Journal of General Virology (2002), **83**, 1735–1743. Printed in Great Britain

Uncoupling of virus-induced inflammation and anti-viral immunity in the brain parenchyma

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Non-neuroadapted influenza virus confined to the brain parenchyma does not induce antigen-specific immunity. Nevertheless, infection in this site upregulated major histocompatibility complex (MHC) class I and MHC class II expression and recruited lymphocytes to a perivascular compartment. T cells recovered from the brain had an activated/memory phenotype but did not respond to viral antigens. In contrast, T cells recovered from the brain after infection in a lateral cerebral ventricle, which is immunogenic, showed virus-specific responses. As with infectious virus, influenza virus-infected dendritic cells elicited virus-specific immunity when inoculated into the cerebrospinal fluid but not when inoculated into the brain parenchyma. Thus, inflammation and dendritic cell function were both uncoupled from immune priming in the microenvironment of the brain parenchyma and neither was sufficient to overcome immunological privilege.

Introduction

Invading pathogens typically trigger co-ordinated inflammatory and immune responses. This is achieved through antigen non-specific inflammatory signals stimulating both the afferent and the efferent arms of the immune response and, through immune effector mechanisms, promoting inflammation (Mims, 1982). In particular, dendritic cells respond to inflammatory stimuli by maturation and migration to lymphoid tissue, where they play a major role in the initiation of antigen-specific immunity (Steinman, 1991; Mellman & Steinman, 2001). Thus, the presence of an inflammatory response to infection generally correlates with specific immunity. The situation may be different in the central nervous system (CNS).

The prolonged survival of allogeneic tissue grafts in the brain implies a degree of immune privilege (Brent, 1990). The rejection of an intracerebral graft can be triggered by a subsequent extracerebral graft from the same donor, so this privilege probably reflects a lack of immune priming by antigens sequestered in the brain (Medawar, 1948). Mycobacterial antigens also appear to be much less inflammatory in the brain parenchyma than in extracerebral sites, probably due to a lack of immune priming (Matyszak & Perry, 1998). We have previously used a stereotactically guided, small volume inoculation of a non-neuroadapted strain of influenza virus to confine virus infection to the parenchymal substance of the brain and thus to demonstrate that such an infection does not lead to immune priming (Stevenson *et al.*, 1997a). Infection with the neuroadapted influenza virus A/WSN in the same site also appears not to prime efficiently but, nevertheless, elicits an intracerebral inflammatory response (Stevenson *et al.*, 1997b). However, because this virus spreads throughout the brain (Stuart-Harris, 1939; Takahashi *et al.*, 1995) and reaches the cerebrospinal fluid (CSF) (Stevenson *et al.*, 1997b), it is unclear whether such inflammation represents the early signs of an immune response consequent upon an extracerebral seeding of infection. Here, we have analysed the local response to infection with a non-neuroadapted influenza virus, which does not produce infectious virions in the brain and thus does not spread. This has allowed us to exclude the possibility of extracerebral infection.

Soluble protein antigens injected into the brain parenchyma can reach the deep cervical lymph nodes (Cserr & Knopf, 1992) and stimulate immune responses (Gordon *et al.*, 1992). The lack of immune priming by cell-associated antigens such as influenza virus thus suggests that deficient antigen-presenting cell function in the brain is an important factor in immune privilege.

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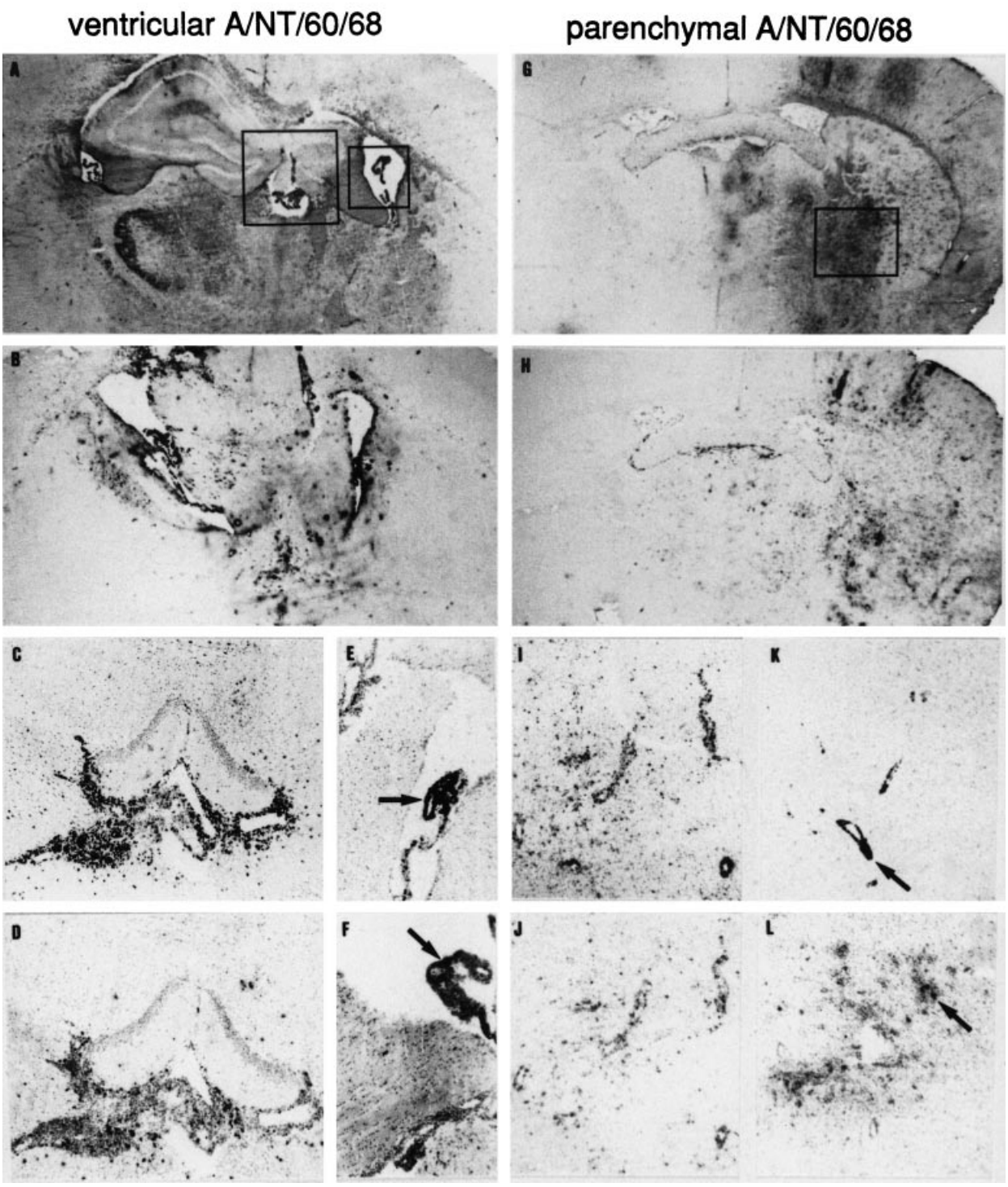

