Ciba Foundation Symposium 187

VACCINES AGAINST VIRALLY INDUCED CANCERS

1994

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VACCINES AGAINST VIRALLY INDUCED CANCERS

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Contents

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Introduction

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This meeting has several aims. Some of the questions to which we will try to find answers during the next few days are:

1) What might the immune system recognize on (virally induced) tumour cells?

2) What sort of immune effector cell is necessary for tumour cell recognition/killing to occur?

3) How might such immune effector cells be induced by vaccination or otherwise?

4) How could the tumour or its environment 'neutralize' such effector cells?

5) What measures can be taken to overcome this local immunosuppression?

Tumour immunology is once again in the news; advances in the molecular and cell biology of tumours have led us to realize that there may be specific antigenic targets on the surface of most tumour cells. A tumour cell expressing a defined viral antigen with multiple non-self epitopes should be an easier target for the induction of specific immunity than a spontaneously arising tumour which may differ from 'self' only in the level of expression of, or in point mutations in, one or more housekeeping genes. In addition, the targets for the virally induced tumours will be the same from patient to patient, MHC (major histocompatibility complex) restriction of epitope selection permitting. For most other tumours, although there are some common themes such as the frequently occurring point mutations in p53, the target is a moving one. So, whatever the problems of tumour-specific immunotherapy for virally induced tumours, there will be even more for other tumours.

Experimental evidence supporting a role for immune surveillance in human cancer

It is worthwhile at this point to reassure ourselves, if we can, that tumour-specific immunity has some basis in fact. Four observations would support the existence of tumour immunosurveillance:

1) An increased incidence of tumours in immunosuppressed patients.

2) An MHC association of specific tumours, implying holes in the repertoire.

3) Simultaneous regression of an existing tumour at multiple sites, as is sometimes seen with warts.

4) Effective tumour immunotherapy.

My remarks today will be limited to humans, as the experimental tumours of animals are clearly different in their natural history. It is worth commenting in passing that if we were to use these criteria to define the role of immunity in the control of infectious agents (for tumour above simply read virus), then all human infections would fail to fulfil criterion 2, and many would fail 3 and 4. So I shall confine my remarks to the increased incidence of tumours in immunosuppressed patients.

Experiments of Nature and of the medical profession can tell us something about the role of the immune system in tumour control. Of the major congenital abnormalities in the human immune system, only common variable immune deficiency is associated with significant long-term survival without replacement treatment. In this disorder, there is a lack of induced antigen-specific humoral immunity. There is also a well recognized increase in tumours of the lymphoreticular system, which may be a consequence of the underlying molecular lesions giving rise to the immunodeficiency, but there are no striking increases in other tumours in these patients, though I'm not aware of a good systematic case-controlled study. The majority of patients with this disorder are now treated with immunoglobulin replacement therapy; one could argue that passive transfer of any pooled immunoregulatory immunoglobulin might be enough to substitute for specific humoral immunity in tumour protection.

Induced long-term suppression of the cellular immune system occurs commonly in two situations, during the administration of immunosuppressive drugs and after infection with human immunodeficiency virus (HIV-1). The increased incidence of tumours in HIV infection is well documented and while some of these tumours may be a consequence of the potential oncogenic activities of HIV-1 itself, some are less easy to explain in this way. The short period between the onset of significant cellular immunosuppression and death from the consequences of infection, coupled with the younger age of many of the patients with HIV-1 infection and the significantly lower risk of tumours in this age group, make data on the incidence of individual tumours in HIV-1 infection difficult to acquire and interpret. However, in addition to Kaposi's sarcoma, the incidence of three tumours with a 'definite' viral association is significantly increased: human papillomavirus-associated carcinoma of the cervix and anal canal, and lymphoreticular malignancy associated with Epstein–Barr virus.

Immunosuppression is routinely induced as part of therapy after allografting and allograft recipients show a striking rate of tumour development. Caution is required in the interpretation of these data: azathioprine was until recently a routine part of such immunosuppressive therapy and it's potential promotion of DNA mutations and DNA demethylation should not be overlooked. The data from the Australian and New Zealand Dialysis and Transplant Registry,

prepared by Dr Ross Shiel and his colleagues, show that the rate of tumour development after transplant is linear and rapid (Shiel 1989). The increase in relative risk is not seen for all tumours but occurs particularly for those with a presumed viral origin.

The linear relation of tumour development with time suggests that whatever the effect of immunosuppression may be, it seems to be working both early and late in the battle against the tumour cell. On the one hand, there is no lag time before the rate of tumour development increases after immunosuppression; on the other, the effect is sustained. If we believe that carcinogenesis is a multistep accumulation of genetic errors, this suggests that whenever immune surveillance is effective throughout the process of error accumulation, if it once fails, it fails because the tumour evades the immune surveillance permanently. It's not as if there are a large number of potential tumours lurking, suppressed but not destroyed, that appear when the immune system controls are removed.

