JOURNAL OF VIROLOGY, Jan. 1997, p. 145–151 Vol. 71, No. 1
0022-538X/97/$04.00+0

The Immunogenicity of Intracerebral Virus Infection Depends on Anatomical Site

P. G. STEVENSON,*[1] S. HAWKE,[1]† D. J. SLOAN,[2] AND C. R. M. BANGHAM[1]‡

Nuffield Department of Medicine[1] and Nuffield Department of Surgery,[2]
John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Received 29 July 1996/Accepted 24 September 1996

The brain parenchyma affords immune privilege to tissue grafts, but it is not known whether the same is true for intracerebral viral infections. Using stereotactically guided microinjection, we have confined infection with influenza virus A/NT/60/68 to either the brain parenchyma or the cerebrospinal fluid (CSF). A/NT/60/68 infection in the CSF elicited a comparable immune response to intranasal infection, with the production of antiviral serum antibody, priming of antiviral cytotoxic T-cell precursors, and an antiviral proliferative response in the draining lymph nodes. The response to virus in the CSF was detectable sooner after inoculation than the response to intranasal virus and also involved a prolonged production of virus-specific immunoglobulin A in the CSF. In contrast, there was no detectable immune response to virus infection in the brain parenchyma by any of the parameters measured for at least 10 days after inoculation. Over the next 80 days, 46% of the mice given parenchymal virus developed low-level immune responses that did not involve CSF antibody production, while the remaining 54% had no detectable response at any time. Thus, a virus infection confined to the parenchymal substance of the brain primed the immune system inefficiently or not at all.

An immunological difference between the brain and other anatomical sites was first demonstrated by intracerebral transplantation (3). Allogeneic skin or neural grafts that are rejected in conventional extracerebral sites survive in the brain, although the rejection of an intracerebral graft can be triggered by rejection of an equivalent extracerebral graft. Thus, there is a lack of immune priming by intracerebral grafts rather than a resistance to primed immune effectors. There are regional differences in the degree of intracerebral immune privilege; high degrees of major histocompatibility complex mismatch between donor and recipient lead to a chronic, low-grade rejection of grafts in the cerebral ventricles but not of those in the brain parenchyma (22).

Intracerebral immune privilege is not due to a lack of lymphatic drainage from the brain (6). Despite the absence of specialized lymphatic vessels, there is an efficient functional drainage from the cerebrospinal fluid (CSF) to the deep cervical lymph nodes via the nasal submucosa (2). Extracellular fluid from the brain parenchyma also reaches the nasal submucosal lymphatics, either through the CSF or along perivascular sheaths (5). Consistent with this lymphatic drainage, intracerebral inoculation gives a greater serum antibody response than extracerebral inoculation for a variety of inert antigens, including a bacterial toxin (17), xenogenic erythrocytes (17, 18, 28), and xenogenic albumin (9, 11, 18). Xenogenic albumin in the brain parenchyma elicits a delayed and diminished immune response compared to the same antigen in the CSF but still a greater response than in extracerebral sites (9).

However, it is difficult to extrapolate from proteins, erythrocytes, or tissue grafts to infectious pathogens; differences in the size, availability for degradation, and capacity for cell binding of antigens are all important considerations in their immunogenicity. Of particular relevance to immune evasion by intracerebral viruses is the lack of resident dendritic cells in the brain parenchyma (12), since they play a key role in initiating immune responses to cell-associated antigens in other organs (23). Many common viruses can infect the central nervous system and cause disease, but the importance of the site of intracerebral infection to any immune response elicited remains unknown.

Influenza virus, apart from being a common respiratory pathogen, has been associated epidemiologically with neuropsychiatic disease in humans (19, 29), and the avian influenza virus strains remain an ever-present potential source of new neurovirulent epidemics (14, 27). Nonneurovirulent influenza viruses such as A/NT/60/68 infect cells in the mouse brain and produce viral proteins but undergo only a single abortive replication cycle (21) and thus remain confined to the site of inoculation (7). They thus provide a suitable tool with which to explore regional differences in the immunogenicity of intracerebral virus infections.

Large-volume intracerebral inoculations are not contained within a precise anatomical site and leak out of the brain parenchyma into the CSF and bloodstream (4, 16). Such inoculations may force antigens into extracerebral sites by pressure-dependent routes that do not contribute to normal lymphatic drainage. We have used stereotactic guidance and an injection volume of 0.5 μl to confine infection with influenza virus A/NT/60/68 to either the lateral cerebral ventricle (CSF) or the anterior caudoputamen (brain parenchyma). Thus, we have been able to determine how the intracerebral location of a virus infection may influence its capacity to avoid immune priming.

