The Immunology of Cytomegalovirus Infection

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Persistent virus infections are often advanced as possible causes for diseases of unknown aetiology. This is logical enough, given the evidence that persistent virus infections in animals, and in certain specific instances in humans, may indeed be associated with various chronic diseases. There are a number of possible strategies for pursuing research in this area: (a) to look for new viruses in diseases of unknown aetiology; (b) to ask whether a particular known virus is associated with a particular disease; (c) to endeavour to learn as much as possible about the biology of a given persistent virus, and see what one learns about its association with disease. Options (a) and (b) are relatively 'high risk', and while well worth pursuing, the third option is perhaps safer and will more predictably yield useful information.

In the last few years we have been studying human cytomegalovirus (HCMV), attempting to understand how the virus persists in the normal host, and how perturbation of this relationship leads to disease.

The Clinical Problem

Primary infection with HCMV in the normal subject is usually asymptomatic but may be associated with the syndrome of infectious mononucleosis (about 10 per cent of infectious mononucleosis is said to be due to HCMV and associated with a negative monospot test). In immunosuppressed subjects, however, primary HCMV infection can produce severe illness, with dissemination and involvement of lungs, liver, gut, eye and other sites. In pregnancy, primary maternal infection is more likely to lead to congenital infection in the neonate than to reactivation of maternal infection[1]. Following primary infection, the virus persists in the host, as do the other herpes viruses. In contrast to herpes simplex and varicella zoster, which establish classical latency in neuronal cells, it now seems more likely that Epstein-Barr virus (EBV), and probably HCMV as well, may replicate at low level for much of the time in the healthy carrier without producing symptoms. EBV persists in B cells and probably in oropharyngeal epithelial cells[2] but the exact site(s) at which HCMV persists in the normal infected individual is still uncertain. The virus probably persists in epithelial cells in the salivary gland and in the kidney; although it is commonly stated that HCMV persists in lymphocytes and/or macrophages, the hard evidence for this is lacking. Major reactivation and dissemination may occur if the host is immunosuppressed. Iatrogenic immunosuppression in the context of organ or bone marrow transplantation has been the most frequent setting for severe HCMV infection, but HCMV is now also one of the most frequent causes of morbidity and death in patients with the acquired immunodeficiency syndrome (AIDS)[3].

In many ways HCMV is the most enigmatic of the herpes viruses, and it is partly for this reason that it has been a 'soft candidate' for implication in diseases of unknown aetiology. HCMV has been rather speculatively associated with a number of such diseases, from atherosclerosis to disorders of the central nervous system. As with other herpes viruses, HCMV is also a candidate oncogenic virus and has been proposed as a cause of African Kaposi's sarcoma. These various disease associations will all remain speculative until more convincing data emerge, particularly from careful DNA hybridisation studies.

Virology and Molecular Biology

Some knowledge of the molecular virology of HCMV is an essential prerequisite to understanding its immunology and biology. HCMV is the largest of the human herpes viruses, with a linear double-stranded DNA genome of about 235 kilobases (Fig.1). This has the capacity to code for up to 150 proteins but as yet the precise number and nature of these proteins is unresolved. However the complete nucleotide sequence of the virus will probably be known soon and this will enable much of the protein structure to be deduced from the primary sequence. Most work on the molecular biology of the herpes viruses has been done with herpes simplex, so more is known about it than about the other herpes viruses, for which it tends to serve as a prototype.

As with other herpes viruses, synthesis of the virus-specified proteins occurs in a regulated cascade, expression of one set of proteins being essential for expression of the next (Fig.1). The immediate early (IE) proteins are expressed shortly after the virus infects a cell. Some of them are 'trans acting factors' that can stimulate transcription of the genes for the virus IE proteins (see below), and, although this is presumably their true function, they can also stimulate transcription of inducible cellular genes[4]. Some of the IE proteins are DNA binding proteins, and the 'trans' refers to their ability to activate transcription from transcription units on duplex DNA other than that on which they themselves are encoded. The virus early proteins are then expressed: little is known of their function, but one of them is the virus DNA