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Potential antigenic targets on Epstein-Barr virus-associated tumours and the host response

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Abstract. There is considerable variation in the degree of expression of viral genes among different tumours associated with Epstein–Barr virus (EBV). Immune control of tumours in immunosuppressed patients (immunoblastic lymphomas) can be exercised through a range of epitopes from cytotoxic T lymphocytes (CTL) covering the full spectrum of latent EBV gene products. A subunit vaccine based on an EBV CTL epitope from one of the latent genes is about to undergo human trial. The options for immune control of Burkitt's lymphoma are more restricted. Antigen expression is limited to a single nuclear antigen, EBNA1, and Burkitt's lymphoma cells are unable to process EBV latent antigens, presumably because of a transcriptional defect in *TAP1* and *TAP2* genes. In contrast with earlier suggestions that EBNA1 is not a target for CTL, there is a class II-restricted epitope within EBNA1. EBV-infected B cells are unable to process this epitope endogenously. The most promising strategy for developing a vaccine against these tumours is to use a single subunit vaccine that incorporates multiple CTL epitopes from several human pathogens.

1994 Vaccines against virally induced cancers. Wiley, Chichester (Ciba Foundation Symposium 187) p 4–20

Epstein–Barr virus (EBV), a herpesvirus widespread in human populations, is the aetiological agent for infectious mononucleosis and has been implicated in the pathogenesis of an increasing number of human malignancies of lymphoid (both B and T cell) and non-lymphoid origin (Table 1) (Epstein & Achong 1986). The distribution of the EBV-associated paediatric B cell malignancy, endemic Burkitt's lymphoma, is strongly focused in Africa and Papua New Guinea. The association of the non-endemic Burkitt's lymphomas (both sporadic cases and those related to AIDS—acquired immune deficiency syndrome) and EBV is not as strong. More differentiated EBV-associated B cell lymphomas occur in immunocompromised individuals (immunoblastic lymphomas). These tumours are particularly important in patients after transplant operations and in AIDS

TABLE 1 Antigen expression in tumours associated with Epstein–Barr virus

Tumour	*EBV antigens in tumour*	*% EBV positive*
Burkitt's lymphoma		
Endemic	EBNA1	>95
Sporadic	EBNA1	25
In AIDS patients	EBNA1	40
Immunoblastic lymphomas		
In PTLD patients	EBNA1-6	100
In AIDS patients	LMPs 1,2	
Nasopharyngeal carcinoma		
	EBNA1	100
	LMPs 1,2	
Hodgkin's disease		
	EBNA1	40
	LMPs 1,2	
T cell lymphoma	EBNA1	10
	LMPs 1,2	

EBNA, Epstein–Barr nuclear antigen; LMP, latent membrane protein; PTLD, post-transplant lymphoproliferative disease.

patients (Cleary et al 1986). The major EBV-associated tumour of epithelial origin is nasopharyngeal carcinoma, which occurs in 1 to 2% of the southern Chinese population. There is an increasing realization that EBV may also be associated with some forms of Hodgkin's disease and of T cell lymphoma.

The virus has two major target tissues, B lymphocytes and squamous pharyngeal epithelium. In normal B cells, the infection is predominantly latent and results in their transformation into lymphoblastoid cell lines; in epithelium, the infection is predominantly lytic, with complete replication of the virus linked to ordered squamous epithelial differentiation (Allday et al 1988). The latent viral proteins expressed in lymphoblastoid cell lines include six nuclear antigens (EBNA 1-6) and two transmembrane proteins, LMP1 and 2. The replicative proteins include early antigen, membrane antigen and viral capsid antigen complexes. EBV strains have been classified as A-type or B-type on the basis of the divergence within the open reading frames encoding EBNAs 2, 3, 4 and 6. Although A-type EBV is the predominant virus found systemically in healthy virus carriers, there is an apparent increased incidence of B-type virus in lymphocytes from individuals subjected to various forms of immunosuppression. The pattern of latent viral antigen expression in the EBV-associated tumours

is variable and ranges from a complete array, as seen in lymphoblastoid cell lines, to the single nuclear antigen, EBNA1 (Table 1).

Immune control of EBV-associated tumours

There is an emerging view that the protective EBV immune response is T cell mediated (Rickinson et al 1992). We have at least a partial understanding of the cytotoxic T lymphocyte (CTL) controls that are exercised over latently infected B cells *in vivo* and that are a potential defence against virally associated lymphomas (Moss et al 1992). By contrast, we are only beginning to understand the role that the CTL response may play in controlling EBV-infected epithelia that are undergoing viral replication. Recent evidence from our laboratory has demonstrated that lytic antigens may be a target for immune recognition during acute infectious mononucleosis (unpublished observations) but their potential as targets on tumour cells is unproven. In considering the potential targets for immune control of EBV-associated tumours, it is convenient to divide the tumours into three categories according to the degree of EBV antigen expression.

Immunoblastic lymphomas

Immunoblastic lymphomas are frequently present as multifocal lesions within lymphoid tissues and/or in the central nervous system; they are classified histologically as immunoblastic or polymorphic B cell lymphomas. Analysis of immunoglobulin gene rearrangement or isotype expression indicates that even within a single patient, individual tumour foci tend to be distinct, each being composed of one or a small number of unique B cell clones.

