Original investigations

Autoimmunity caused by host cell protein-containing viruses

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Abstract. Autoreactive T cells specific for myelin basic protein (MBP), a major component of central nervous system (CNS) protein, are frequently found in blood and cerebrospinal fluid of patients with postinfectious encephalomyelitis. This autoimmune syndrome is a CNS complication after infections with a number of different enveloped viruses, e.g. mumps, measles, rubella, influenza and varicella. However, the pathophysiological mechanism leading to this breaking of natural self tolerance in the course of viral infection remains an enigma. A long-lasting hypothesis has suggested that incorporation of cellular (self) proteins into the envelope of budding viruses might be a possible mechanism leading to autosensitization. In a model study we demonstrate here that vesicular stomatitis virus (VSV), grown in myelin protein-expressing cell cultures, is highly efficient in triggering T cell responses to MBP in vitro and can prime autoreactive T cell immune responses in vivo. On the basis of these findings, we suggest that incorporation of CNS membrane components into the viral envelope and subsequent priming of self-reactive immune responses might be the common pathogenic mechanism underlying the postinfectious encephalomyelitis syndrome.

Introduction

Virus infections are often accompanied by transiently appearing self-reactive T cell responses, which might occassionally be even high enough to cause destruction of self components, i.e. to manifest clinical signs of an autoimmune disease. In fact, experimental models of virus-mediated autoimmune diseases are still nowadays the only satisfying models to exemplify naturally occurring induction of autoimmunity [5, 13, 21, 24]. However, the exact mechanisms leading to the breakdown of natural tolerance in the course of viral infections remain largely obscure.

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Postinfectious encephalomyelitis syndrome (PES) is an infrequent complication after infections with a number of different evenloped viruses [3, 9, 10]. PES is an acute and monophasic disease, where neurological manifestations may be severe and sometimes even with a fatal outcome. Clinical symptomatology comprises seizures and motor deficits, such as hemiparesis, cranial nerve palsies, and cerebellar ataxia (reviewed in [9]). Interestingly, in most cases infectious virus can no longer be isolated from the brain or cerebrospinal fluid of patients with postinfectious encephalomyelitis at the onset of clinical disease. This has led to the assumption that a transient viral infection of the brain, which is per se not exerting a vast destructive effect on the integrity of brain functions, is subsequently followed by an (auto-) immune-mediated destruction of the cerebral tissue [6]. Pathological similarities to experimental allergic encephalomyelitis (EAE) and post-rabies vaccine encephalomyelitis, such as perivenular inflammatory foci in white matter and the spinal cord, give further support to the hypothesis that the disease might have an autoimmune character. Indeed, significant primary T cell responses specific for myelin basic protein (MBP) can frequently be observed in patients with PES, both in peripheral blood mononuclear cells and in cells derived from the cerebrospinal fluid [3, 7, 9, 10, 14, 15]. This high degree of autoreactivity against a particular antigen strongly suggests a crucial role of the autoantigen in pathogenicity.

Among the potential immune mechanisms leading to CNS antigen-directed T cell autoimmunity in PES patients, incorporation of host cell proteins into the envelope of budding viruses was proposed a long time ago to represent the crucial pathogenicity factor [4, 18]. Such a common pathogenic mechanism could explain the puzzling observation that PES is found after infections with a great variety of different enveloped viruses, including influenza, rabies, rubella, measles, varicella, and many others [9], which makes immune cross-reactivity (i.e. molecular mimikry [5, 8]) as the underlying pathogenic principle highly unlikely. The hypothesis of enveloped viruses as carriers of autoantigens gains further support from an older observation that brain tissue infected with viruses exhibits a greatly enhanced encephalitogenic potential, when administered to experimental animals. Furthermore, neuroparalytic reactions have been documented in humans immunized with rabies vaccines prepared from neuronal tissue, which can be regarded as the direct human equivalent to various models of virus-mediated EAE [22].

These observations prompted us to examine the potential of an enveloped virus to transfer incorporated autoantigens in a way that allows accessibility and T cell recognition in vitro and in vivo. We found that vesicular stomatitis virus (VSV), grown in myelin-expressing cell cultures, is extremely efficient in stimulating MBP-specific T cell responses in vitro and can induce autoimmunity in vivo. The results of our model study imply that incorporation of host cell proteins into a budding virus can provide the basis of a subsequent induction of T cell-mediated autoimmunity, an immune mechanism that might define the common pathogenic basis of PES.