Fig. 1. Intracerebral inflammatory response to influenza virus infection. (A, B, G and H) Coronal brain sections (10×) 10 days after influenza virus A/NT/60/68 infection in the site indicated. Adjacent brain sections equivalent to the boxed areas in (A) are shown at 30× in (C) and (D) (left-hand box, third ventricle) and in (E) and (F) (right-hand box, lateral ventricle). Adjacent brain sections equivalent to the boxed area in (G) (the injection site) are shown at 30× in (I–L). (A, G) Anti-MHC class I staining; (B, H) anti-MHC class II; (C, I) anti-CD4; (D, J) anti-CD8; (E, K) anti-B220 (arrows show clusters of positively stained B cells); (F, L) anti-viral ribonucleoprotein (arrows show infected cells). The brains shown are representative of at least 15 individual mice examined with each route of infection.

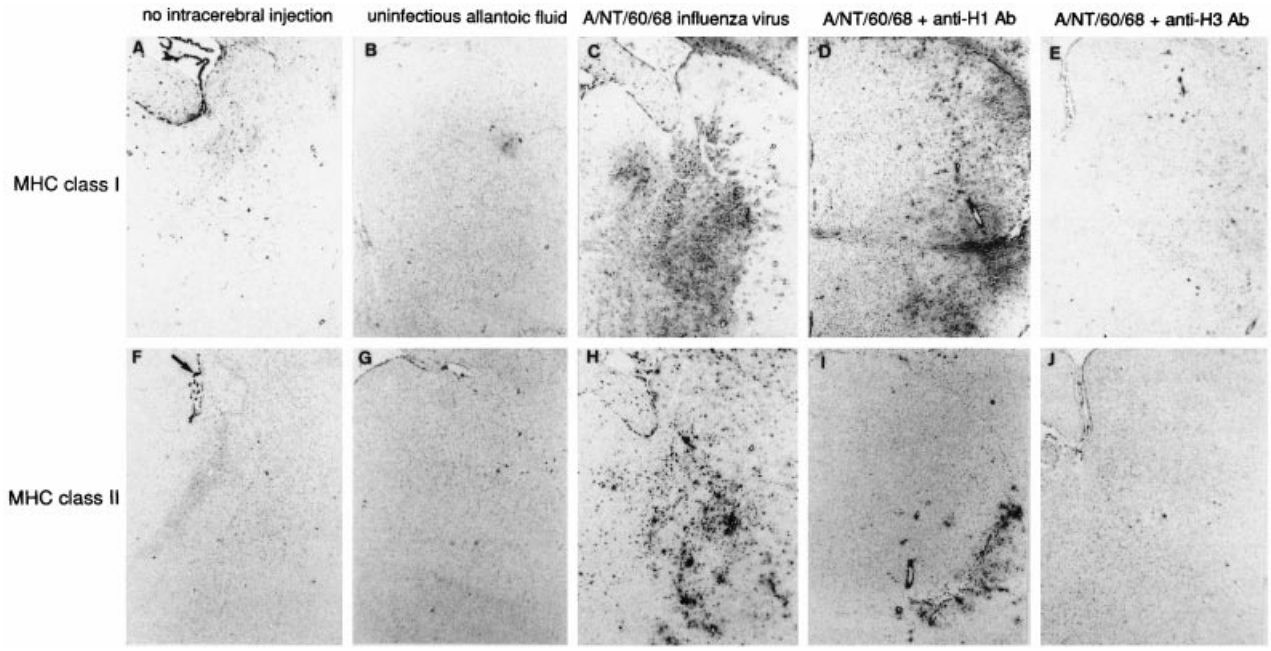

Fig. 2. Prevention of intracerebral inflammation by prior influenza virus neutralization. Influenza virus A/NT/60/68 (H3N2) was incubated with an anti-haemagglutinin H3 MAb (clone BH151) or an anti-haemagglutinin H1 MAb (clone HC132), each as neat infectious allantoic fluid with 10% monoclonal ascites on ice for 1 h prior to intracerebral inoculation. For clone BH151, this was a threefold excess of the dose required to achieve > 90% inhibition of haemagglutination by the same quantity of virus. Clone HC132 inhibited haemagglutination by influenza virus A/PR/8/34 (H1N1) but had no effect on influenza virus A/NT/60/68 (data not shown). (A)–(E) Anti-MHC class I staining; (F–J) anti-MHC class II staining. The arrow in (F) indicates normal MHC class II expression in the choroid plexus. All brains are shown 10 days after the intracerebral inoculations indicated. Consistent results were obtained with at least three individual mice in each experimental group.