Several studies have demonstrated that a full spectrum of latent antigens are expressed in immunoblastic lymphomas. Moreover, the cellular phenotype of these lymphoma cells mirrors that of lymphoblastoid cell lines, with high surface expression of cellular adhesion molecules such as ICAM-1 and LFA-3 as well as cellular activation antigens such as CD23 (Rickinson et al 1992). It seems likely that these lymphomas arise as a result of drug-induced (in patients with post-transplant lymphoproliferative disease) or virally induced (in AIDS patients) immunosuppression of the normal EBV-specific CTL response allowing what is essentially uncontrolled proliferation of lymphoblastoid cells *in vivo*. Clearly, there is a wide range of potential targets for immune recognition of these tumours.

Given what we now know about processing of endogenously synthesized proteins to small peptide fragments and their presentation on the cell surface as a complex with HLA class I antigens, it is clear that viral antigens have the potential to provide target epitopes for a specific CTL response. It has been suggested that these peptides are transported into the endoplasmic reticulum by a pair of transporters associated with antigen processing (TAP1 and TAP2).

TABLE 2 Defined epitopes from Epstein–Barr virus recognized by cytotoxic T lymphocytes

Allele	*EBV antigen*	*Epitope sequence*	*Type specificity*
DR1	EBNA1	TSLYNLRRGTALA	A & B
B18	EBNA2	TVFYNIPPMPL	A
A2	EBNA2	DTPLIPLTIF	A
A2	EBNA3	SVRDRLARL	A & B
B8	EBNA3	FLRGRAYGL	A
B8	EBNA3	QAKWRLQTL	A
?	EBNA3	HLAAQGMAY	A
B35	EBNA3	YPLHEQHGM	A
A11	EBNA4	IVTDFSVIK	A
B44	EBNA6	ENLLDFVRF	A & B
A24/B44	EBNA6	KEHVIQNAF	A
B27	EBNA6	RRIYDLIEL	?
A2.1	LMP2A	CLGGLLTMV	A & B
B27	LMP2A	RRRWRRLTV	?

Much of what is known of the role of EBV latent antigens as a target for CTL has been derived by studying the response in healthy EBV-seropositive individuals. The level of EBV-infected B cells in these individuals is controlled by virus-specific CTL. Immunoblastic lymphomas displaying the same array of latent antigens are, presumably, also targets for these effector cells.

Target antigens for the EBV-specific CTL response have been localized within EBNAs 1-6 and LMPs 1 and 2 using bulk T cell lines and EBV-specific CTL clones (Table 2) (Khanna et al 1992, Murray et al 1992). This work can be summarized as follows. Firstly, all of the defined antigens are included within the latent antigens. This does not infer that the density of epitopes within the replicative proteins is likely to be low; it probably reflects the ease of activating a memory response to the latent EBV antigens using lymphoblastoid cell lines compared with activating a response to the replicative proteins. It seems likely that CTL epitopes within the replicative proteins will be defined when an antigen-presenting cell capable of expressing a full complement of replicative proteins is found. Secondly, some of the epitopes are present on both A- and B-type transformants, while others are A-type specific (Table 2). There seems no indication at this stage that the site of CTL epitopes is preferentially located at regions of diversity between A- and B-type EBV. Thirdly, although there is no evidence so far for disease-specific EBV strain variation at the site of CTL epitopes, there is some indication of mutations in CTL epitopes in viral isolates from some geographical locations (de Campos-Lima et al 1993). The relevance

of these mutations to immune evasion by these viral strains will require an analysis of a range of CTL epitopes restricted through different alleles of the major histocompatibility complex.

It is assumed that the sensitivity of immunoblastic lymphomas to EBV-specific CTL will be similar to that of lymphoblastoid cell lines, because the phenotype of each is identical. Although this has not yet been tested, it is well established that relaxation of immunosuppressive therapy (with a presumptive rise in the level of EBV-specific CTLs) can lead to regression of these lymphomas. However, formal proof that the specific CTLs that control the latent EBV infection in healthy individuals are capable of recognizing immunoblastic lymphomas will require adoptive transfer of cultured CTLs. EBV-positive polyclonal B cell lymphomas in *scid* mice regress after transfer of virus-specific CTLs (Cooper et al 1992).

Endemic Burkitt's lymphoma

Analysis of productively rearranged immunoglobulin loci in Burkitt's lymphoma has confirmed the monoclonal nature of this tumour. The c-*myc* oncogene (chromosome 8) is regularly translocated to either the immunoglobulin heavy chain locus (chromosome 14) or one of the light chain loci (chromosomes 2 and 22). In contrast to the immunoblastic lymphomas, fresh biopsies and early passage cell lines from Burkitt's lymphomas do not express EBNAs 2-6 and LMP, thus limiting the potential target antigens to EBNA1. These cell lines are referred to in the literature as group I Burkitt's lymphoma cell lines. Continued *in vitro* culture of Burkitt's lymphoma cells in some cases results in the expression of high levels of EBNA 2–6 and surface adhesion molecules (referred to as group II or III).