Material and methods

Virus

Throughout the study the Indiana serotype of VSV was used. For virus purification from infected glia cell cultures, medium was harvested 10 h post infection, pooled and filtered twice through

a 0.22-µm diameter pore-size Millipore filter. A volume of 8 ml of supernatant was layered on top of a 4-ml cushion of 20% (w/w) sucrose in PBS and centrifuged for 3 h at 37 000 rpm at 4 °C in a Beckman SW40 rotor [16]. The pellet was resuspended in culture medium, subjected to several pulses in a waterbath sonicator and virus concentration was assessed in a plaque test using HeLa cells. Virus adjusted to a concentration of 5×10^6 PFU/ml was UV inactivated.

T cell culture

The T cell lines utilized in this study were MBP- and proteolipid protein (PLP)-specific CD4$^+$ T cells of SJL mouse or Lewis rat origin, which were established according to standard protocols as described recently [19]. Briefly, lymph node cells from donors immunized with 100 µg purified MBP emulsified in complete Freund's adjuvant (CFA) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Boehringer, Mannheim), L-glutamine, L-asparagine, 2-mercaptoethanol, sodium pyruvate and antibiotics for 4–5 days in the presence of 10 µg/ml (for protein antigen) or 100 ng/ml (for peptide antigen) of the respective antigen, and then expanded in growth medium containing interleukin-2 (IL-2) (10% supernatant from concanavalin A (Con A)-stimulated cell cultures) for 6–9 days. T cells underwent weekly cycles of antigen-supplemented stimulation medium, containing irradiated syngeneic thymocytes as antigen-presenting cells (APCs), followed by a subsequent expansion in IL-2-supplemented medium.

Ex vivo proliferative responses were analyzed by cultivating $1\times10^5–2\times10^5$ red blood cell-depleted spleen cells per well in round-bottom 96-well culture plates in the presence of MBP (20 µg/ml), ovalbumin (20 µg/ml), VSV (10 µg of total viral protein/ml), PLP peptide (100 ng/ml), or Con A (1 µg/ml). Mitogen-stimulated cultures were labelled for the last 8 h of a 72-h culture period and antigen-stimulated cultures for the last 8 h of a 104-h cultivation and proliferation was assessed by standard liquid scintillation technique.

Glia cell culture

Oligodendrocyte-enriched cell cultures were established according to the method of McCarthy and De Villis [7], with minor modifications. Lewis rat pups, 2–3 days old, were decapitated, meninges were dissected away, and the cerebral hemispheres were mechanically disrupted. Primary glia cells were seeded into poly-L-lysine-coated culture dishes in basal medium of Eagle medium supplemented with 10% heat-inactivated FCS and 10% Con A supernatant as a rich source of IL-2. IL-2 has recently been shown to stimulate the proliferation and differentiation of oligodendroglia [2]. Three weeks following explantation, cells were characterized by immunocytochemistry; the main cell types comprised GFAP$^+$ astrocytes type I (25–40%), A2B5$^+$ astrocytes type II (1–5%), W2/25$^+$ microglia cells (1–5%) and GalC$^+$ oligodendrocytes (60–75%) (data not shown). At this stage of development the vast majority of oligodendrocytes produce MBP, and MBP-expression levels reach more than 90% of the levels seen in young animals in vivo. Myelin-forming glia cells were infected with VSV at 10 PFU/cell; after virus was adsorbed for 60 min, serum-free medium was exchanged and supernatants were collected after 12–15 h.

Primary cultures of type-1 astrocytes were established from newborn Lewis rats as previously described [20]; more than 98% of cells were GFAP$^+$.

Detection of MBP in VSV virions

Oligodendroglia-grown VSV (1×10^{10} PFU/ml) and HeLA cell-grown VSV were either disrupted and then spotted three times onto the same dot of a PVDF membrane (Bio-Rad, USA). Purified rat MBP was used as positive control. Filters were saturated for 1 h at room temperature in PBS containing 5% defatted dry milk, then incubated overnight at 4 °C with supernatant of rat monoclonal antibodies (mAbs) specific for rat MBP. After washing, filters were incubated with peroxidase-conjugated anti-rat IgG and IgM (Jackson Immunoresearch). After another washing, the filters were incubated in 0.5% mg/ml diaminobenzidine, 0.037% H_2O_2 and 0.03% $CaCl_2$.