While the brain parenchyma lack classical dendritic cells (Hart & Fabre, 1981), the bone marrow-derived perivascular microglial cells (Hickey & Kimura, 1988) resemble immature antigen-presenting cells (Carson *et al.*, 1998) and can be induced to differentiate towards a dendritic cell phenotype *in vitro* (Aloisi *et al.*, 1999; Santambrogio *et al.*, 2001). $CD11c^+$ microglia (Fischer *et al.*, 2000; Serafini *et al.*, 2000) also share some features with dendritic cells and can prime naive T cells *in vitro* (Fischer & Reichmann, 2001). However, there is clearly a functional deficit *in vivo*, which presumably applies both to the carriage of cell-associated antigens from the brain parenchyma to lymph nodes and to the priming of intracerebral T cells *in situ*. Either should suffice to overcome immune privilege.

Two possibilities, not mutually exclusive, are that bone marrow-derived microglia are not functionally equivalent to dendritic cells *in vivo* and that the microenvironment of the brain parenchyma does not support immune priming by any cell type. Influenza virus-infected dendritic cells are known to stimulate potent immune responses *in vitro* (Macatonia *et al.*, 1989; Nonacs *et al.*, 1992) and *in vivo* (Hamilton-Easton & Eichelberger, 1995; Lopez *et al.*, 2000). Thus, we have used *ex vivo* dendritic cells infected with influenza virus to explore the possibility that professional antigen-presenting cells delivered to the brain parenchyma might overcome the barrier to immune priming.

Methods

■ **Mice and viruses.** C57BL/10 mice were obtained from Harlan, housed under UK Home Office-approved conditions and used at 8–10 weeks of age. Influenza virus A/NT/60/68 was grown and titrated by standard methods (Stevenson *et al.*, 1997a). The infectious allantoic fluid used for all intracerebral inoculations contained 1×10^4 haemagglutination units (HAU)/ml, 1×10^8 p.f.u./ml and 1×10^{10} electron microscopic virus particles/ml. Influenza virus (5 HAU) was administered under brief general anaesthesia as either 0·5 µl in the lateral cerebral ventricle or anterior caudoputamen or 30 µl intranasally. All intracerebral injections were performed under stereotactic guidance, as described previously (Stevenson *et al.*, 1997a). To harvest cells, anaesthetized mice were exsanguinated by trans-cardiac perfusion with 30 ml heparinized PBS. Spleens and lymph nodes were disrupted into single-cell suspensions and used directly. Lymphocytes in brain or lung homogenates were recovered by centrifugation (1000 ***g*** for 15 min) on 60/40% Percoll gradients (Hawke *et al.*, 1998).

■ **Preparation of dendritic cells.** Dendritic cells were purified from mouse spleens by standard methods (Austyn *et al.*, 1983; Kupiec-Weglinski *et al.*, 1988). Briefly, low-density cells were enriched from splenocytes by centrifugation onto a cushion of dense (1·08 g/ml) BSA. These cells were adhered to plastic for 3 h at 37 °C. Non-adherent cells were removed by pipetting and discarded. After overnight culture at 37 °C, cells were detached by pipetting and readhered to plastic for 2 h to remove macrophages. The proportion of dendritic cells [major histocompatibility complex (MHC) class II^+, $B220^-$, $Mac\text{-}1^-$, $CD3^-$ and Fc $receptor^-$] in the final non-adherent cell population was 50–70%.

Table 1. Functional analysis of T cells recovered from infected organ homogenates 10 days after inoculation of 5 HAU influenza virus A/NT/60/68 by the route indicated

Cells were recovered by Percoll density gradient centrifugation from homogenates of brain (after ventricular or parenchymal infections) or lung (after intranasal infection). Total cell numbers isolated per infected organ are the mean ± SEM of results from 8 to 10 pairs of mice for each route of immunization, pooled from three separate experiments. Peripheral blood from pairs of naive mice ($n = 6$) analysed at the same time contained 26·8 ± 3·0% $CD4^+$ T cells and 15·5 ± 1·9% $CD8^+$ T cells, with a CD4:CD8 ratio of 1·8 ± 0·1. Although a greater proportion of the intracerebral T cells were $CD4^+$ after ventricular virus inoculation, this was not statistically significant ($P = 0·13$ by paired t-test, comparing the CD4:CD8 ratios of the intracerebral cells isolated after ventricular virus infection with those in peripheral blood). Cytotoxicity before restimulation was tested in parallel with proliferation using cells pooled from the same mice for each assay. At the same time, cells were restimulated *in vitro* for 5 days with virus-infected feeder cells before testing cytotoxicity again. Proliferation index = mean response to influenza-infected targets (c.p.m.)/mean response to uninfected targets (c.p.m.). The mean ± SEM proliferation indices from three pairs of mice for each route of immunization are shown. Positive responses to virus-infected feeders were typically 1000–1500 c.p.m., with responses to unpulsed feeders of 50–150 c.p.m. Cytotoxicity is shown as the net percentage of specific lysis [percentage of specific lysis with NP (immunodominant viral nucleoprotein peptide-pulsed target cells) or Flu (influenza virus A/NT/60/68-infected target cells)] − (percentage of specific lysis with untreated target cells). Specific lysis of untreated targets did not exceed 10%. Means from three pairs of mice per route of immunization are shown, with SEM < 3·0 for each mean. Effector:Target (E:T) ratios were based on the total viable cells (excluding Trypan blue) harvested from 40/60% Percoll gradients or from restimulation cultures. ND, Not done.