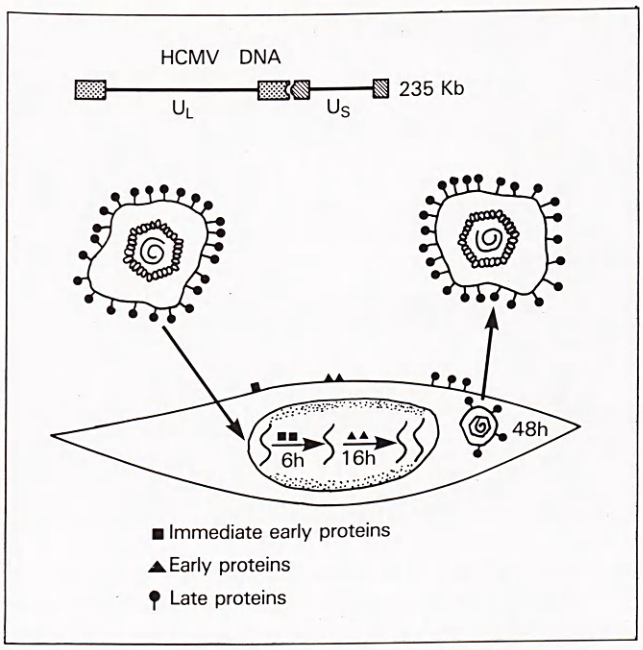

Fig. 1. *Schematic view of genome and protein synthesis of human cytomegalovirus. The virus has a linear double-stranded DNA genome divided into long and short unique DNA sequences (U_L and U_S) which are each bounded by inverted repeat sequences. Within the infected cell the proteins specified by the virus are expressed in three sequential phases, the immediate early and early proteins are* only *expressed in the cell, and virus DNA replication cannot occur until they have been expressed. Following virus DNA replication the late proteins are expressed: they are the only proteins actually present in the virus particle. The times refer to the approximate timing of expression and progeny virus production in tissue culture.*

polymerase. Unlike herpes simplex, HCMV does not encode its own thymidine kinase, and this explains its lack of sensitivity to acycloguanosine (Acyclovir). The next event is replication of the virus DNA, following which the virus late proteins are expressed: the late proteins are the structural proteins of the virus particle. It is important to emphasise that the IE and early proteins are only expressed in the infected cell and not in the virion particle itself, which is particularly pertinent when considering the specificity of the immune response. Little is known of the precise molecular events in the host which determine reactivation of latent virus, or affect the subsequent transcription of the virus genome, although they are likely to be of great importance in the pathogenesis of HCMV infection.

Immunology

The presence of serum antibody is used as a marker of infection with the herpes viruses (as it is for other viruses) and thus as evidence for the carriage of latent virus. Antibody is probably important in neutralising virus in the fluid phase and perhaps in diminishing the severity of primary infection. Yet immunoglobulin deficiency is not associated with particularly severe herpes virus infections, and reactivation occurs in seropositive subjects despite the presence of serum antibody. Hence it is generally assumed that T cell immunity is likely to be of more importance in controlling these large DNA viruses within the infected host. Evidence to back this assumption comes from the considerable body of work on EBV: normal subjects seropositive for EBV have a high frequency of memory cytotoxic T cells in their peripheral blood, which show specificity for EBV-transformed B cells. These T cells are presumed to play an important role *in vivo* by preventing the continued growth of EBV-transformed B cells[5].

In attempting to understand the immunological control of HCMV it seems more logical to examine T-cell responses in the asymptomatic persistently infected host rather than in patients with symptomatic HCMV infection in whom such responses are presumably likely to be defective. In order to determine the nature and specificity of circulating memory T cells responding to HCMV in normal seropositive subjects, we used the technique of secondary *in vitro* stimulation with antigen and subsequent expansion of the responding cells in interleukin-2 (IL-2) dependent culture. IL-2 is a growth factor released by stimulated T cells which permits the continued growth of antigen-activated T cells (and is in this sense an autocrine growth factor); this allows the accumulation of sufficient T cells for their function to be assessed and also permits attempts at their cloning[6]. We were especially interested to see if cytotoxic T cells specific for HCMV were present, because of the evidence on EBV already mentioned and because the role of this effector T cell has been particularly well studied in experimental models of virus infection[7].

