that LCMV-primed T-cells from H-2 heterozygous mice ("AxB" F1) which would kill virus (v) infected A and B targeT-cells partitioned into "clonotypes" lytic for A+v alone or B+v alone following further stimulation in irradiated, virus-infected mice of type A or B, respectively (5). Later variants of "two recep-

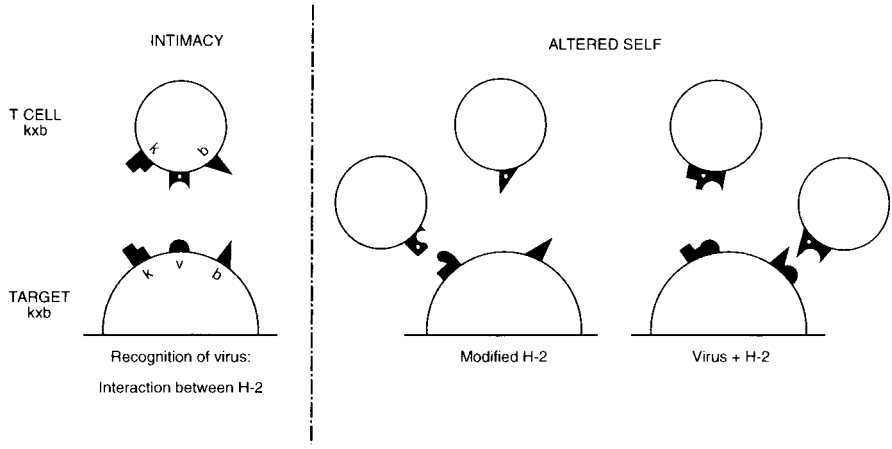

Figure 1. This formulation of the "single T-cell receptor altered self" hypothesis was published in the second article that appeared in *Nature* (4), about a year after the initial experiment showing MHC restriction of CTL activity. In this paper we discredited the "intimacy" model, which argued that there might be some form of "like-like" interaction between H-2 glycoproteins expressed on the T lymphocyte and the target cell. The experiments, and the intellectual context, are dealt with in much greater detail in the accompanying article by Rolf Zinkernagel (35).

tor" models got around this problem by arguing that the postulated recognition molecules seeing A or B molecules were themselves clonally excluded.

The "single TCR-altered self" hypothesis allowed us to assign a biological role to the strong transplantation antigens, and to explain alloreactivity, Ir gene hierarchies, and the extreme polymorphism of the MHC. We believed from the outset that we had found the mechanistic basis of immunological surveillance of self, though we used the term in a somewhat different context (5,6,38,39) from that employed by Lewis Thomas and Burnet in their discussion of susceptibility to cancer (40). Many people did not accept the "altered-self" idea, including some of our colleagues in the JCSMR group (34). It was to remain controversial, and regarded as heretical by most T-cell immunologists for the next 10 years or so (discussed in, 41,42). The "two receptor" models that were generally favoured continued, however, to have the problem that they offered no satisfactory generalization that would accommodate alloreactivity and the Ir gene mechanism.

The resolution of the dilemma came with the finding of A.R. Townsend, A.J. McMichael and colleagues that the class I MHC molecules are presenting viral peptides processed via the "endogenous" pathway (43). This, together with the publication of the 3-dimensional structure of a class I MHC molecule by P. Bjorkman, J. Strominger and D. Wiley (44), and the definition of the two chain TCR by M. Davis, S. Hedrick, T. Mak, J. Allison, P. Marrack, J. Kappler, S. Tonegawa and others (45), put the final nail in the coffin of the various "dual receptor" TCR models. The recent characterization of the tertiary structure of the TCR provides a very satisfactory conclusion to the debate that started with the "altered self" hypothesis (46,47).

THE LCMV MODEL AND T-CELL TARGETING *IN VIVO*

The MHC-restriction of CTL effector function indicated from the outset that the virus-immune T-cell must interact directly with the virus-infected target cell. This had already been obvious for alloreactivity, but no one had suggested that the strong transplantation antigens were in any way involved in the immune response to pathogens. Thinking about CMI in infectious diseases had been very much constrained by experiments with *Listeria monocytogenes*, which emphasized the need for T-cells in macrophage recruitment and activation (10). The numbers and the status (in terms of heat shock-protein mRNA expression) of monocyte/macrophages that localize to the lungs of mice infected with an influenza A virus are, for example, clearly a function of the concurrent $CD4^+$ and/or $CD8^+$ T-cell response (48). Macrophages activated during the T-cell-mediated elimination of LCMV or ectromelia virus can rapidly destroy *L. monocytogenes* (49).