		Route of infection					
		Ventricular		Parenchymal		Intranasal	
Total cells (1×10^{-5})		2·9 ± 0·7		1·8 ± 0·3		2·1 ± 0·4	
CD4 (%)		34·6 ± 4·0		17·8 ± 2·8		22·8 ± 2·2	
CD8 (5)		13·1 ± 2·1		13·0 ± 1·8		30 ± 1·6	
CD4:CD8 ratio		3·2 ± 0·7		1·7 ± 0·2		0·7 ± 0·1	
Proliferation index		15·9 ± 2·5		1·2 ± 0·2		5·2 ± 1·8	
Cytotoxicity before restimulation	E:T ratio	NP	Flu	NP	Flu	NP	Flu
Brain/lung	20:1	13·5	11·6	−2·3	−3·2	10·6	17·2
	5:1	2·7	2·8	−0·3	−0·2	3·8	10
Cytotoxicity after restimulation	E:T ratio	NP	Flu	NP	Flu	NP	Flu
Brain	30:1	50·4	40·3	−0·1	0·3	ND	ND
	6:1	35·6	23·9	−0·4	−0·5	ND	ND
Lymph node	30:1	34·2	22·6	−0·6	3·1	ND	ND
	6:1	16·1	13·8	−1·7	−1·8	ND	ND
Spleen	30:1	45·2	30·9	−1·2	−1·2	ND	ND
	6:1	43·1	25·8	−0·5	−2·5	ND	ND

These cells were infected for 2 h with influenza virus A/NT/60/68 (2 p.f.u. per cell) and washed three times prior to intracerebral microinjection, as described above. Cell-free virus was undetectable by plaque assay in the intracerebral inoculum.

■ **ELISA.** Influenza virions were purified from infectious allantoic fluid by sucrose density gradient centrifugation, washed in PBS, lysed with detergent and adsorbed overnight onto Nunc Polysorb immunoplates (Life Technologies). Virus-specific IgG, IgA and IgM were assayed as described previously (Stevenson *et al.*, 1997a).

■ **Restimulation and assay of $CD8^+$ cytotoxic T lymphocytes (CTL).** Single-cell suspensions from lymph nodes (5×10^5 cells/ml) or cells from infected brains (5×10^4 cells/ml) were restimulated with virus-infected feeder cells (1×10^6 cells/ml) in 2 ml cultures. Spleen cells (1×10^6 cells/ml) were restimulated with virus-infected feeder cells (3×10^5 cells/ml) in 15 ml cultures. Syngeneic feeder spleen cells were incubated with 400 µl influenza virus A/NT/60/68-infected allantoic fluid per 1×10^7 cells at 37 °C for 1 h, irradiated (20 Gy) and washed twice in complete medium before use. Cytotoxicity was tested after 5 days of culture in complete medium at 37 °C with 5% CO_2. ^{51}Cr-labelled EL-4 cells (100 µCi/1×10^6 cells) were either incubated with complete medium alone, pulsed with an *H-2D*^{b}-restricted influenza virus nucleoprotein epitope (1 µM ASNENMDAM) or infected with influenza virus A/NT/60/68 (5 p.f.u. per cell). After 1 h at 37 °C, the target cells were washed twice in complete medium and incubated with CTL for 4 h before harvesting supernatants for scintillation counting. Chromium release from the targets alone was 5–15% of the release with Triton.

■ **Proliferation assay.** Lymphocytes were aliquotted into triplicate cultures (2×10^3 cells per well) in Terasaki plates (20 µl per well) or 96-well plates (2×10^5 cells per well) with irradiated (20 Gy) naive feeder spleen cells (2×10^4 or 2×10^5 per well, respectively). Feeder cells were either untreated or infected with influenza virus, as for CTL restimulation.

After culture for 72 h at 37 °C with 5% CO_2 using complete medium but with 1% normal mouse serum substituted for 10% foetal calf serum, 0·1–1·0 μCi [^{3}H]thymidine was added to each well and the cells were harvested for scintillation counting 18 h later.

■ **Flow cytometry.** Lymphocytes were incubated for 15 min on ice with 5% normal mouse serum and 5% normal rat serum followed by a 30 min incubation with rat anti-mouse antibodies as follows: anti-CD4, KT6-phycoerythrin (Serotec); anti-CD8, KT15-FITC (Serotec); anti-CD62L, biotinylated MEL-14; anti-CD44, biotinylated IM7.8.1; anti-CD49d, biotinylated PS/2; or anti-CD25, Quantum Red-3C7 (Sigma). After washing in PBS/FCS (1%), streptavidin–Quantum Red (Sigma) was added for 15 min followed by a further wash. Samples were analysed on a FACSort instrument using CELLQUEST, version 1.1 (Becton-Dickinson).