MATERIALS AND METHODS

Mice. C57BL/10 mice were bred at Harlan U.K. Ltd. (Bicester, United Kingdom) and kept under standard Home Office-approved conditions at the Biomedical Services Unit, John Radcliffe Hospital, Oxford, United Kingdom. Intracerebral injections were performed on 6- to 10-week-old mice, matched for age and sex within each experiment. These was no difference in experimental outcome between males and females or across the age range used.

* Corresponding author. Phone: 1865-221784 Fax: 1865-222502.
† Present address: Department of Clinical Neuroscience, Charing Cross and Westminster Medical School, London W6 8RF, United Kingdom.
‡ Present address: Department of Immunology, St. Mary's Hospital, London W2 1PG, United Kingdom.

Viruses. Influenza virus A/NT/60/68 was obtained from A. R. Douglas (National Institute for Medical Research, London, United Kingdom) and was grown in 10- to 12-day-old embryonated hen eggs. The virus titer in infectious allantoic fluid was measured by agglutination of chicken erythrocytes and by plaque assay on MDCK cells (1). The same stock allantoic fluid was used for all experiments, with aliquots kept at −80°C and thawed only once before being discarded. The standard 0.5-μl inoculum of neat infectious allantoic fluid contained 5 hemagglutination units (HAU), 5×10^4 PFU, and 5×10^6 electron microscopic viral particles.

Virus administration. Intracerebral injections were performed with the mice immobilized in a stereotactic frame with the tooth bar at a level 3 mm below the interaural line. The injection coordinates relative to bregma, confirmed by histological inspection, were posterior 0.5 mm, lateral 0.8 mm, and depth 1.7 mm for ventricular injections and anterior 1.0 mm, lateral 2.25 mm, and depth 2.5 mm for parenchymal injections. Mice were anesthetized for the procedure with Hypnorm (fentanyl/fluasinone; Janssen Pharmaceuticals, Wantage, United Kingdom) and Hypnovel (midazolam; Roche, Welwyn Garden City, United Kingdom). A hole was made in the skull by using a dentist's drill, and the virus suspension was injected slowly by using a 26S-gauge positive-displacement Hamilton syringe. The skin was sutured after a slow withdrawal of the needle, and all mice made an uneventful recovery. Intracerebral A/NT/60/68 infection in either site was not associated with significant clinical illness. Intranasal influenza virus was administered under brief ether anesthesia in a volume of 30 μl.

Antibody assays. Serum and CSF anti-A/NT/60/68 antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). Influenza virus was concentrated from infectious allantoic fluid by sucrose density gradient centrifugation (1) and coated overnight (400 ng/well) at 4°C onto 96-well Nunc Polysorb immunoplates (Life Technologies, Paisley, United Kingdom) in sodium bicarbonate (50 mM, pH 9.7)-sarcosyl (0.025%) buffer. Coated plates were washed four times with 0.05% Tween in double-distilled H_2O, and nonspecific protein binding was blocked by incubation for 20 min with 2% bovine serum albumin in phosphate-buffered saline–Tween (0.05%)–EDTA (1 mM). After two further washes, duplicate fivefold serum dilutions (100 μl/well) were incubated for 1 h at room temperature followed by five washes. All serum dilutions started at 1/30 and all CSF dilutions started at 1/200 unless otherwise stated. The secondary antibody conjugate was then incubated for 1 h at room temperature followed by a further five washes. The secondary antibodies used were as follows: total immunoglobulin G (IgG), horseradish peroxidase (HRP)-coupled goat anti-mouse Fcγ (Sigma Chemical Co., Poole, United Kingdom); IgM, HRP-coupled goat anti-mouse Fcμ (Sigma); and IgA, HRP-coupled goat anti-mouse Fcα (Sigma). *o*-Phenylenediamine dihydrochloride (1 mg/ml; Sigma) in citrate-phosphate buffer (0.1 M, pH 5.5) was used as the HRP substrate, the reaction was terminated with 3 M HCl, and the optical density (OD) was read at 492 nm. Standard immune and naive sera were included on each plate to allow comparison on a plot of OD versus $\log_{10}$ (concentration). Titers were calculated relative to the immune standard for an equivalent OD reading, with this standard assigned an arbitary number of titer units for each immunoglobulin class, chosen such that the background OD with 1/30 naive mouse serum was equivalent to a titer of 1 U. Log_{10} titers were compared statistically by paired *t* test.