We found that when peripheral blood mononuclear cells from seropositive subjects were stimulated *in vitro* with HCMV antigen, which consisted of virus particles, the T-cell lines generated were of helper (T4) phenotype and did not kill HCMV-infected target cells. However, if HCMV infected fibroblasts were used as the stimulating antigen, most of the responding T cells were of the T8 phenotype. These T cells were cytotoxic, killing HCMV-infected fibroblast targets and showing the property of HLA restriction; i.e. they would only lyse virus-infected target cells with which they shared class I MHC antigens, the hallmark of virus-specific cytotoxic T cells. They also killed cells which had been infected with HCMV for only six hours[8] (Fig. 2). These experiments suggested that there were memory T cells in the peripheral blood of normal seropositive people which were directed predominantly at the virus early and/or immediate early proteins which are only expressed in the infected cell and not in the virus particle itself. In further experiments we found that such T-cell lines could lyse target cells treated with phosphonoformate, an inhibitor of the virus DNA polymerase which prevents virus DNA replication and expression of the late proteins (and is now incidentally marketed as a drug for the treatment of HCMV—Foscarnet). The experiment provides further evidence that T cells are directed to those proteins which are only expressed in the infected cell. In what are probably analogous results, it has recently been reported that a high proportion of cytotoxic T cells in mice infected with mouse CMV show

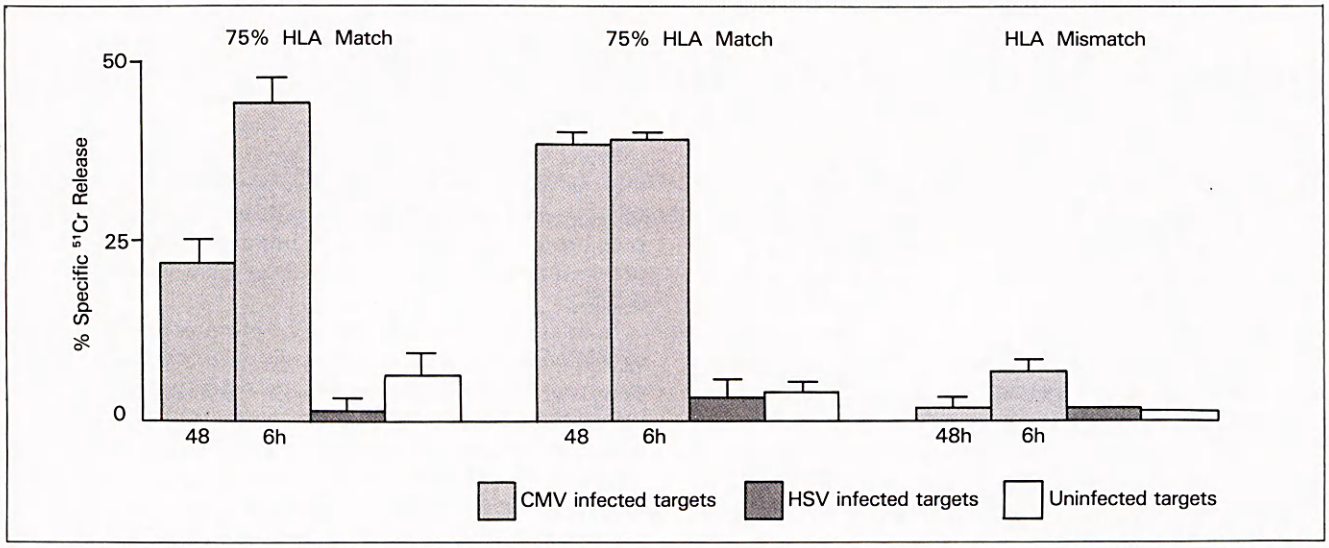

Fig. 2. *An example of the specificity of a cytotoxic T-cell line established in response to HCMV from a normal seropositive donor, assessed by release of radioactive chromium from target cells. This T-cell line kills HCMV infected cells, including those which have only been infected for six hours, but does not kill herpes simplex virus infected cells. Only HLA-matched HCMV-infected cells are killed.*

specificity for the immediate early protein of that virus[9].

T cells with this sort of specificity could obviously provide a 'surveillance' mechanism for eliminating HCMV-infected cells before release of progeny virus, but more work will be needed to determine if this is what actually happens *in vivo*. There is work showing that HCMV-specific cytotoxic T cells may be detected directly in the peripheral blood of patients (bone marrow transplant recipients) with active HCMV infection, and that their presence correlates with recovery[10]. However, one problem with this sort of direct approach, without secondary stimulation *in vitro,* is that it is difficult to get enough cells to analyse their specificity for particular viral proteins.

The existence of cytotoxic T cells does not necessarily imply that other T-cell-dependent mechanisms are unimportant in host defence. Helper T-cell lines with similar specificity for the HCMV early proteins can also be established from normal seropositive subjects, as might be expected. In addition to providing help for the development of other effector T-cell responses, such helper cells could in theory have an effector function. T cells mediating delayed hypersensitivity (DH) are important in resistance to herpes virus infections in experimental models in mice[11]; DH is probably mediated principally by helper T cells releasing gamma interferon which activates macrophages at the site. It is difficult to study DH in human systems because this would have to be done mainly *in vitro,* and so far we do not know how important it is in HCMV, or in any human virus infection.