■ **Immunohistochemistry.** Acetone-fixed frozen sections (7–10 μm) were preincubated in 10% rabbit serum, and stained with the following rat anti-mouse antibodies: anti-CD8, YTS169.4; anti-CD4, GK1.5; anti-MHC class I, M1-42.3.9.8; anti-MHC class II, M5/114.15.2; and anti-B220, RA3-6B2 (Serotec). A mouse-absorbed, biotinylated, rabbit anti-rat serum (Dako) was used as the secondary antibody, followed by streptavidin–biotin–peroxidase complexes (Vector Laboratories). For anti-virus staining, the sections were preincubated in 10% goat serum and then incubated with an anti-influenza virus ribonucleoprotein rabbit serum (Reinacher *et al.*, 1983) followed by a peroxidase-coupled goat anti-rabbit serum (Vector Laboratories). All antibody incubations were for 1 h at room temperature and sections were washed three times in PBS after each incubation. Endogenous peroxidase activity was blocked with 0·3% hydrogen peroxide in methanol after the primary antibody incubation. Diaminobenzidine was used as the peroxidase substrate and haematoxylin was used as the counterstain.

Results

Inflammation after virus inoculation into different intracerebral sites

Influenza virus A/NT/60/68 (5 HAU) was inoculated into either a lateral cerebral ventricle, the anterior caudoputamen, or the lung. We have previously demonstrated that small volume (< 1 μl) inoculation of influenza virus into the anterior caudoputamen (parenchymal substance of the brain) does not induce detectable immune priming (Stevenson *et al.*, 1997a). Here, we looked at the local consequences of equivalent infections. Surprisingly, mice given virus into either the CSF (Fig. 1A–F) or the brain parenchyma (Fig. 1G–L) all showed a marked upregulation of intracerebral MHC antigen expression (Fig. 1A, B and G–H) together with a local infiltration of T and B cells (Fig. 1C–E and I–J). The lymphocytic infiltrates were predominantly perivascular. After intraventricular virus inoculation, periventricular infiltrates were also prominent, particularly for the $CD4^+$ T cell subset. In all brains, the extent of observed MHC class I and II upregulation extended considerably beyond the extent of infection, although with parenchymal infection, the choroid plexus and contralateral hemisphere appeared normal (Fig. 1G–H).

No bacterial or fungal growth was apparent after incubation of the influenza virus A/NT/60/68 (H3N2)-infected allantoic fluid at 37 °C in standard tissue culture or bacteriological media. To establish that the intracerebral inflammation was due to infection with influenza virus rather than a contaminating virus, infectious allantoic fluid was treated with an anti-influenza virus haemagglutinin monoclonal antibody (MAb) before intracerebral inoculation. Prior anti-haemagglutinin H3 neutralization but not incubation with a control anti-haemagglutinin H1 MAb entirely prevented the upregulation of MHC class I and class II expression (Fig. 2) and any lymphocytic infiltration (data not shown) associated with intracerebral A/NT/60/68 injection. Uninfected allantoic fluid also caused no significant intracerebral inflammation (Fig. 2). Thus, the upregulation of MHC expression and recruitment of lymphocytes depended on the presence of infectious influenza virus.

Influenza virus is generally a lytic virus. This probably explains the destruction of ependymal cells lining the cerebral ventricles (Fig. 1F) and the loss of cells from around the site of parenchymal infection (Fig. 1L, compare with Fig. 2). The crucial deficit in neuronal infection is a lack of haemagglutinin processing, such that any virions produced cannot enter new cells (Schlesinger, 1950). Plaque assays and egg infection assays from days 1–7 after infection failed to recover infectious

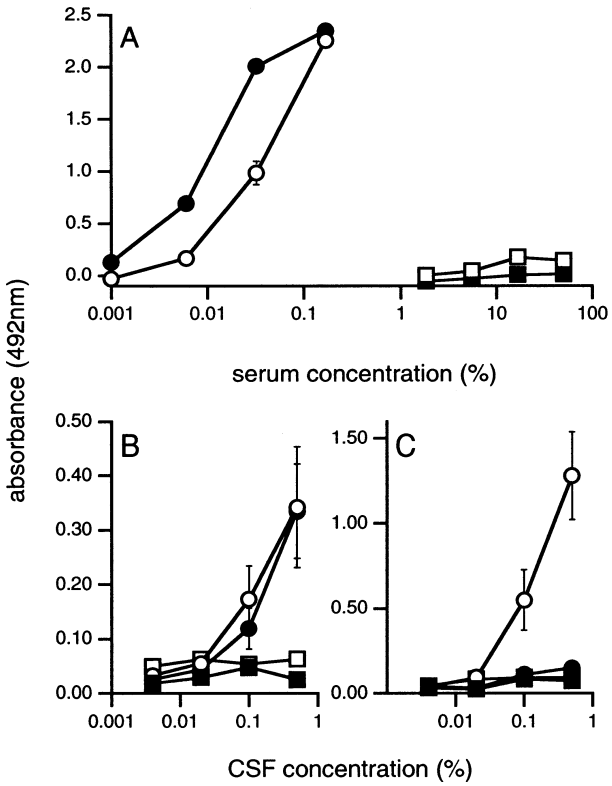

Fig. 3. Serum and CSF antibody levels 10 days after intracerebral or intranasal influenza virus A/NT/60/68 infection. Each graph shows virus-specific antibody in samples taken from naive mice (□) or from mice 10 days after infection with 5 HAU influenza virus A/NT/60/68, either intranasally (●), into the CSF (○) or into the brain parenchyma (■). (A) Serum IgG. Each line shows the mean ± SEM of results from a representative three mice tested on the same ELISA plate. (B) CSF IgG. (C) CSF IgA. Each line shows the mean ± SEM of results from six representative mice. Absorbance values were corrected between trials by reference to a standard immune serum included on each ELISA plate.