The MHC-restriction findings with *in vitro* CTL assays were quickly translated into the *in vivo* situation. One of the reasons that the LCMV model was so powerful was that we had a very clean *in vivo* system for analysing an inflammatory process. Unlike many viruses, LCMV causes little damage and the levels of "background" cellular infiltration independent of the T-cell response (3,50) are low (Fig. 2). Combining this with the capacity to quantitate

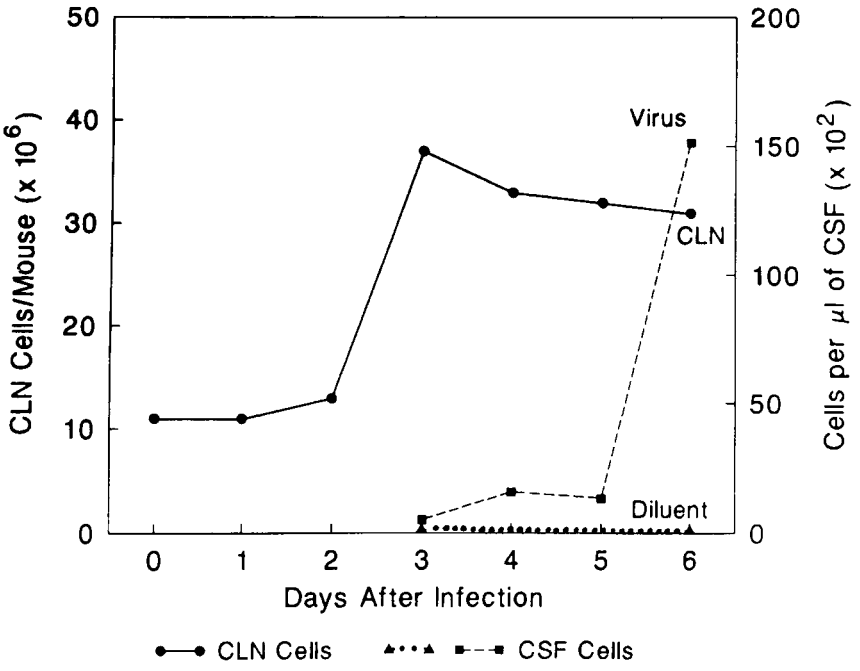

Figure 2. The finding that led to the discovery of MHC-restriction was the demonstration that the inflammatory population recovered from the CSF of mice with clinical LCM contains potent CTL effectors (22). The data shown are from a much later experiment (50), which illustrates the massive influx of cells (dotted line) into the CSF accompanying the onset of CTL activity between days 5 and 6 after infection of the central nervous system. Also shown (solid line) is the preceding increase in cellularity (4-fold) of the cervical lymph nodes (CLN).

very accurately by counting cells in the CSF allowed us to determine rapidly by adoptive transfer experiments that the same rules apply for T-cell recognition *in vitro* and *in vivo*. This was shown for differences in MHC haplotype, particular MHC class I alleles and for H-2 mutant mice (34,51,52).

The interpretation that the T-cell must bind directly *in vivo* to the virus-infected target rather than just to an appropriate antigen-presenting stimulator cell has, however, continued to be subject to challenge. The alternative idea is that cytokines released as a consequence of such interactions are the key effector molecules, an idea that seems to work for the control of a hepatitis virus transgene by interferon-mediated mechanisms (53). So far, this hepatitis model seems to be unique. Adoptively transferred LCMV-immune T-cells only caused severe meningitis in virus-infected, chimeric mice when the appropriate MHC restriction element was present on virus-infected epithelial cells in the brain. Neither secondary stimulation of the virus-specific CTL populations in lymphoid tissue, nor interaction with inflammatory monocyte/macrophages in the site of pathology, were alone sufficient to cause the massive cellular extravasation characteristic of LCM (54–56).

Perhaps the final indication that the LCMV-immune effector CTL must interact directly with virus infected CNS epithelia *in vivo* has come from experiments with perforin -/- mice, which failed to develop the classical symptoms of T-cell-mediated immunopathology (57). Cytokine-mediated mechanisms may, however, be responsible for the chronic wasting disease (58–60) that develops in LCMV-infected CD8 "knockout"(-/-) mice and $CD8^+$ T-cell-deficient mice (61) that are -/- for β2-microglobulin (β2-m), the light chain of the MHC class I glycoproteins. Both these genetically- manipulated mouse strains mice are unable to clear LCMV, leading to a persistent confrontation between virus-infected stimulators and the immune $CD4^+$ population. The studies with LCMV, and other experiments with respiratory viruses (see below), indicate that the effector mechanisms used by $CD4^+$ and $CD8^+$ T-cells to deal with viruses are fundamentally different.