Burkitt's lymphoma provides the most amenable model for experimental analysis of the role of specific CTLs in the control of tumour cells. The model is supported by the fact that Burkitt's lymphoma cells carrying the relevant translocation have been established in culture, while lymphoblastoid cell lines have been independently derived from normal circulating B cells from the same patient by infection with EBV *in vitro*. Thus, it is possible to compare the sensitivity to immune lysis of tumour-derived and non-tumour-derived tissue from the same patient (Khanna et al 1992). Although only a limited number of Burkitt's lymphoma patients have been studied thus far, no detectable EBV-specific CTL dysfunction is evident. However, *in vitro* studies have shown that group I EBV-positive Burkitt's lymphoma cells are highly resistant to virus-specific CTL lysis (Fig. 1). Several possible mechanisms have been suggested to explain this resistance. The low expression of adhesion molecules and HLA class I alleles might contribute to the escape of Burkitt's lymphoma cells from immune recognition. However, down-regulation of these molecules on Burkitt's lymphoma cells has been shown *not* to provide an absolute barrier to tumour cell recognition by virus-specific CTLs (Fig. 1).

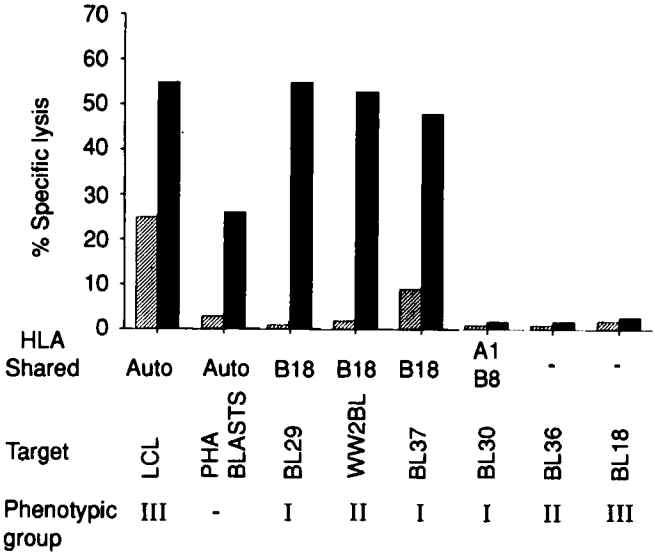

FIG. 1. Recognition of autologous lymphoblastoid cell lines (LCL), autologous phytohaemagglutinin-treated blasts (PHABLASTS) and Burkitt's lymphoma cell lines (BL29, WW2BL, BL37, BL30, BL36 and BL18) by the HLA B18-restricted CTL clone LC27 with (■) or without (▨) the relevant specific peptide epitope, TVFYNIPPMPL. Reproduced with permission from Khanna et al (1993). Copyright 1993, The Journal of Immunology.

A second possibility is that there is a defect in the expression of TAP1 and/or TAP2. Defects in this process represent a potential risk, since it is an essential link in CTL-mediated immune surveillance. This mechanism is supported by the observation that many Burkitt's lymphoma cell lines are unable to stimulate either an allospecific or a virus-specific CTL response *in vitro* (unpublished observations). Recent studies from our laboratory have established that Burkitt's lymphoma cells cannot process intracellular antigenic determinants efficiently and that these cells do not transcribe *TAP1* and *TAP2* properly (R. Khanna, C. A. Jacob, V. Argaet, A. Apolloni, Q. Y. Zang, M. Masucci & D. J. Moss, unpublished work). This suggests that Burkitt's lymphoma cells are unable to transport peptide epitope from the cell cytosol into the endoplasmic reticulum. Transfection of Burkitt's lymphoma cells with a minigene expression vector encoding an EBV epitope fused to an endoplasmic reticulum translocation signal sequence restored CTL recognition (Fig. 2) and the ability to activate a specific CTL response (Khanna et al 1994).

EBNA1 is the only antigen invariably expressed in all EBV-associated tumours. It has generally been considered that this antigen does not include CTL epitopes (Klein 1989). This hypothesis provides a convenient explanation

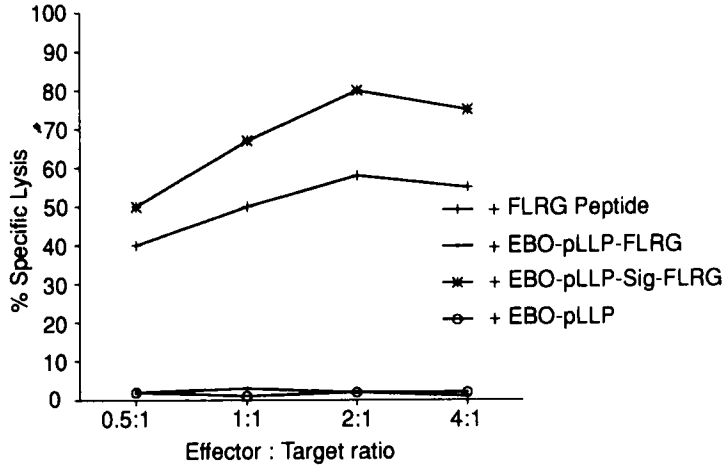

FIG. 2. Recognition of cells from the Burkitt's lymphoma cell line, BL30, by the EBV-specific CTL clone, LC13. This clone recognizes the peptide sequence FLRGRAYGL (FLRG) which is included within EBNA3. The BL30 cells were transfected with the expression vector alone (EBO-pLLP) (○), the vector encoding the epitope FLRG (•) or a vector encoding this peptide preceded by the adenovirus E3/19 kD protein signal sequence (*).