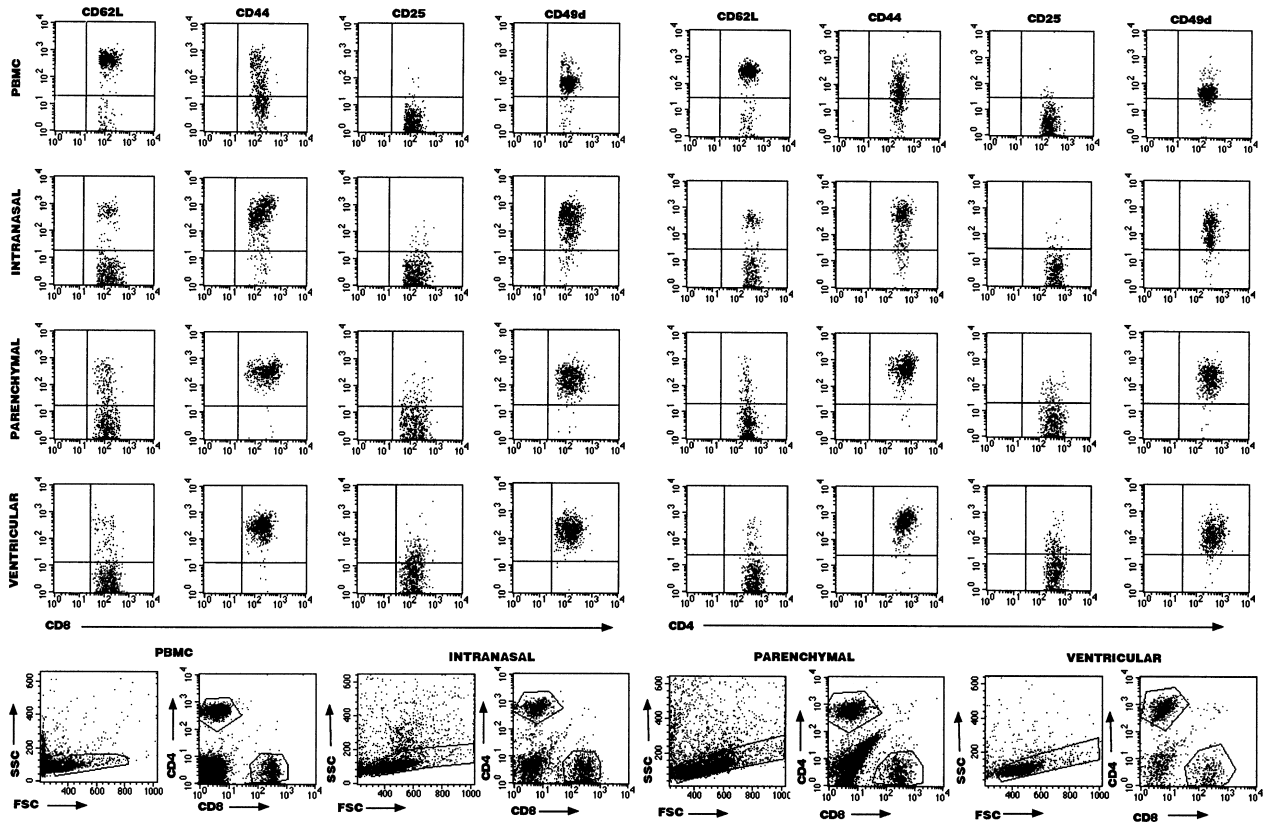

Fig. 4. Cytofluorometric analysis of T cells recovered from infected organs 10 days after intracerebral or intranasal influenza virus A/NT/60/68 infection. Cells were pooled from four mice for each route of immunization and peripheral blood mononuclear cells (PBMCs) from untreated mice were analysed in parallel for comparison. The bottom row shows the ungated populations (FSC, forward scatter; SSC, side scatter) and staining of the gated lymphocyte populations for CD4 and CD8. The upper rows show at least 300 gated $CD4^+$ (right four columns) or $CD8^+$ (left four columns) cells stained with a third colour, as indicated. The horizontal marker shows the upper limit of third colour intensity in each gated CD4 or CD8 population with isotype control antibodies. The data shown are from one of three equivalent experiments.

virus from inoculated brains (limit of detection 10 infectious units per brain). Viral antigen, detectable by immunostaining, and viral mRNA, detectable by RT–PCR, persisted for approximately 2 weeks after ventricular infection and approximately 1 month after parenchymal infection (data not shown). The extent of histological inflammation declined with the decline in surviving viral antigen.

Analysis of infiltrating lymphocytes

After parenchymal virus infection, the presence of infiltrating lymphocytes in the absence of detectable anti-viral serum antibody suggested that these cells had been recruited without an antigen-specific immune response and, thus, that they were not virus-specific. To test this possibility, T cells were recovered from infected brains 10 days after ventricular or parenchymal influenza virus infection or from infected lungs 10 days after intranasal virus inoculation and were assayed for virus specificity *in vitro* (Table 1). The cells recovered after ventricular or intranasal, but not after parenchymal, virus infections showed virus-specific proliferation and cytotoxicity. Virus-specific CTL precursors could be recovered from the brain, spleen and deep cervical lymph nodes after ventricular or intranasal infections but not after parenchymal infection (Table 1). All mice given intracerebral virus were checked for the presence of virus-specific serum antibody. As observed previously (Stevenson *et al.*, 1997a), virus-specific antibody was undetectable in the serum or the CSF after parenchymal virus inoculation (Fig. 3). Thus, there was no evidence of an antigen-specific immune response to parenchymal influenza virus infection, either systemically or locally, within the CNS.