THE NON-SELF COMPONENT: ANALYSIS WITH RESPIRATORY VIRUSES

The division of labor in the LCMV experiments at the JCSMR was that Rolf Zinkernagel did the *in vitro* CTL analysis, while I was responsible for the *in vivo* immunopathology experiments and for writing the manuscripts with, of course, the benefit of his immediate and constant critique. I thus used respiratory infection with the parainfluenza type 1 virus, Sendai virus, to develop a facility with the CTL assay (62), and to confirm that MHC restriction was true for more than LCMV and the poxviruses (27, 32, 63). I returned to the Sendai model (64, 65) years later at St Jude Children's Research Hospital, principally because of the molecular virology expertise available in the laboratory of A. Portner.

When I moved to the Wistar Institute in mid-1975, it quickly became apparent that the animal facility was not sufficiently secure to allow extensive experimentation with LCMV, which can easily induce clinically "silent" infections

in mouse colonies. The CTL response to Sendai virus was being studied by others at the Wistar, so I took the opportunity to collaborate with W.E. Gerhard on the analysis of T-cell-mediated immunity to the influenza A viruses (Fig. 3). Walter Gerhard had learned his influenza virology at the Basel Institute for Immunology from Stephen Fazekas de St Groth (1), who had formerly been a leading figure in Fenner's virology department at the JCSMR.

The aim was to exploit "recombinant" influenza A viruses to dissect the other half of the T-cell specificity equation, for viral components rather than for MHC glycoproteins. The so-called recombinants result from growing two different influenza A viruses concurrently in the one cell (66). The eight segments of the influenza genome re-package to give novel variants, the mechanism responsible for the phenomenon of "antigenic shift". The principal focus of immunology-related influenza research, which tends to focus on vaccine studies, was on the viral hemagglutinin (H) and neuraminidase (N) glycoproteins. These virion surface proteins are subject to the antibody-mediated selection pressure that results in "antigenic shift," a further mechanism that can give rise to novel pandemic strains.

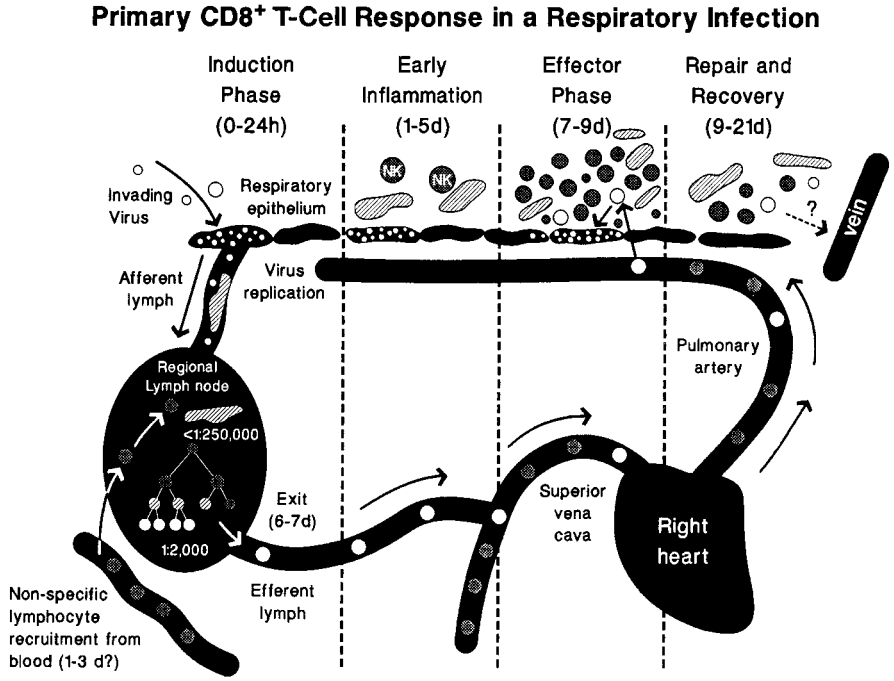

Figure 3. The various events occurring in the respiratory tract and regional lymph nodes are illustrated for mice infected with an influenza A virus, or with Sendai virus (64,98,83). Infectious virus cannot generally be recovered from the lung subsequent to day 9 after infection. The $CD8^+$ and $CD4^+$ T-cells are responsible for most of the monocyte/macrophage recruitment (48). The $CD8^+$ set predominates in the inflammatory exudate, but most of these lymphocytes are memory T-cells specific for other antigens. Probably <1:100 are reactive to the inducing pathogen (99). Potent CTL effectors, and large numbers of cytokine-producing cells, are found mainly in the virus-infected lung.