for the growth of EBV-associated tumours in individuals who are not overtly immunosuppressed and has led to speculation that EBV latency is maintained in a long-lived B cell population expressing only EBNA1 (Klein 1989). However, we have recently identified a class II-restricted CTL epitope within EBNA1 (Fig. 3) (unpublished observations). One of the most important features of this epitope is that it is apparently not processed in B cells but is recognized by specific CTLs only after exogenous addition of peptide epitope (Fig. 3). Thus, neither lymphoblastoid cell lines nor B cell blasts infected with a recombinant vaccinia virus expressing EBNA1 are recognized by these EBNA1-specific CTLs. The frequency of CTL precursors recognizing this epitope suggests that reactivity is part of a secondary rather than a primary response. It is interesting that this epitope is included in the EBNA1 DNA-binding region, which may limit transport of the epitope into the endoplasmic reticulum. The translocation of EBNA1 into the nucleus is dependent on a nuclear localization sequence within the protein. It has been suggested that the strong DNA binding in this region explains the failure of this antigen to be processed. Support for this concept has recently been provided by the observation that DNA-bound EBNA1 is resistant to degradation by proteases (Shah et al 1992). An effective test of this hypothesis will be to determine whether mutations or deletions in the nuclear localization sequence restore class II processing of this epitope.

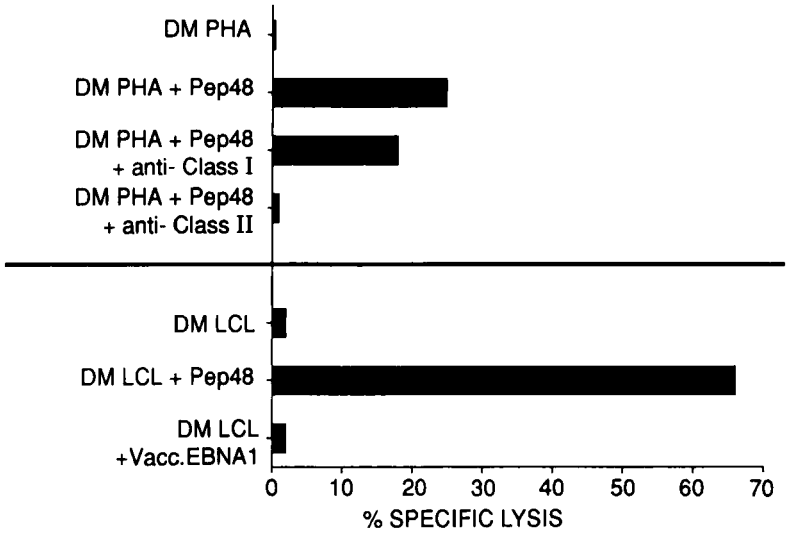

FIG. 3. Recognition of EBNA1 CTL epitope, TSLYNLRRGTALA (Pep48). Autologous phytohaemagglutinin (PHA)-treated blasts and lymphoblastoid cell lines (LCL) were used as targets alone or after pretreatment with Pep48. Autologous lymphoblastoid cell lines were also used as a target after infection with a recombinant vaccinia virus encoding EBNA1 (Vacc.EBNA1). CTL lysis of peptide-coated PHA blasts was inhibited by anti-class II but not anti-class I antibody. This CTL clone failed to recognize the epitope when processed endogenously by the autologous lymphoblastoid cell lines or cells infected with Vacc.EBNA1.

General considerations for EBV vaccination

In the last few years, efforts to develop vaccines have concentrated on the use of a subunit preparation of gp340 (recombinant and affinity purified), the EBV surface glycoprotein carrying the antibody neutralization determinant. Surprisingly, protection mediated by this vaccine does not appear to depend on antibodies, leaving unresolved the precise protective mechanism. Latently infected B cells in humans do not express gp340 but are controlled in healthy seropositive individuals by $CD8^+$ CTL specific for EBNAs 2-6. The Queensland Institute of Medical Research is currently conducting a human trial using the CTL peptide epitope FLRGRAYGL in a water-in-oil adjuvant containing helper components to test the feasibility of such vaccine formulations and ultimately to establish whether such CTL vaccine can protect against infectious mononucleosis and post-transplant lymphoproliferative disease.

Because of HLA diversity, several CTL epitopes restricted by the most common alleles must be combined to protect a high proportion of any given population. Such combination may simply be achieved by mixing peptides; however, an alternative approach has been to join multiple minimal CTL

epitopes together in a synthetic polyepitope protein, which is then delivered by an appropriate vector or adjuvant. In recent experiments, using a recombinant vaccinia virus that incorporates many of the CTL epitopes listed in Table 2, we have found that each $CD8^+$ CTL epitope within the construct was efficiently presented to its restricting allele. There thus appears to be no requirement for specific amino acid sequences flanking the minimal $CD8^+$ CTL epitopes to direct the proteolytic processing events. This result adds considerable impetus to the concept of developing a single subunit vaccine that incorporates multiple CTL epitopes from several human pathogens.