Flow cytometric phenotype of infiltrating lymphocytes

Despite their differences in specificity, the infiltrating T cells recovered after parenchymal or ventricular virus infections showed little difference in surface phenotype (Fig. 4). In each case, the predominant pattern observed was $CD44^{hi}$/$CD62L^{lo}$/$CD49d^{hi}$, implying a memory or activated state. There was little or no evidence of naive ($CD44^{lo}$/$CD62L^{hi}$/$CD49d^{lo}$) T cells entering the brain in response to influenza virus infection. The T cells recovered after ventricular

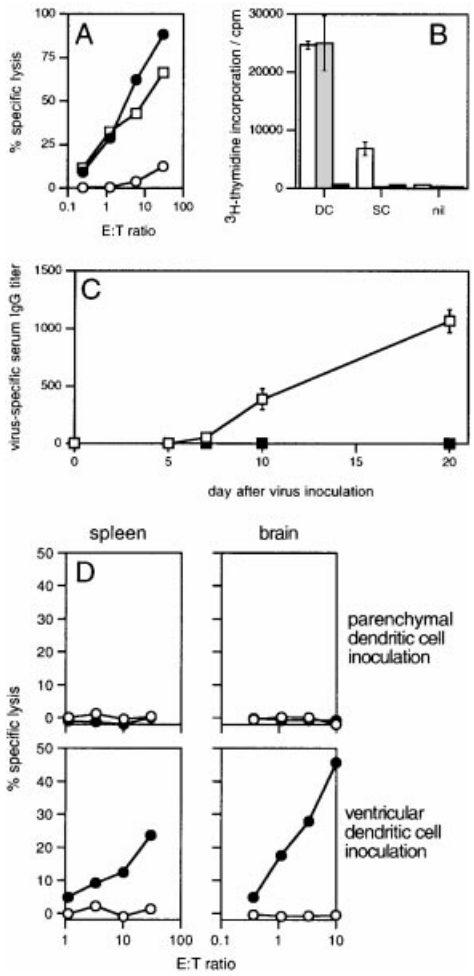

Fig. 5. Influenza virus-infected dendritic cells stimulate virus-specific immunity after extracerebral inoculation. (A, B) Spleen cells enriched for dendritic cells and infected *ex vivo* with influenza virus A/NT/60/68 are immunogenic *in vivo*. (A) A population of cells (5×10^4 cells) highly enriched for splenic dendritic cells were infected with influenza virus and inoculated by footpad injection. After 10 days, spleen cells from inoculated mice were restimulated *in vitro* and assayed for cytotoxicity against syngeneic ^{51}Cr-labelled target cells that were uninfected (○), infected with influenza virus A/NT/60/68 (□) or pulsed with the viral nucleoprotein peptide ASNENMDAM (●). (B) Mice were inoculated subcutaneously into the footpad with 5×10^4 influenza virus-infected cells, either those enriched for dendritic cells (DC) or non-adherent spleen cells (SC). After 10 days, popliteal lymph node cells were incubated *in vitro* with influenza virus-infected (grey bars) or uninfected (black bars) naive irradiated syngenenic spleen cells and, after 3 days, assayed for proliferation by [^{3}H]thymidine uptake. As a positive control, 10 μg/ml phytohaemagglutinin was added to cultures with uninfected targets (open bars). Nil, feeder cells only. Each bar shows the mean ± SD of results of triplicate cultures. (C) 1×10^5 spleen cells highly enriched for dendritic cells were infected with influenza virus A/NT/60/68 and inoculated with 0·5 μl into either the anterior caudoputamen (■) or the lateral cerebral ventricle (□). Virus-specific serum IgG, in arbitrary units, was determined by ELISA. Each point shows the mean ± SEM of results of six individual mice. One of three equivalent experiments is shown. (D) The same mice as in (C) were assayed for virus-specific CTL precursors by restimulation *in vitro* with influenza virus A/NT/60/68-infected syngeneic target cells and ^{51}Cr-release assay with either unpulsed (○) or ASNENMDAM peptide-pulsed (●) target cells. Lymphocytes for restimulation were pooled from three individual mice and either recovered from brain homogenates by centrifugation on discontinuous Percoll density gradients or taken directly from single-cell spleen homogenates, as indicated. In each case, lymphocyte viability was checked by Trypan blue exclusion just prior to assay. One of three equivalent experiments is shown.

infection did have a higher proportion of CD25$^+$ (23%) and CD62L^{lo} (87%) cells than those recovered after parenchymal virus infection (13 and 76%, respectively), suggesting a greater degree of acute activation. This was perhaps consistent with an ongoing immune response only after influenza virus inoculation into the CSF. The T cells recovered from virus-infected lungs were mostly of the CD8$^+$ subset but were otherwise similar to those from infected brains in having an activated/memory phenotype. Because the lung receives a dual blood supply from the bronchial and pulmonary arteries, complete perfusion of this organ was difficult to achieve and the small numbers of CD44^{lo} and CD49d^{lo} T cells isolated were probably intravascular contaminants. In no experiment were significant numbers of naive phenotype T cells isolated from influenza virus-infected brains. T cells recovered from uninfected brains were also uniformly CD44^{hi}CD49d^{hi} (data not shown).