We thought that, by using serologically distinct variants such as the H1N1 and H3N2 viruses which do not cross-neutralize, we would be able to map CTL activity to H or N, both of which are expressed on the plasma membrane of the virus-infected cell. The aim then was to use "drifted" variants to map fine specificity. The initial experiments were done by my first graduate student, R.B. Effros and were later pursued by my second student J.R. Bennink. Much to our surprise, Rita found that the CTL response for these two viruses was almost totally cross-reactive (67,68). Though we were unaware of it at the time, similar findings were being recorded in B.A. (Ita) Askonas' laboratory at the National Institute for Medical Research in London (69). Our findings with LCMV had induced Ita to drop her long-term studies of the B cell responses to concentrate on virus-specific T-cell mediated immunity. Her laboratory was to make an enormous contribution over the next 10 years.

Though we failed at this stage to define the nature of the antigenic entity recognized by the influenza specific CTL, these early experiments further supported the conclusion from the MHC restriction analysis that the specificity profiles of T and B lymphocytes are fundamentally different (34,70). In addition, we quickly established that exposure to any one influenza A virus will prime T-cell memory for a secondary response (Fig. 4) to any other in-

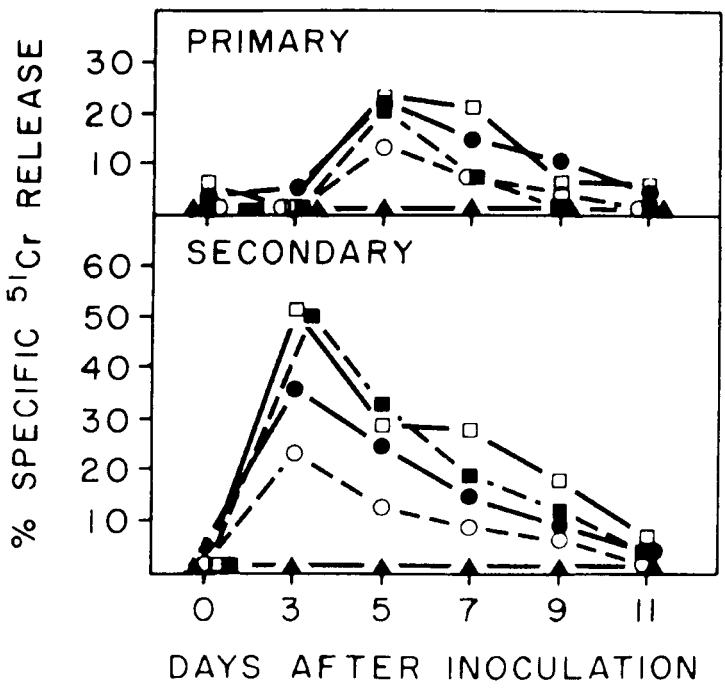

Figure 4. Immunologically naive (primary) CBA/J (H-2^k) mice, or mice that had been infected with the PR8 (H1N1) influenza A virus 52 days previously (secondary), were challenged with the HKx31 (H3N2) influenza A virus (68). Spleen cells were assayed (100:1) at the intervals shown on H-2 compatible targets infected with a range of influenza A viruses, or with an influenza B virus (triangles). Both the enhanced kinetics and potency of the recall response are very apparent. Later experiments with HKx31 infection of C57BL/6J (H-2^b) mice have shown very little CTL activity in lymphoid tissue following primary exposure (98).

fluenza A virus (68,71). This meant that infection with a "drifted" or "shifted" influenza A virus in an adult person is likely to be proceeding in the context of a secondary CTL response and a primary humoral response. The albeit imperfect protection conferred by influenza-specific memory T-cells (72–74) probably explains why young to middle-aged adults are generally much less likely to die from influenza than are small children or elderly people. These results were quickly confirmed for other viruses which had, after the influenza experiments, also been found to induce CTL specificity profiles that were not predicted by serological analysis (75).