Conclusions

Any vaccine preparation aimed at the EBV-associated tumours will need to consider the pattern of EBV gene expression and tumour immune evasion mechanisms. In infectious mononucleosis and immunoblastic lymphomas, the full array of latent antigens are potential antigenic targets, whereas in Burkitt's lymphoma and nasopharyngeal carcinoma, targets are often restricted to EBNA1. In addition a strategy to overcome the processing defect in Burkitt's lymphoma will need to be devised.

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DISCUSSION

Liew: Denis, with the T cell epitope FLRGRAY (Table 2), is the MHC restriction monomorphic or polymorphic?

Moss: We're starting by asking a simple question: can we induce a CTL response with a single peptide? Clearly, a successful vaccine will never ultimately be based on a single peptide epitope. At the same time, we are looking at stitching peptide epitopes together rather than using a vaccine based on a cocktail of individual peptide epitopes. We are trying to define the conditions under which a single peptide epitope will activate a CTL response *in vivo*. We've used the epitope I described because we know more about that one than about any of the other epitopes and it's restricted through the allele HLA B8, which tends to be invariant.

de Thé: When you said polyclonal tumours, I think it would have been preferable to speak of polyclonal lymphoid proliferation, not tumours.

Secondly, I would like to refer to another EBV-associated tumour that has a much greater world-wide importance, namely nasopharyngeal carcinoma. Could you comment on the possibility of finding some specific CTL response in this case?

Moss: Most people in the field of cancers associated with EBV have tended to work with the easy models, because we have the correct target cell that can be easily manipulated in the laboratory—the lymphoblastoid cell line and the Burkitt's lymphoma line. The problem once you start to talk about a T cell response to nasopharyngeal carcinoma is that it is very difficult to grow these cells *in vitro* and those that do grow tend to lose EBV antigens quite rapidly. So there is really a black box in terms of the T cell response to nasopharyngeal carcinoma. Until we can overcome that technical hurdle of efficiently growing them in the laboratory and maintaining the same sort of phenotype as they do *in vivo*, we shall be fumbling in the dark for quite some time.

de Thé: What are the chances of developing a therapeutic vaccine? As you well know, for nasopharyngeal carcinoma we have a marker which can be used

for early detection in the population at risk, namely the expression of IgA antibody to VCA/EA (viral capsid antigen/early antigen) and to the Z (Zebra) protein.

Moss: One of the reasons that I'm pessimistic about a vaccine to any of the EBV-associated tumours, apart from the immunoblastic lymphomas, is that I suspect that you are going to need to induce some form of sterile immunity before they will work. In the case of immunoblastic lymphomas, and particularly in cases of infectious mononucleosis, really all you're trying to do is prevent disease. I suspect that even a vaccine that gives quite a mild response will prevent these diseases. Of the patients who acquire their primary EBV infection in adulthood, only about 50% come down with clinical infectious mononucleosis. To shift the equilibrium from 50% who contract clinical symptoms to 5% after administration of a vaccine is not unrealistic. We are certainly not trying to develop a vaccine that will induce sterile immunity. But once you're talking about Burkitt's lymphoma and nasopharyngeal carcinoma, maybe even Hodgkin's disease, I suspect that we're looking at something that may require much more solid immunity.

Arrand: We might naively think that if we have an effective vaccine against mononucleosis, then we may be preventing or at least delaying the primary infection by the virus. If that's true (it's not relevant to a therapeutic vaccine here), there is such a long time lag for the development of nasopharyngeal carcinoma that even if we cannot completely prevent the infection but only delay it for 20 years or whatever, then as long as the time lag stays the same, we could prevent a lot of cases of nasopharyngeal carcinoma.

Moss: I'm pessimistic about that, because once EBV gets into that first cell, particularly if it is some sort of long-lived B cell, I suspect that the dynamics of latency have already been set up. So you are talking about the induction of a fairly long-lived sterile immunity, aren't you?

Arrand: It's a question of whether we really can prevent that primary infection. I agree that once the virus has got in and has established itself, then we are not going to have any effect. But if we can prevent that establishment, and the virus–tumour relationship is true, we might prevent development of the cancer. But we don't really know whether we can actually prevent that infection. I don't think we will know until we do the trials.

Moss: The cottontop marmoset model (Morgan et al 1988) doesn't really tell you much about that, because it doesn't establish a latent infection.

Arrand: We know that you have to give a huge dose of virus in order to induce the tumour in the animals. If you give a lesser dose, you don't get the tumour. You may, when using a vaccine, be just neutralizing some of the virus and thereby reducing that input dose to a level below which the virus can have an effect.

We've been alluding mostly to a sort of humoral immunity against gp340. In some of the experiments in the tamarin model, particularly those done with vaccinia recombinants, although protection was observed there was no detectable

gp340 antibody in the animal. This suggests that there may be some other mechanism working—whether it's CTL or not I don't know.