Non-Specific Immunity

Natural killer (NK) cells are found among peripheral blood mononuclear cells and are so called for their ability to kill virus-infected (and tumour) cells without showing conventional immunological specificity or memory. Their cytotoxic activity is enhanced by interferons and by interleukin-2. The extent to which they are primarily of monocyte or T-cell lineage is uncertain: however, a proportion of NK cells do have T-cell markers, and the balance of current evidence favours their being more related to T cells. There is increasing evidence from experimental models that NK cells have a role in limiting the severity of virus infection in the period immediately following infection, particularly before the development of specific T-cell immunity; this has been shown better for mouse CMV infection than for other viruses[12].

We have also looked at the stage at which CMV infected cells become susceptible to being killed by natural killer cells. We found that when CMV infected cells expressed the virus early antigens they became markedly more susceptible to being killed by NK cells[13] (Fig. 3). The actual target structures recognised by NK cells have not been defined, but are almost certainly not viral proteins; it is more likely that NK cells recognise some normal cell surface component whose expression is modified or enhanced by the infecting virus[14]. The importance of NK cells in the containment of human virus infections remains to be determined, although it seems unlikely that it will be more than adjunctive to that of specific T-cell immunity.

CMV and Immunosuppression

There is experimental evidence that mouse CMV can exert immunosuppressive effects by infecting macrophages. There is somewhat anecdotal clinical evidence that HCMV may also produce immunosuppression; it is said that patients with active HCMV infection are more prone to infection with other opportunists such as fungi.

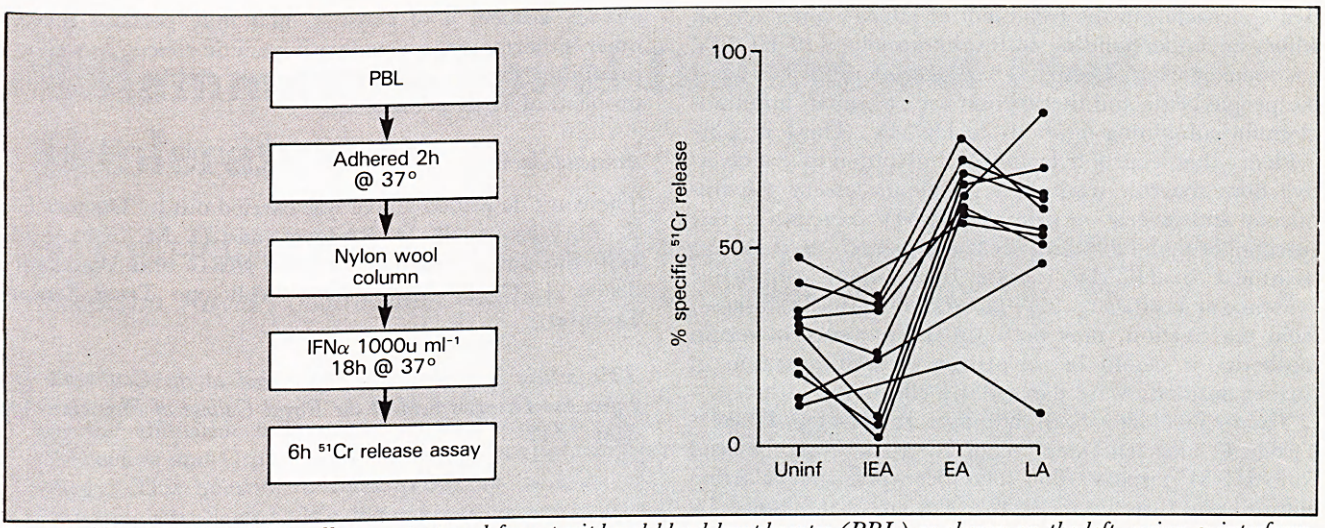

Fig. 3. *Natural killer (NK) cells were prepared from peripheral blood lymphocytes (PBL) as shown on the left, using α interferon (IFNα) to enhance their killing capacity. Their ability to kill HCMV-infected cells was then assessed in a chromium release assay. HCMV-infected cells became more susceptible to being killed by NK cells at the stage when the virus early proteins or antigens (EA) were expressed. Uninf = uninfected; IEA = immediate early antigen; LA = late antigen.*