Neutralizing antiviral antibodies were measured by hemagglutination inhibition (HAI) assay (1). Twofold triplicate serum dilutions were made from an initial 1/5 concentration and were incubated for 30 min at room temperature with 5 HAU of A/NT/60/68 virus. Chicken erythrocytes (Serotec, Kidlington, United Kingdom) were then added (0.5%, final concentration), and the presence or absence of agglutination was scored after a further 30 min. The HAI titer was taken as the reciprocal of the last dilution inhibiting erythrocyte agglutination by the virus in at least two of three wells.

Cytotoxic T-lymphocyte (CTL) restimulation. Spleens were aseptically removed, pooled from pairs of mice, and disrupted into single-cell suspensions. Naive syngeneic feeder spleen cells were incubated for 1 h at 37°C with 200 HAU of influenza virus A/NT/60/68 per 10^6 cells in RPMI (Life Technologies), irradiated (20 Gy), and washed twice in RPMI supplemented with 50 μM 2-mercaptoethanol, 60 μg of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, and 10% fetal calf serum (FCS; Globepharm, Esher, United Kingdom) (complete medium). Responder spleen cells (10^6/ml) and feeder spleen cells (3×10^5/ml) were cultured for 5 days at 37°C with 5% CO_2 in 15 ml of complete medium before testing of cytotoxicity (24).

CTL assay. EL-4 target cells (*H-2*^{b}) were labelled with ^{51}Cr (100 μCi; Amersham International, Amersham, United Kingdom) for 1 h at 37°C, washed once, and then incubated with medium alone, pulsed with the *H-2D*^{b}-restricted immunodominant influenza virus nucleoprotein peptide (1 μM ASNENMDAM, kindly provided by N. Groome, Oxford Brookes University, Oxford, United Kingdom) (25), or infected with A/NT/60/68 virus (500 HAU/10^6 cells). After 1 h at 37°C, the targets were washed twice in complete medium and incubated (10^4 EL-4 cells/well) with effector cells for 4 h before harvesting of supernatants for scintillation counting. Targets with effectors were measured in duplicate, and target controls with Triton or medium alone were measured in quadruplicate. The percent specific lysis was calculated as 100 × (release by CTL − release by targets alone)/(release by Triton − release by targets alone). The ^{51}Cr released with medium alone was 5 to 15% of that released by Triton.

Proliferation assay. Lymph nodes were aseptically removed, pooled from pairs of mice, and disrupted into single-cell suspensions. Lymph node cells (2×10^5) were mixed with irradiated syngeneic feeder spleen cells (5×10^5) in quadruplicate 150-μl cultures. Feeder cells were either unpulsed, infected with influenza virus A/NT/60/68 as for CTL restimulation, or mixed with phytohemagglutinin (PHA) at a final concentration of 10 μg/ml (Sigma). The response to PHA gave an indication of the total number of potentially responsive cells; 10 μg/ml elicits an optimal mitogenic response in this assay system. Culture conditions were as for CTL restimulation (above) except that 1% naive homologous mouse serum was used in place of 10% FCS. After 3 days, 1 μCi of [^{3}H]thymidine (Amersham International) was added to each well, and the cells were harvested for scintillation counting of incorporated label 18 h later.