Moss: Did that apply to recombinant gp340 as well?

Arrand: No. If you give the protein, you do see the neutralizing antibody response.

Doherty: Marek's disease in chickens is caused by a herpesvirus. How well does the vaccine against that protect and how does it protect?

de Thé: The vaccine (and naturally attenuated Turkey herpesvirus) does protect at nearly 100%. It does not prevent primary infection by Marek's disease virus but it prevents the disease.

Doherty: Has anyone ever let the chickens live long enough to see whether the vaccine protects for the full lifespan?

de Thé: If I recall correctly, yes, the vaccine does protect the chickens for their whole lifespan.

Let us go back to EBV. I think there is a big difference between the development of Burkitt's lymphoma and of nasopharyngeal carcinoma with regard to primary EBV infection. Very early EBV infection is directly related to the risk of developing Burkitt's lymphoma, but this does not appear to be the case for nasopharyngeal carcinoma. Therefore, theoretically one could imagine that a vaccine that delays primary infection could be effective in Burkitt's lymphoma, but probably not for nasopharyngeal carcinoma. In the latter tumour, it seems that the virus' oncogenic potential is not related to primary infection but probably to an event that takes place much later, possibly in precancerous lesions induced by environmental chemical carcinogen; in such a case, the viral effect could be the last step towards carcinoma. Then, only a therapeutic vaccine would be effective.

Arrand: Many patients destined to develop nasopharyngeal carcinoma show high levels of IgA against EA and VCA. To my mind, the fact that you are getting an increase in antibody against the replicative antigens suggests that somewhere there's a productive cycle going on which is somehow needed for the development of the tumour. Rather than use a therapeutic vaccine, could we use something like acyclovir, which dampens down replication? You could have an antibody screen to detect active replication, then use chemotherapy to dampen it. Has anybody tried that?

de Thé: It's very difficult, because of ethical problems. Out of the subpopulation in China which have IgA antibody (about 5% of individuals aged above 35 years) and who are prone to develop nasopharyngeal carcinoma, only 5–7% do develop the tumour. Ethically, you cannot intervene with a strong drug in all the people when only a few percent will develop the disease.

Doherty: What is the stem cell in nasopharyngeal carcinoma, is it an epithelial cell?

de Thé: Yes.

Frazer: What viral proteins obligatorily have to remain in the stem cell? What are they doing there? Can they be kicked out by the tumour cell once the clone has got some way down the track, so that if you started using immunotherapy, the first thing to arise would be a clone without viral proteins?

Rickinson: We don't fully understand the relationship between the epithelial and the lymphoid infections. We don't know which is the primary cell that is targeted by orally transmitted virus. We don't understand whether viral replication, either in a B cell or in an epithelial environment, is critical to the establishment of latency/persistence because the experiments cannot be done. If there is an early replicative phase, either in a mucosal B cell or in an epithelium, then targetting against replicative antigens might actually prevent colonization of the B cell system, which is what you want to do to prevent virally associated B cell malignancies.

There's no evidence that the virus is latent in epithelium. When we look at epithelial lesions—the classic one is hairy leukoplakia—we see expression of all the lytic genes and, surprisingly, also EBNA-1. Therefore, we think EBNA-1 is not only a latent gene, it's also a lytic cycle gene. All the lytic antigens and EBNA-1 are expressed in the outer layers of hairy leukoplakic lesions, but if you look by any marker, and we have some very potent markers now for latency, such as the small EB ER ? RNAs, you see no expression in the basal layers. However hard you look, there is no evidence that there is persistence where there is a genuine replicative lesion. There is no evidence that the stem cell compartment, i.e. the basal layer, is infected. So although it makes biological sense to envisage such persistence in epithelium, there is no evidence that it actually happens.

Doherty : If that's the case, you have to say that the infected B cells are constantly bringing the virus to the epithelium. Yet isn't it very difficult to reactivate the virus from peripheral blood lymphocytes?

Rickinson: If you take a virally transformed B cell line, it's difficult to reactivate it into lytic cycle *in vitro*. But we showed many years ago that if you take virus-carrying cells from peripheral blood, the nature of whose infection we don't really understand, and put them into tissue culture, many of them go into lytic cycle, release transforming virus and you get cell lines coming out from co-resident B cells infected by the virus *in vitro*. So it is possible to deliver a trigger, albeit something non-physiological like putting the cells into tissue culture, and activate the lytic cycle. There's strong evidence now that the true reservoir of latency is in the lymphoid system from the work of Gratama et al (1988) on bone marrow transplant recipients and that of Yao et al (1989) on virus carriers treated with acyclovir.

Once the virally infected cell gets into memory, I like to think that it's then under the normal physiological control of the B cell system. We know that memory cells are probably triggering back into the lytic cycle occasionally *in vivo*. So you might conceive of a situation where local infiltration of a mucosa

delivers a signal which activates viral replication; that would be a very efficient mechanism for re-establishing foci of productive infection for viral transmission.

Doherty: What happens in patients who don't have any B cells?