Histology

Rats were anesthesized with 10 mg/kg, i.p. pentobarbital and perfused with 4% formalin. Brain and spinal cord were removed and fixed for another 48 h in formalin. The histological examination was subsequently performed according to standard protocols.

Results and discussion

To evaluate the capacity of viruses to transfer host cell proteins in a manner that allows accessibility to T cell recognition, we tested oligodendroglia-grown VSV in a functional T cell proliferation assay. Oligodendrocyte-enriched Lewis rat glia cell cultures were infected with the Indiana strain of VSV. VSV was chosen as a model for enveloped viruses in our experimental approach, since the virus replicates efficiently in vitro in myelin-forming oligodendrocytes from Lewis rats [3]. In addition, there is no evidence of VSV exhibiting any cross-reactivity with myelin proteins, as has been suggested for structural proteins of other enveloped viruses such as measles or rubella [8]. Furthermore, the incorporation of a significant proportion of host cell surface proteins into budding VSV virions has been documented [16]. In the present study, VSV was allowed to replicate in oligodendrocyte-enriched cultures for 12–15 h and purification of released viruses from pooled supernatants was performed as described previously [16].

A murine $CD4^+$ T cell line specific for rat MBP was incubated with soluble purified antigen or was alternatively confronted with oligodendroglia-passaged VSV in the presence of macrophage-enriched thymocytes as APCs. VSV grown in myelin protein-expressing glia cells ("VSV*") turned out to be highly efficient in eliciting proliferation of MBP-specific T cells. As few as 1.25×10^6 PFU/ml (Fourfold diluted virus preparation) gave rise to a marked T cell proliferation, comparable to the T cell response induced with 200 ng/ml of soluble MBP (Fig. 1 a). Virtually identical results were obtained when a Lewis rat-derived T cell line raised against guinea pig MBP and cross-reacting with rat MBP was used as responder cell (data not shown). To confirm the specificity of this response, several controls were performed. (1) VSV grown in non-myelin-forming cells (HeLa) could not activate MBP-specific T cells, demonstrating that viral proteins themselves are not responsible for the stimulatory effect (data not shown). (2) VSV grown in HeLa cells, mixed with an appropriate lysate of disrupted myelin protein-expressing cells and then purified the same way could likewise not elicit an MBP-specific response ("VSV copurified", Fig. 1). (3) When oligodendroglia-grown virus was pre-adsorbed for 90 min on Lewis rat astrocytes to allow virus attachment and the supernatant was subsequently tested in proliferation assays, T cell responses were almost completely abrogated ("VSV-preabsorbed", Fig. 1).