There have been more concrete reports of monocytes from patients with primary HCMV infection having a reduced ability to support the lectin-induced proliferation of human peripheral blood T cells[15]. We have examined possible mechanisms by which HCMV might produce such effects, by infecting monocytes with HCMV *in vitro;* these HCMV-infected monocytes were no longer able to produce interleukin-1 (IL-1) activity[16]. IL-1 is required as a second signal for T-cell activation during antigen presentation, and has an increasingly recognised number of other biological activities[17]. Further experiments showed that this loss of IL-1 activity was due to the release of an inhibitor of IL-1 from the virus-infected cells, abrogating the action of IL-1 on the responding cells[16] (Fig. 4). This IL-1 inhibitor appears to be a normal cellular protein whose release is induced by the virus. Despite this clear biological consequence of HCMV infection, the infected monocytes show no evidence of virus replication and there is little or no expression of viral proteins, so the molecular mechanism of the effect remains uncertain.

The whole question of the extent to which HCMV infects immunocompetent cells (lymphocytes and macrophages) is controversial. It seems clear that normal resting lymphocytes and monocytes/macrophages do not replicate the virus but there are reports that expression of the CMV major IE protein occurs in a small proportion (a few per cent) of peripheral blood mononuclear cells, following their infection *in vitro*[18]. It is possible that such limited expression of virus proteins could have functional consequences, for instance by virtue of the 'trans acting' factors discussed above. However, it is important to emphasise that there is no firm evidence as yet that any of these cells are a normal site of latency for HCMV.

In addition to these possible effects on macrophages, there is one report that HCMV can also act as a polyclonal B cell activator[19]. If confirmed it would be of considerable interest.

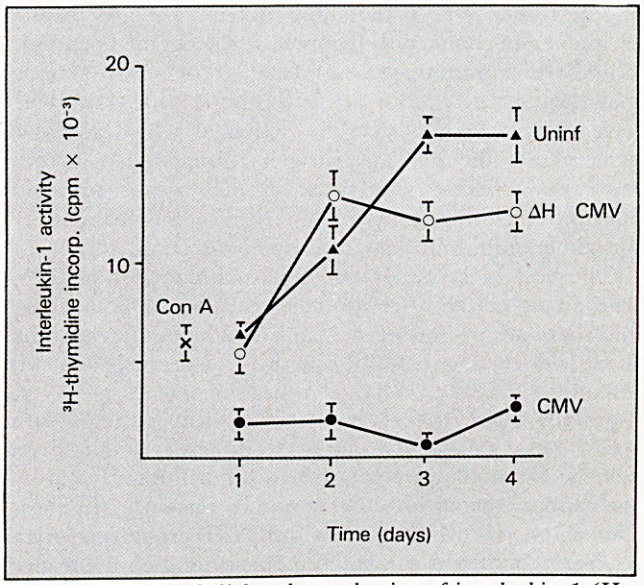

Fig. 4. *HCMV abolishes the production of interleukin-1 (IL-1) activity from normal human monocytes. IL-1 activity was constitutively produced by normal monocytes maintained in culture for 1-4 days (▲). Production of IL-1 was not affected by infection of the monocytes with heat-inactivated HCMV (○), but was abrogated by infection with live virus (●). See text for further details. Uninf = uninfected; Con A = concanavilin A; ΔH = heat inactivated.*

Implications for Therapy

In the absence, at least until very recently, of effective specific chemotherapy, there have been several alterna-

tive approaches to the treatment of HCMV infection on which an understanding of the immunology of HCMV has some bearing. There has been considerable interest in the prophylactic and therapeutic use of human immunoglobulin containing antibody to HCMV. There is some evidence that its prophylactic administration to seronegative bone-marrow transplant recipients lessens the frequency and severity of primary HCMV infection in this susceptible population[20]. It has also been used for the treatment of HCMV disease but without conclusive evidence of its efficacy. Indeed, as severe HCMV disease, from reactivation, may occur in the presence of serum antibody, it would be surprising if administration of further antibody were particularly effective.

There have also been attempts to develop HCMV vaccines. The 'vaccines' reported to date are derived from HCMV isolates that have been passaged in tissue culture and are used as live vaccines; it should be emphasised that there is no marker for determining attenuation in such viruses. They have been administered to susceptible seronegative subjects before renal transplantation but there is no conclusive evidence that they prevent infection with wild type HCMV[21]. There are theoretical objections to the use of live persistent virus vaccines that will presumably persist for life in the recipient and could conceivably have oncogenic potential. Therefore sub-unit vaccines (devoid of virus genetic material) seem a more attractive possibility, but their development demands a knowledge of which HCMV proteins are the relevant antigens. Finally, we do not know how effective any such vaccines are in inducing effector T-cell immunity, or indeed whether this matters for conferring immunity to primary infection.