Dendritic cell inoculations

The fact that local inflammation and lymphocyte recruitment occurred in the absence of immune priming implied that these events were insufficient to overcome immune privilege. This raised the question as to what other features of the brain parenchyma were maintaining immune privilege. Since classical dendritic cells are conspicuously lacking from the brain parenchyma, we next addressed the possible contribution of a lack of suitable antigen-presenting cells. Thus, we introduced influenza virus-infected dendritic cells into this site to try to generate an immune response. Dendritic cells were purified as low-density, partially adherent cells from naive mouse spleens and infected *in vitro* with influenza virus just prior to inoculation. Initial experiments confirmed that such a population was capable of immune priming after subcutaneous footpad injection. Virus-specific T cells were present in lymphoid tissue (Fig. 5A, B) and virus-specific antibody was present in serum (data not shown).

Influenza virus-infected dendritic cells were then introduced by small volume sterotactic injection into the lateral cerebral ventricle or the brain parenchyma. Virus-specific serum IgG titres indicated systemic immune priming after injection into a lateral cerebral ventricle (Fig. 5C). This was in accordance with a previous report that intrathecally injected dendritic cells can cause some lymphocyte infiltration into the CNS (Carson *et al.*, 1999). However, dendritic cells injected into the anterior caudoputamen elicited no detectable serum antibody response (Fig. 5C). Virus-specific antibody was also undetectable in the CSF after parenchymal dendritic cell injection (data not shown). Finally, virus-specific CD8$^+$ T cell precursors could be recovered from the brain and spleen after dendritic cell injection into the CSF but from neither site after dendritic cell injection

into the brain parenchyma (Fig. 5D). Thus, the presence of antigen-presenting cells in the brain parenchyma was not sufficient to overcome the barrier to immune priming.

Discussion

Influenza virus infection confined to the brain parenchyma elicited no detectable immune response but, nevertheless, caused a massive upregulation of intracerebral MHC antigen expression together with local lymphocyte recruitment. T cells recovered from the brain after parenchymal infection, unlike control populations, showed no recognition of influenza virus antigens *ex vivo*. Thus, the local inflammatory response was uncoupled from immune priming. We hypothesized that the infiltrating cells were not primed because of a lack of appropriate intracerebral antigen-presenting cells. However, influenza virus-infected dendritic cells, which were immunogenic when inoculated into the footpad or the CSF, did not prime virus-specific immune responses from the brain parenchyma. We conclude that, while the brain can provide inflammatory signals to attract primed lymphocytes, there is a specific microenvironmental deficit that prevents initial priming.

The brain has historically been viewed as a site of decreased immune reactivity and inflammation. However, there is still controversy over the factors involved; for example, whether a lack of immune priming or a defect in intracerebral T cell function is the crucial influence (Streilein, 1993). We have investigated immune privilege using intracerebral challenge with viral antigens, since immune evolution has probably been driven by the requirement to detect and eliminate such parasites. While intracerebral autoimmunity may be downregulated by lymphocyte apoptosis (Ford *et al*., 1996; Gold *et al*., 1997), this does not seem to be a major feature of influenza virus infection. Indeed viable, activated, virus-specific $CD8^+$ T cells can persist long-term in the CNS (Hawke *et al*., 1998). Brain gangliosides have been reported to inhibit T cell proliferation (Irani *et al*., 1996) but again we have found no evidence for this during influenza virus infection: virus-specific intracerebral T cells proliferate *in vivo* (Stevenson *et al*., 1997c) and *in vitro* (Stevenson *et al*., 1997a; Hawke *et al*., 1998). Overall, anti-viral T cells appear to function very effectively in the brain. Here, intracerebral virus infection recruited primed T cells even when they were not virus-specific (Table 1, Fig. 4). The focus in understanding CNS immune privilege is thus shifted onto the generation of primed T cells and onto the antigen-presenting cell.

Cells with a dendritic cell-like surface phenotype accumulate in the brain during chronic toxoplasma infection (Fischer *et al*., 2000) and experimental allergic encephalomyelitis (Serafini *et al*., 2000). The precise function of these cells *in vivo* remains unclear. Even though influenza virus infection stimulated a local inflammatory response in the brain parenchyma (Fig. 1), it did not stimulate any resident dendritic cell-like antigen-presenting cells to generate detectable immunity. And, while influenza virus-infected dendritic cells are normally highly efficient at presenting viral antigens *in vivo*, these were ineffective when inoculated into the brain parenchyma (Fig. 5). This suggested that it is not the lack of dendritic cells but an inappropriate environment for their function that prevents immune priming. It may be that the brain microenvironment fails to support normal dendritic cell migration. A lack of suitable anatomical pathways, chemokines or adhesion molecules could all contribute. The end result is that inflammation can be driven locally but that priming requires extracerebral antigen.

What are the potential implications of the uncoupling of inflammation and immunity in the brain parenchyma? Congenital infections and the retrograde transport of virions along peripheral neurons are both mechanisms by which a virus may reach the brain while avoiding extracerebral immune priming. Viruses may also express genes uniquely in the brain during a period of latency in this site. A lack of immune priming could then contribute to the persistence of an intracerebral, inflammatory focus. The immune response to unrelated extracerebral infections could have significant effects on antigen non-specific intracerebral inflammation by increasing the circulating pool of activated cells capable of entering the brain. Clearly, a priority with any intracerebral inflammatory disease is to consider not only antigen-specific immunity but also the potential role of non-antigen-specific responses to cryptic infections.

P. G. Stevenson was supported by the Medical Research Council (UK) and the Oxford University Medical Research Fund. S. Hawke was supported by the Multiple Sclerosis Society of Great Britain and Northern Ireland. Thanks are due to Dr C. Bangham for helpful discussion, Dr A.R. Douglas for anti-influenza haemagglutinin MAbs and Dr C. Scholtissek for anti-influenza ribonucleoprotein rabbit serum.

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Received 12 November 2001; Accepted 19 February 2002