Finally, we tested whether oligodendroglia-grown VSV could evoke T cell responses against another myelin antigen, PLP. Both myelin proteins are equally abundantly expressed in myelin synthesizing oligodendrocytes, but they show fundamental differences with respect to their biochemical properties; whereas MBP is a peripheral membrane protein, apposited to the cytosolic side of the membrane, PLP is an integral membrane protein with several transmembrane domains [23]. When testing PLP-specific T cells in the presence of oligodendroglia-grown virus, no stimulation was observed (Fig. 1 b). Interestingly, this result parallels the situation in PES, where patients have been shown to exhibit a specific T cell reactivity for MBP but not for PLP [7].
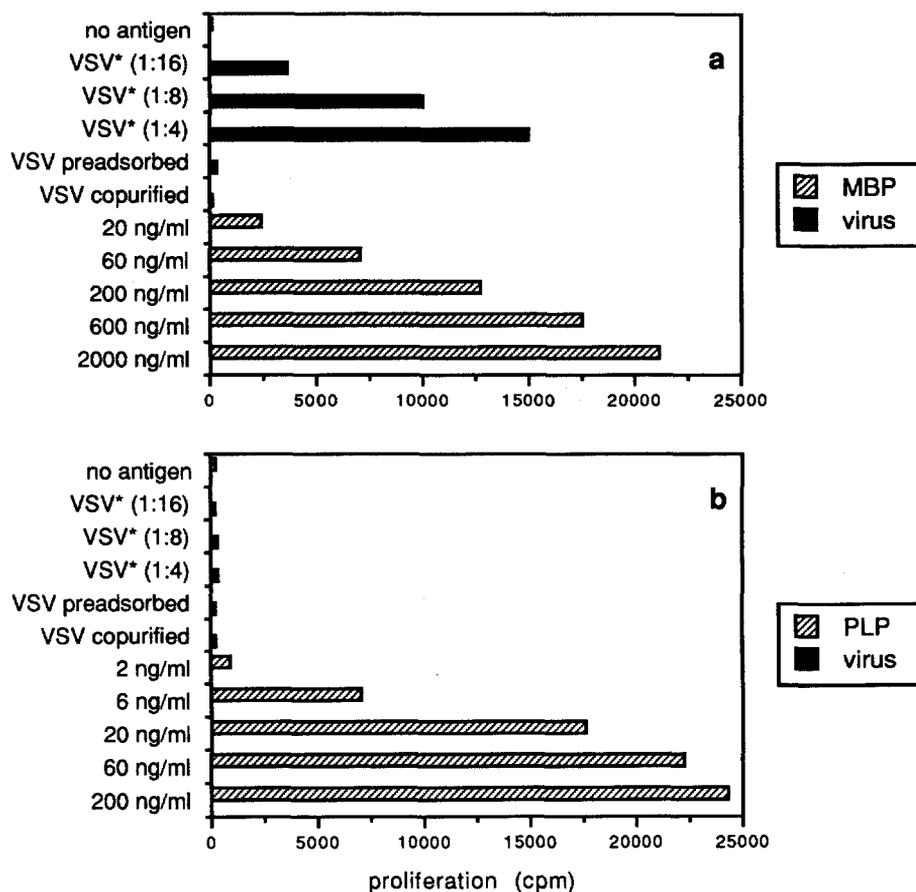
Fig. 1a, b. Proliferation of myelin basic protein (MBP)- or proteolipid protein (PLP)-specific T cells to soluble autoantigens and oligodendroglia-grown vesicular stomatitis virus (VSV). SJL mouse T cell lines specific for rat MBP (**a**) or PLP (**b**) were established as described recently [19]. T cells (3×10^4/well) were incubated with various doses of soluble MBP or a synthetic PLP oligopeptide [single letter amino acid (aa) code: QAHSLERVCHCLGKWLGHPDK; aa 130–150] or oligodendroglia-grown VSV ("VSV *"; with the respective dilution of a stock solution indicated) in the presence of macrophage-enriched irradiated syngeneic thymocytes (2×10^5/well). HeLa cell-grown VSV, which was copurified with supernatants from oligodendrogial cell cultures as described in Material and methods ("VSV-copurified"), served as negative control. As a further control, the oligodendroglia-grown VSV stock preparation was preabsorbed for 90 min on Lewis rat astrocytes to allow virus attachment, and the supernatant was subsequently tested in the same proliferation assay ("VSV-preabsorbed"). After an incubation for 24 h, cultures were pulsed with [^{3}H] thymidine for the following 12 h and proliferative responses were assessed using standard liquid scintillation technique

We next investigated whether oligodendroglia-grown VSV is able to prime T cells for MBP in vivo. For this purpose, rats were inoculated with oligodendroglia-grown virus by intraperitoneal injection. To avoid any undesired effect of a synthetic viral infection, the virus was inactivated by UV treatment before application. T cells from immunized animals displayed a statistically significant re-
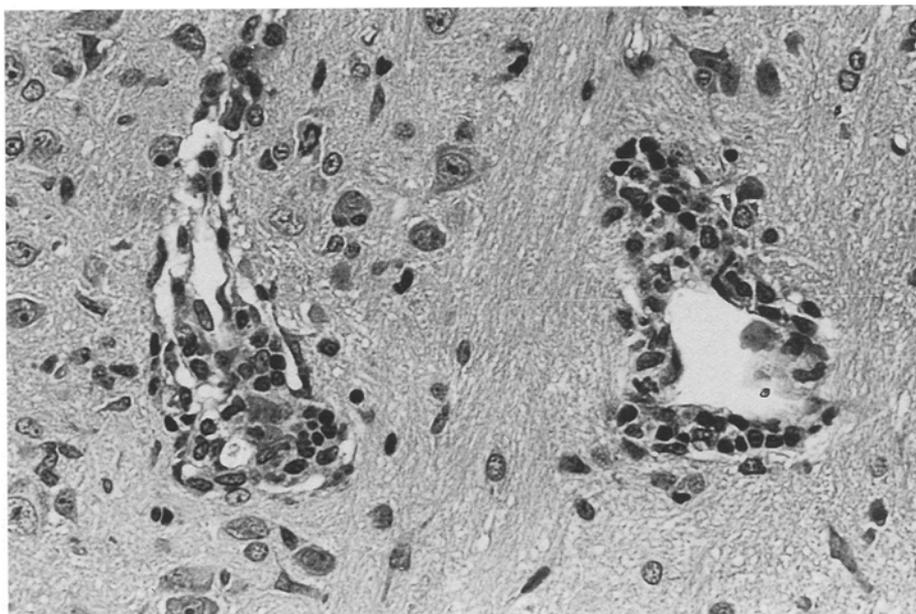
Fig. 2. Perivascular EAE-like lesions in the basal cortex of Lewis rats after T cell transfer. Animals received 5×10^7 activated T lymphocytes from an animal immunized with UV-inactivated oligodendroglia-grown VSV after one single in vitro stimulation with rat myelin basic protein. Five days after T cell transfer, rats were perfused and tissue specimens from the brain and spinal cord were embedded in paraffin for 1-μm sections. Inflammatory lesions were predominantly localized in the leptomeninges and the gray matter of the spinal cord, but similarly found in the brain parenchym. Histocytologically, there were mononuclear and neutrophilic cell infiltrates. Paraffin-embedded section, hematoxylin-eosin staining, $\times100$