Other significant experiments that were done with the influenza model at about this time were the extension of MHC-restriction to the rat model (76) and, most importantly, the clear demonstration by A.J. McMichael (from the Askonas laboratory) that this is also true for humans (77). Further pursuit of this unpredicted cross-reactivity for the influenza viruses led to A. Townsend's (again from the Askonas laboratory) seminal finding (43,78) that MHC class I restricted T-cells are responding to a peptide derived from the influenza nucleoprotein (NP), which finally explained the molecular mechanism underlying our "altered self" hypothesis (Fig. 1) and started the whole field of antigen processing in the "endogenous" compartment. Analysis at the Wistar Institute by Jack Bennink and J.R. Yewdell with recombinant vaccinia viruses also showed that much of the CTL recognition in the influenza model is directed at internal components of the virus (79).

Latterly, the non-lethal respiratory infections have emerged as the experimental system of choice for analysing localised, transient viral infections (80–83). These models have the advantage that it is easy to obtain both the regional lymphoid tissue, and the inflammatory cells from the pneumonic lung by bronchoalveolar lavage (BAL). There is also no obvious way that either the virus or the viral genome can persist after infection with such negative strand RNA viruses (84), which is important when we are considering the difficult area of T-cell memory (85).

Some recent experiments addressed again the question of T-cell targeting *in vivo* that we had analysed earlier with the LCMV model (Fig. 2). Using adoptive transfer protocols and bone marrow radiation chimeras made between β2-m -/- and +/+ mice, we showed that the clearance of Sendai virus depends on the $CD8^+$ effector T-cells interacting directly with virus-infected, MHC class I^+ respiratory epithelium (86). The opposite conclusion was reached concerning the capacity of $CD4^+$ T-cells (87–89) to control influenza virus infection in the absence of the $CD8^+$ subset: expression of the MHC class II glycoproteins that target the $CD4^+$ T-cells is not essential in the respiratory tract (90). More recent experiments performed by David Topham are confirming the theme developed by Walter Gerhard (91) that complete clearance of the virus by $CD4^+$ T-cells requires the concurrent presence of antibody-forming cells.

QUANTITATING INFLAMMATION AND T-CELL-MEDIATED IMMUNITY

The essence of the virology-based approach to pathogenesis has always been quantitation, a theme that was started by Mac Burnet with his various plaque assays and continued by Cedric Mims for the fluorescence microscopic localization of virus-infected cells in different organ sites (9). Measuring the inflammatory process that is the consequence of CMI has, however, traditionally been (at best) semi-quantitative, The classical delayed-type hypersensitivity foot-pad swelling assay is a very blunt instrument.

Analysing inflammation

My early experience in ultrastructural pathology and immunocytochemistry (92,93) resulted in an intense curiosity concerning the nature of the lymphocyte populations that invade into tissue sites of virus growth (Fig. 5). The