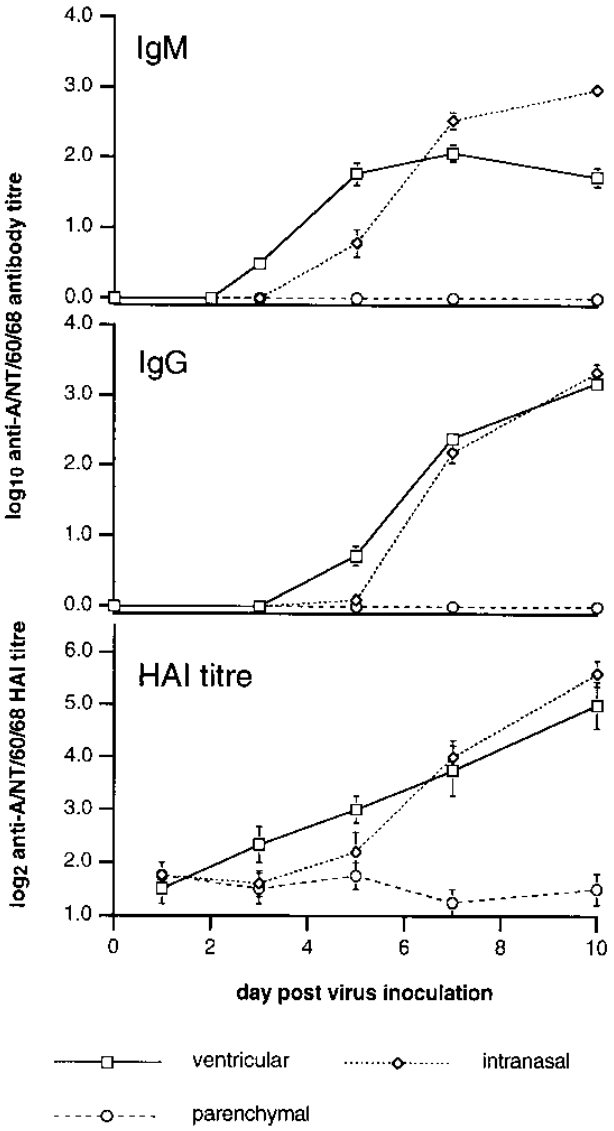

FIG. 1. Early serum anti-A/NT/60/68 antibody responses after intranasal, parenchymal, or ventricular virus inoculation. Intranasal or ventricular but not parenchymal virus inoculations led to the production of specific serum antibody. Means and standard errors of 12 mice per time point from four trials are shown for IgG and IgM titers and for 6 mice per time point from two trials for HAI titers. Log_2 (titer) = 1.6 is the lower limit of HAI assay sensitivity.

Immunohistochemistry. Brains were dissected free of the cranium and outer meninges and quickly frozen in liquid nitrogen. Cryostat sections (7 to 10 μm) were air dried and fixed in acetone for 10 min before staining. All subsequent steps were carried out at room temperature. The sections were preincubated in 10% goat serum and incubated for 1 h with an anti-influenza virus ribonucleoprotein rabbit serum (20), kindly provided by C. Scholtissek (Department of Virology, Justus Liebig University, Giessen, Germany). An HRP-conjugated goat anti-rabbit serum (Vector Laboratories, Peterborough, United Kingdom) was used as the secondary antibody. The sections were washed three times in phosphate-buffered saline with 1% FCS after each incubation. Endogenous

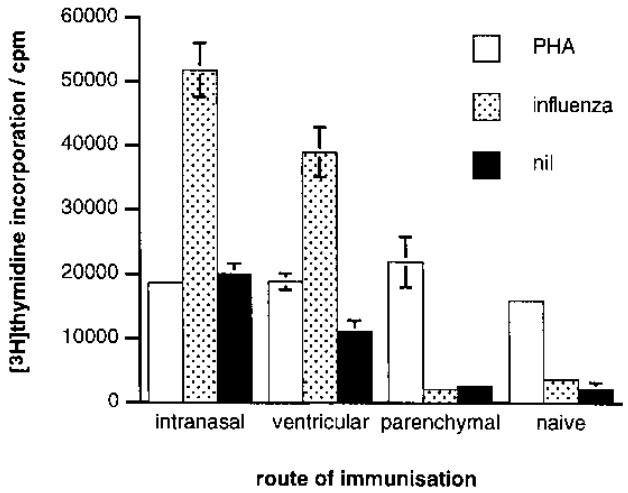

FIG. 2. Proliferative response of cells from the draining lymph nodes 10 days after intranasal, parenchymal, or ventricular virus inoculation. Deep cervical lymph nodes were taken from mice given parenchymal and ventricular virus, mediastinal lymph nodes were taken from mice given intranasal virus, and both sets of lymph nodes (pooled) were taken from naive mice. Cells were pooled from pairs of mice, and each bar shows the mean and standard error of three pairs.

peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol after incubation with the primary antibody. Diaminobenzidine (Sigma) was used as the HRP substrate, sections were counterstained with hematoxylin (Sigma), and slides were mounted with DPX (Merck Ltd., Poole, United Kingdom).

RESULTS

Early antibody response to virus in different sites. Mice were infected with 5 HAU of influenza virus A/NT/60/68 in either the left lateral cerebral ventricle (CSF), the left anterior caudoputamen (brain parenchyma), or the lung. The efficacy of immune priming by each route was determined over the following 10 days by ELISA measurement of total antiviral IgG and IgM and HAI assay of neutralizing antiviral antibody (Fig. 1). Ten days after intranasal or ventricular A/NT/60/68 infection, virus-specific IgG was detectable at a serum dilution of $1/10^5$; 10 days after parenchymal infection, virus-specific IgG was undetectable at a serum dilution of 1/2.