sponse against MBP but not against PLP or a third-party antigen (ovalbumin; Table 1), indicating that MBP-specific autosensitization had occurred. The magnitude of autoreactivity is compatible with levels of MBP-directed T cell responsiveness described in other rat models of virus-induced CNS autoimmunity, such as coronavirus-induced encephalopathy [24], subacute measles encephalomyelitis [13], or in Borna virus disease [21]. Adoptive transfer of these T cells after in vitro restimulation (5×10^7 cells transferred cells/animal) resulted in typical EAE-like symptoms in recipient Lewis rats (weight loss, unsteady gait and loss of tail tonus; symptoms were found in eight out of ten animals tested). Histological examination revealed perivascular cell infiltrations predominantly in the brain stem, with most severe signs of inflammation in the medulla oblongata and in the spinal cord (Fig. 2). T cells from control animals, which were immunized in parallel with "VSV-copurified", only showed a responsiveness for the viral antigen but not for autoantigens (Table 1) and were unable to cause any clinical or histological manifestations of EAE after adoptive transfer (ten out of ten animals tested, data not shown).

In animal models of EAE, priming of autoreactive T cells against MBP normally requires a quite rigid manipulation of the immune system with applications of high doses of heterologous "auto"-antigen and emulsification in CFA. Only

Table 1. Oligodendroglia-grown VSV can prime for myelin basic protein T cell responses in vivo

Animal no.	No antigen	RBP	VSV	PLP	OVA
O.g. VSV group [a]					
1	912±179	2907±211	5182±366	877±87	744±89
2	1844±277	4336±523	8101±589	1643±145	1471±133
3	1644±127	4420±288	8392±624	1570±112	1507±77
4	1175±188	3947±362	6129±301	1027±84	979±69
Control group [b]					
1	1241±106	989±76	7155±443	1104±125	1041±119
2	1418±155	1187±131	8551±487	1302±154	1293±205
3	860±54	611±47	5661±172	789±41	721±118
4	1582±132	1233±146	8014±556	1419±101	1449±71

RBP, Rat myelin basic protein; VSV, vesicular stomatitis virus; PLP, proteolipid protein; OVA, ovalbumin; O.g., oligodendrocyte-grown
[a] Lewis rats were immunized with rat myelin basic protein-containing VSV (twice with 1×10^9 PFU, with an interval of 7 days between the two injections) by intraperitoneal injection of UV-inactivated virus. Fourteen days following the first immunization, proliferative responses to viral- and autoantigens of T lymphocytes pooled from mesenterical lymph nodes and spleen from one individual animal were tested. Aliquots of 1×10^5 cells/well were incubated for 96 h in the presence of the indicated antigens. Proliferation was assayed by measuring the incorporation of [³H]thymidine for the following 8 h. Data are given as cpm and are representative for a group of ten animals
[b] Control animals were immunized with the same amount of inactivated copurified VSV (i.e., VSV grown in HeLa cells, mixed with disrupted MBP-expressing oligodendrocyte cultures and then purified; see legend to Fig. 1). Proliferative responses were assessed as described above.

under these highly artificial circumstances can self tolerance be abrogated. In contrast, the close vicinity of highly immunogenic viral glycoproteins to certain self antigens in a viral envelope seems obviously to exert a "natural adjuvant effect" and consequently lowers the threshold for initiating autoreactivity. It can be assumed that this effect is even more significant in a naturally occurring infection with an actively replicating virus.