The best prospect for effective treatment for HCMV will almost certainly come from specific chemotherapy, and there are promising new antiviral agents such as the nucleoside analogue 9-(1,3 dihydroxy-2-propoxy-methyl) guanine (DHPG). What is becoming clear, however, especially from the experience of treating patients with AIDS who have active HCMV disease, is that even specific chemotherapy is likely to be of limited value in the absence of an effective immune response. In these patients the HCMV disease is likely to recrudesce as soon as chemotherapy is stopped, emphasising the importance of intact T-cell immunity in containing virus replication at local sites and preventing dissemination. Using interleukin-2, attempts have been made to restore T-cell responses to HCMV in AIDS patients, but although IL-2 may improve their T-cell responses *in vitro,* it has not yet proved effective as an 'immunomodulator' *in vivo*[22].

There is clearly still much to be learned, particularly in molecular terms, about the relationship between HCMV and the persistently infected human host. New knowledge should tell us how HCMV produces the diseases we already suspect it of causing, and may perhaps reveal other hitherto unsuspected pathogenetic effects, as well as providing further basic information on the fascinating problem of virus persistence.

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References

1. Stagno, S., Pass, R. F., Dworsky, M. E. *et al.* (1982) *New England Journal of Medicine,* **306,** 945.
2. Sixby, J. W., Nedrud, J. G., Raalo-Traub, N., Hanes, R. A. and Pagano, J. S. (1984) *New England Journal of Medicine,* **310,** 1225.
3. Hirsch, M. S., Schooley, R. T., Ho, D. D. and Kaplan, J. C. (1984) *Review of Infectious Diseases,* **6,** 726.
4. Everett, R. D. (1984) *The Embo Journal,* **3,** 3135.
5. Rickinson, A. B., Yao, Q. Y. and Wallace, L. E. (1985) *British Medical Bulletin,* **41,** 75.
6. Smith, K. A. and Ruscetti, F. W. (1981) *Advances in Immunology,* **31,** 137.
7. Zinkernagal, R. M. and Doherty, P. C. (1979) *Advances in Immunology,* **27,** 52.
8. Borysiewicz, L. K., Morris, S., Page, J. D. and Sissons, J. G. P. (1983) *European Journal of Immunology,* **13,** 804.
9. Reddehase, M. J., Keil, G. M. and Koszinowski, U. H. (1984) *European Journal of Immunology,* **1,** 56.
10. Quinnan, G. V., Kirmani, N., Esber, E. *et al.* (1981) *Journal of Immunology,* **126,** 2036.
11. Nash, A. A. (1985) *British Medical Bulletin,* **41,** 41.
12. Bukowski, J. F., Warner, J. F., Dennert, G. and Welsh, R. M. (1985) *Journal of Experimental Medicine,* **161,** 40.
13. Borysiewicz, L. K., Rogers, S., Morris, S., Graham, S. and Sissons, J. G. P. (1985) *Journal of Immunology,* **134,** 2695.
14. Borysiewicz, L. K., Graham, S. and Sissons, J. G. P. (1984) *Immunobiology,* **167,** 182.
15. Carney, W. P. and Hirsch, M. S. (1981) *Journal of Infectious Diseases,* **144,** 47.
16. Rodgers, B. C., Scott, D. M., Mundin, J. and Sissons, J. G. P. (1985) *Journal of Virology,* **54,** in press.
17. Dinarello, C. A. (1984) *Review of Infectious Diseases,* **6,** 51.
18. Rice, G. P. A., Schrier, R. D. and Oldstone, M. B. A. (1984) *Proceedings of the National Academy of Sciences,* **81,** 6134.
19. Hutt-Fletcher, L. M., Balachandran, N. and Elkins, M. H. (1983) *Journal of Experimental Medicine,* **158,** 2171.
20. Winston, D. J., Pollard, R. B., Ho, W. G. *et al.* (1982) *Annals of Internal Medicine,* **97,** 11.
21. Plotkin, S. A., Friedman, H. M., Fleisher, G. R. *et al.* (1984) *Lancet,* **2,** 528.
22. Rook, A. H., Masur, H., Land, C. *et al.* (1983) *Journal of Clinical Investigation,* **72,** 398.