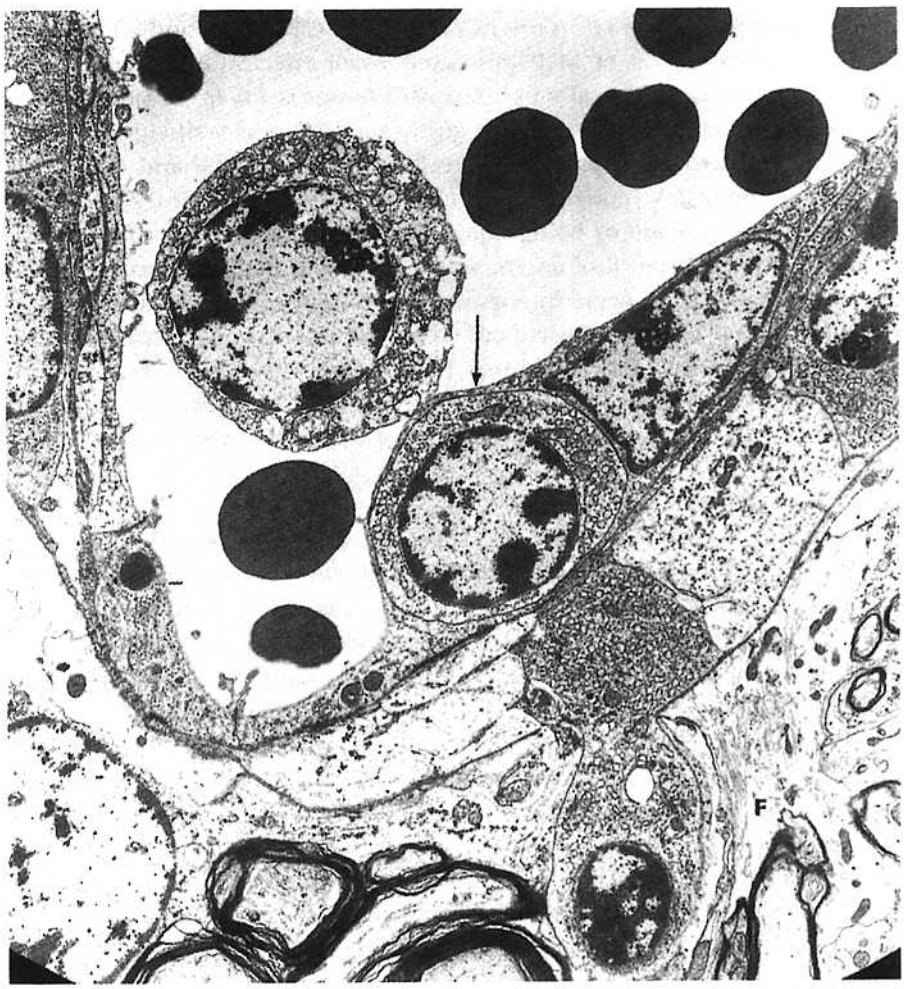

Figure 5. My interest in the nature of virus-specific CMI was stimulated by the experimental studies of louping-ill encephalomyelitis that Hugh Reid and I did with sheep at the Moredun Research Institute, Edinburgh (92–94). The electron micrograph shows cells of undetermined identity extravasating from a capillary into the brain parenchyma. Lymphocyte invasion into tissue sites of virus growth is the central feature of CMI.

experiments that H.W. Reid and I did with the sheep louping-ill (tick-borne flavivirus) model at the Moredun Research Institute in Edinburgh led to the conclusion that antibody forming cells were extravasating into the CNS and were responsible for a substantial, *in situ* virus-specific Ig response (92, 94). Much later studies by D.E. Griffin have shown that such locally-produced antibody controls Sindbis virus infection of mouse brain (95).

The dual frustrations with morphological approaches are the lack of functional analysis and the difficulty of quantitation, though my current perception is that there is a great need to focus more attention on the anatomical localisation of events in immunity that is only possible with microscopy-based protocols. My early attempts at quantitating inflammatory pathology caused me to seize on the CSF tap technique that Richard Carp (15) had developed to look for enzymatic activity in CSF from scrapie-infected mouse brain and apply it to the study of viral meningitis (Fig. 2). Experiments with this simple model induced us to develop the LCMV-specific CTL assay, and allowed the later *in vivo* dissection of MHC-restricted T-cell effector function. The capacity to measure statistically significant differences for quite small effects enabled us to operate with unique sensitivity in the *in vivo* situation (3, 34). However, because the approach was technically demanding and only really useful with the LCMV model, nobody else adopted this analytical system. The other, great advantage of being able to obtain inflammatory cells directly from the CSF (or from the lung by the BAL) is that the lymphocytes do not need to be freed from tissue by enzymatic techniques. This has been of great benefit for later experiments concentrating on flow cytometric analysis and FACS separation to define functional T-cell subsets (56, 96, 97), experiments that were first started for LCMV (with R. Ceredig in Canberra) and have been a major focus of the respiratory infection studies (98,99) in Memphis (Fig. 3).

Measuring the T-cell response

Though the *in vitro* ^{51}Cr release CTL assay provides numbers, the precursor (p) frequencies that give comparable levels of lytic activity (both during the course of *in vivo* infection or as a consequence of *in vitro* culture) can vary enormously (100,101). The potency of the $CD8^+$ CTL set in the BAL population from mice with influenza pneumonia is, for example, apparently equivalent by day 10 after infection for $CD4^+$ T-cell deficient MHC class II -/- mice and for MHC class II +/+ controls (101,102). However, the CTLp numbers in both the BAL and regional lymph nodes are much lower for the MHC class II -/- mice, and virus clearance may be slightly delayed. The much smaller CTLp pool in the MHC class II -/- mice is almost totally consumed to provide the level of CTL effector function needed to deal with the infection. A conclusion based on the CTL data alone that the absence of the $CD4^+$ set does not greatly modify the magnitude of the $CD8^+$ T-cell response would thus be misleading. Careful, kinetic analysis is essential in these *in vivo* pathogenesis studies.