In an attempt to detect MBP associated with VSV virions, protein dot blots were performed. We could demonstrate MBP in ether-disrupted VSV virions by a modified dot blot assay (Fig. 3) only after further concentrating the virus to 10^{10} PFU/ml. The protein concentration of MBP/10^{10} PFU was estimated to be in the range of 0.5–1.0 ng/ml. This shows that extremely low amounts of antigen, when contained in a "virus carrier", can give rise to a substantial T cell activation. Although it is difficult to directly compare the effect of membrane-bound protein versus soluble protein on the basis of this semiquantitative approximation, it can be estimated that APCs present virus-associated MBP at antigen concentrations of at least 10^{-5} lower than required for presentation of free soluble antigen.

A number of mechanisms have been suggested for the induction of autoimmune reactions in the course of viral infections. The most often discussed postulates comprise molecular mimicry based on the homology between antigenic determinants of viruses and host cells [5], generation of anti-idiotypic responses, and polyclonal activation of B cells and T cells by virus-evoked cytokine release. All of these mechanisms can hardly explain the phenomenon of PES, where an astoundingly
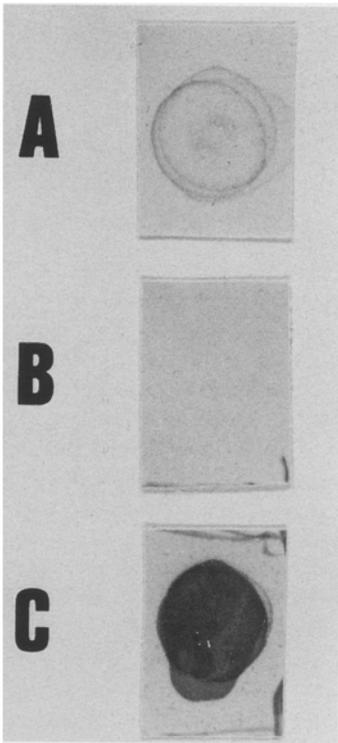
Fig. 3A – C. Detection of MBP in VSV virions. Oligodendroglia-grown VSV or HeLa cell-grown control virus was prepared as described in detail in Material and methods. For detection of MBP antigen, virions were ether-disrupted, dotted onto a PVDF membrane and incubated with a mixture of three MBP-specific monoclonal antibodies. **a** Oligodendroglia-grown VSV; **b** the same amount of HeLa cell-grown VSV; **c** 100 ng/ml of purified soluble rat MBP

large variety of rather distantly related viruses cause an immune response to one and the same autoantigen, MBP. However, incorporation of myelin proteins into viral envelopes could define the common basis leading to MBP-specific autoreactivity in this syndrome. It is nowadays generally assumed that autoimmune diseases in man are entities that are caused by many factors. Since inflammatory demyelinating signs, even after measles or rabies vaccine remain an infrequent and very rare complication, many factors are likely to contribute to disease, such as genetic factors, humoral immune responses and autoreactive T cell responses. For the induction of the latter, which may be a necessary but alone not sufficient event to ultimately manifest clinical symptoms in man, we describe here a new mechanism.

In this report we demonstrate that viruses can transfer an auto-antigen to antigen-presenting cells, leading to the subsequent recognition by MHC class II-restricted T cells. Virus-mediated receptor-specific antigen uptake resulted in enhanced antigen presentation that proved to be orders of magnitude more efficient than the presentation of exogenously added soluble antigen. These results are compatible with the well-known amplification effect by receptor-mediated antigen uptake [11, 12]. In addition, it appears that the close juxtaposition of an autoantigen to foreign viral antigen(s) is sufficient to break T cell tolerance, as shown by the induction of T cell responses in vivo.

The confrontation of the immune system with virus-associated autoantigens might in general have a profound impact on immune responses in virus diseases.

Cellular proteins incorporated into budding viruses could be transfered to other types of cells, thereby shedding a formerly "hidden" (cryptic) self antigen. Association of self components with virus carriers may be found to be of considerable importance, especially in the context of infections with viruses which exhibit a dual specificity for the immune and central nervous system, such as measles, HIV or HTLV-1 [1].

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