Rickinson: In patients in whom you can't find any B cells, you don't see any EBV in the haemopoietic system, using the most sensitive techniques that we have, which are not that sensitive.

Stanley: Alan, is there any evidence that persistence of EBV in nasopharyngeal carcinoma cells is essential for tumour progression?

Rickinson: The virus is always retained in that particular tumour. The level of association is much stronger than it is with Burkitt's lymphoma, for instance. The question is, how does the virus get into the stem cell of the tumour? The idea of B cell–epithelial fusion keeps recurring in the literature. In that context, if you take a virally infected B cell in tissue culture and fuse it with an epithelial cell, you produce a hybrid that expresses precisely those antigens we find in nasopharyngeal carcinoma.

de Thé: In the early 1970s, we observed, using electron microscopy, evidence of cytoplasmic bridges between epithelial and lymphoid cells of normal and tumorous nasopharyngeal mucosa. Alan, could you give the latest data on the expression of viral antigen in the nasopharyngeal carcinoma cell?

Rickinson: I can give you the latest extrapolations! The problem is that a lot of the experiments are done by PCR analysis of RNA, so you don't know whether 100% of the cells are expressing. When we look at nasopharyngeal carcinoma, we always find EBNA1 by immunoblotting and immunohistochemical staining and we see the EBNA-1 mRNA by PCR amplification. We never see the other EBNA proteins or their transcripts. We can just about always amplify transcripts for the latent membrane protein LMP1, but this can be difficult. In some tumours, we can see the LMP1 mRNA-specific PCR products easily and when we look by immunohistology at those tumours, they are the ones where we actually see the protein. There are many other tumours from which you can amplify the transcripts only by heroic efforts; in these, by immunoblotting or immunohistology the LMP1 protein is either very weakly expressed or not expressed at all. For the LMP2 protein, which is another potential target antigen, we can amplify transcripts, but the antisera are simply not good enough to detect the protein. In a way, we have been too dogmatic in distinguishing between the pattern of EBV latent gene expression seen in Burkitt's lymphoma and that seen in nasopharyngeal carcinoma. Burkitt's lymphoma generally expresses only EBNA-1; nasopharyngeal carcinoma can express the latent membrane proteins as well. These represent two ends of a spectrum however; there are many nasopharyngeal carcinomas that are much closer to Burkitt's in that expression of the latent membrane proteins (at least LMP1) is either very low or undetectable. So in terms of immunological recognition and the possibility of cytotoxic T cell therapy, there are quantitative aspects to this that we simply know nothing about.

Campo : You showed (Henderson et al 1991) that as far as B cells are concerned, LMP acts by rescuing them from apoptosis by up-regulating expression of the *bcl-2* gene. Does LMP do the same thing in epithelial cells? If so, could *bcl-2* be a target for intervention?

Rickinson: Up-regulation of *bcl-2* in B cells is a definite consequence of LMP expression, although the kinetics differ with a lot of the other phenotypic changes that the protein induces. We have never seen up-regulation of *bcl-2* in any other cell type. Ironically, if you look at nasopharyngeal carcinoma, where sometimes LMP is expressed, those tumours are always Bcl-2 positive, but so is the stem cell from which the tumour probably arises. There are stem cells in normal basal epithelium which are Bcl-2 positive. I think the expression of Bcl-2 may reflect the fact that nasopharyngeal carcinoma is a tumour of undifferentiated epithelium.

Melief: I have a question regarding the practical applicability of a vaccine against the immunoblastic lymphomas. They arise in the 80% of individuals who started with good protective immunity; immunoblastic lymphomas arise only under conditions of immunosuppression or immunodeficiency disease. Can you under those conditions still expect vaccination to be protective?

Moss: In some scenarios, there isn't any doubt that the EBV-seronegative recipients tend to be at a much higher risk than the EBV-seropositive ones. I am really targetting the individuals who are seronegative. Where I come from, Brisbane, is a centre of liver transplants, for instance. The recipient infants are generally about 15 months old and the majority are EBV seronegative. The occurrence of lymphomas in these transplant kids is now becoming a significant clinical problem. Our intention would be to vaccinate before transplantation. This type of population could well be looked at quite early on in an EBV vaccination programme.

Melief: Is there evidence that CTL responses decline in the seropositives when the patients are immunosuppressed?

Moss: Yes, they certainly do.

Doherty: What happens with CTL memory in people who have latent EBV infection? The sort of viruses that we look at (e.g. Sendai virus, influenza virus) are completely eliminated, at least at the level of viral RNA. In this situation, evidence of T cell memory declines slowly and the T cells gradually lose some of the activation markers that they express for months after the initial priming. Are EBV epitopes being continually expressed?

Moss: Yes. There are several parameters we think tend to be almost set in concrete some time not long after the primary infection. These include the amount of virus released orally for the rest of the person's life and the cytotoxic T cell response.

Doherty: Do you ever find cytotoxic T cell effectors in the blood?

Moss: Not without *in vitro* activation, except during acute infectious mononucleosis. During the acute phase of the disease you can find an activated class I-restricted response, also a class II-restricted response.