We first realised this when we tried to quantitate the Ir gene hierarchy that we discovered for the MHC class I alleles $H\text{-}2K^k$, $H\text{-}2K^b$ and $H\text{-}2D^b$ with the in-

fluenza A viruses (30,31). The situation here is that the CTL response associated with H-2D^b +NP peptide is dominant in mice that express H-2K^b, but apparently absent when H-2K^k is present. The same thing happens with vaccinia virus. Jack Bennink and I tried hard to work out the underlying mechanism, using thoracic duct cannulation and negative selection *in vivo* to remove the alloreactive T-cells (103), a very tedious protocol that we learned from J.A. Sprent. The lack of any useful resolution is a good example of the fact that (though important insights can be generated) molecular mechanisms cannot ultimately be worked out by biological experiments. My current guess is that H-2K^k must greatly out-compete H-2D^b for binding some constant molecule involved in the MHC class I antigen processing pathway (104), though not to the extent that H-2D^b +NP peptide is no longer present at sufficient levels to be recognized by the appropriate effector CTL on an H-K^{k}D^b targeT-cell.

When J. A. Owen joined the laboratory in Philadelphia she took on the task of developing a quantitative limiting dilution analysis (LDA) for determining influenza virus-specific CTLp frequencies (105), an approach that has been central to my research program over the subsequent years. Judy Owen and Michelle Allouche then applied this technique to the H-2K^{k}D^b hierarchy problem and found that, for mice that had been primed a month or more previously, the CTLp numbers specific for influenza virus components expressed in the context of H-2K^k and H-2D^b may be fairly comparable (106). Recent experiments with this experimental system by R.A. Tripp in Memphis indicate that the reason that the H-2K^k-restricted CTL effectors are preferentially generated is that this component of the response emerges more quickly.

The rate problem in immunology

The preceding is a good example of one of the major difficulties that we face in developing a detailed understanding of immunity. How do we measure the true kinetics of responses in terms of lymphocyte generation and loss, and in the context of transit times through the various tissue sites that are sampled? A major insight developed from studies with superantigens was that the majority of the T-cells that proliferate following such stimulation die as a consequence (107,108). We initially thought that this was not the case for "conventional" antigens, such as influenza virus or Sendai virus epitopes, largely because sequential LDA studies indicated a remarkable constancy (Fig. 6) in CTLp frequencies (1:2–3,000 cells in a lymph node) from about day 7 after infection through to long-term memory (85,99,109). This was somewhat different from the situation that was concurrently being described for LDA studies with LCMV by Rafi Ahmed (110), but we agreed that the difference was probably due to the fact that systemic LCMV infection undoubtedly gives a much greater antigenic stimulus.

The thymidine analogue bromodeoxyuridine (BrDU) incorporates into the DNA of multiplying cells and causes the formation of toxic thymidine dimers following exposure to bright light, a protocol that was used many years ago to "suicide" *in vitro*-stimulated T-cells (111). Ralph Tripp found that load-

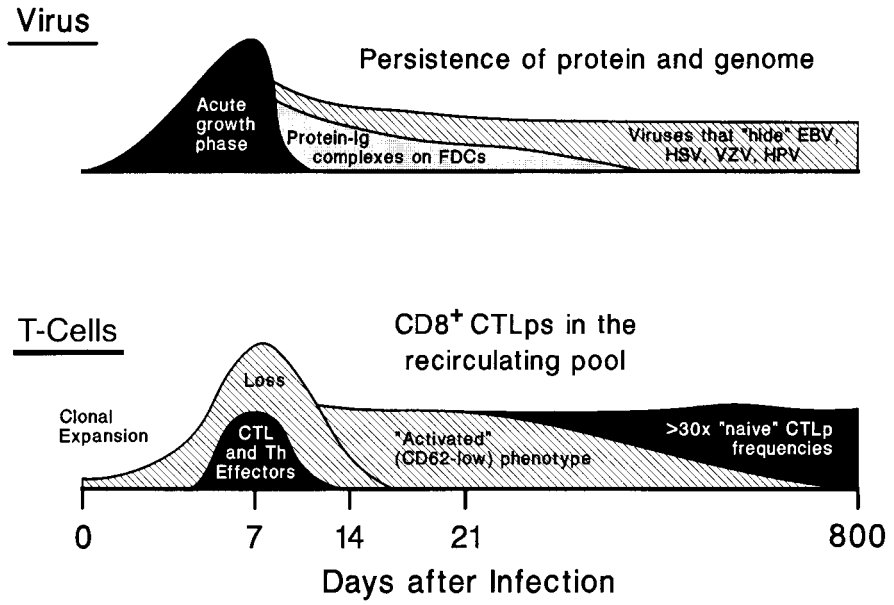

Figure 6. *Virus.* Replication of the negative strand RNA viruses is generally terminated by the immune response within 7–10 days (84,118), with the only trace of the infectious agent generally being the long-term maintenance of protein-Ig complexes on the surface of follicular dendritic cells (110). These do not seem to be necessary for the maintenance of influenza-specific $CD4^+$ T-cell memory (119), but are probably essential for B cell memory. Other pathogens, such as the herpes viruses, may persist in a latent form in neurons or B lymphocytes and periodically reactivate into lytic phase (120).

T-cells. Clonal expansion of the $CD4^+$ THp and $CD8^+$ CTLp during the acute phase of the response is greatly in excess of the numbers required to generate the cytokine-producing TH or effector CTL populations that deal with the infection. Many of the CTLp, in particular, either die or are excreted, and CTLp frequencies may remain remarkably stable thereafter for the life of a laboratory mouse. However, the activation phenotype of these T-cells may (as first shown by Sam Hou, 121) change with time.

ing proliferating T-cells with BrDU *in vivo* led to the same effect when the lymphocytes were exposed to the laser beam of the flow cytometer (112). Use of this protocol indicates that the CTLp numbers generated during the acute phase of the response to an influenza A virus are more than 10-fold in excess of what would be expected from estimates of CTLp frequencies (Fig. 6). Some of these T-cells will give rise to the CTL effectors that are found in the virus-infected respiratory tract, but it seems that many others are lost. Measuring a virus specific $CD8^+$ T-cell response by effector CTL assays takes no account of the CTLp numbers that are generated. However, the more extensive quantitation of CTLp frequencies shows only the balance between CTLp generation and loss, and does not give a true estimate of the real magnitude of an acute host response. This is less a problem with T-cell memory, where the cell populations are turning over at a much slower rate (113).

Homeostasis: on the edge of chaos?

The above experience, together with trying to understand the nature of T-cell memory, has left me with the conviction that the major challenge for cellular immunology is to develop a much clearer understanding of lymphocyte ho-

meostasis (114). In the past, many of us would have followed Burnet's insight that the balance between responsiveness and tolerance is the key question, but recent studies (particularly with transgenic mice) have clarified the issues, blurred the distinction and made the tolerance/response mantra less compelling (115). It could be argued that tolerance/response is simply a more refined statement of the homeostasis problem, but language conditions, perceptions and defining the area in this way focuses attention solely towards antigen and away from the more physiological mechanisms and quantitative considerations that are also likely to be enormously important.

An obsession with homeostasis is dangerous territory, as it has led in the past to some of the least useful and expensive efforts in immunology. Even so, it may be time for our experimentally- based discipline to take greater cognizance of the contribution that can be made by theoreticians, particularly those who are more mathematically inclined. A good example of the way that quantitative analysis can contribute to the development of better predictive models has been provided by recent determinations of the numbers of virus infected cells (116) in people infected with the human immunodeficiency virus (HIV). Influenza virus-specific memory $CD8^+$ T-cells seem to show a remarkable constancy in frequency over a very long period (Fig. 6). Is this an example (117) of a chaotic system? Now that we are starting to generate useful numbers, we need the help of people whose business is numbers.

CONCLUSIONS

The need to deal with pathogens has driven the evolution of the vertebrate immune system, so it should not be surprising that experiments with infectious agents have often illuminated key elements of the underlying mechanisms. The discovery of MHC restriction and the development of the single TCR "altered" self hypothesis is a classical example of how interfacing different scientific disciplines and ways of thinking, an inevitable consequence of studying viral pathogenesis, can lead to a major paradigm shift. The progress that was made over the subsequent 10 years, and the intellectual directions that were followed as a consequence of this simple, operational hypothesis tell us a great deal about the power of ideas. This is not to decry the importance of technology. Our success depended totally on the availability of the ^{51}Cr release CTL assay and inbred mouse strains, both of which had been developed to study alloreactivity. The experiments were done in the context of an experimental framework that was being used to dissect T-cell responsiveness, the immunopathology of LCMV infection, and the nature of CMI to *L. monocytogenes* and ectromelia virus. The local intellectual environment was strong, and heavily focused on the then current forefront of immunology research. Being isolated in those pre-FAX and e-mail days in Australia was a great advantage, as it allowed time to discuss and to think things through. We had outspoken and informed local critics, the freedom and resources to pursue our own ideas and were given full credit for our efforts. Those of us who

have senior roles in science need to do everything possible to ensure that comparable opportunities and environments remain available to young scientists.

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