Early cellular response to virus in different sites. The presence of a virus-specific immune response in the draining lymph nodes was determined 10 days after inoculation by proliferation assay. Virus-specific proliferation was evident with intranasal or ventricular but not with parenchymal virus infections, despite equivalent responses to PHA stimulation (Fig. 2). The high spontaneous proliferation rates seen with mice given intranasal or ventricular virus were probably due to the continued division of cells activated in vivo and to the presence in the responder population of cells presenting viral antigens acquired in vivo. This proliferation provided a further indication of immune activation.

The same mice were tested for the presence of primed CTL precusors 10 days after virus inoculation by restimulating spleen cells in vitro with A/NT/60/68-infected feeder cells prior to cytotoxicity assay (Fig. 3). These results again correlated with the serum antibody titers; virus-specific CTL were primed by intranasal or ventricular infections but not by parenchymal infection. Virus-specific CTL precursors were also detected in the deep cervical lymph nodes of mice given ventricular but not parenchymal A/NT/60/68 (not shown). In six separate trials, there was a consistent agreement between the different immunological parameters; intranasal or ventricular virus always induced specific antibody in the serum, a proliferative response in the lymph nodes, and CTL precursors in the spleen, whereas parenchymal virus failed to induce a response by any of these criteria.

Timing of the immune response to ventricular virus infection. Although ventricular virus infection did not elicit a greater antibody response than intranasal infection, specific serum IgG and IgM were both detectable sooner after virus inoculation into the CSF (Fig. 1). The antiviral antibody response to ventricular influenza virus infection exceeded that to intranasal infection at day 3 ($P < 0.0001$ for IgM) and day 5 ($P < 0.001$ for IgM and $P < 0.001$ for IgG) after virus inoculation.

To determine whether the sensitization of antigen-specific cells in the draining lymph nodes was also more rapid after infection in the CSF, the proliferative responses to ventricular and intranasal virus infections were followed with time after inoculation (Fig. 4). The response to ventricular virus reached high levels by day 3, 2 days earlier than the response to intra-

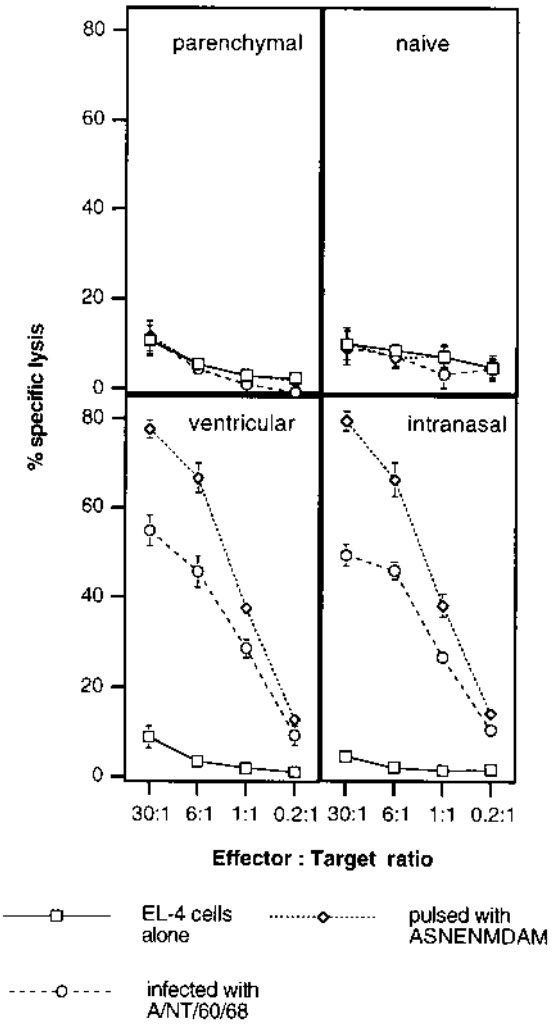

FIG. 3. Cytotoxic activity of restimulated spleen cells 10 days after intranasal, parenchymal, or ventricular virus inoculation. Spleen cells pooled from pairs of mice were restimulated in vitro as described in Materials and Methods before testing of cytotoxicity against EL-4 ($H\text{-}2^b$) target cells. Means and standard errors of three pairs of mice are shown in each case. ASNENMDAM is the $H\text{-}2D^b$-restricted immunodominant influenza virus A/NT/60/68 nucleoprotein epitope.

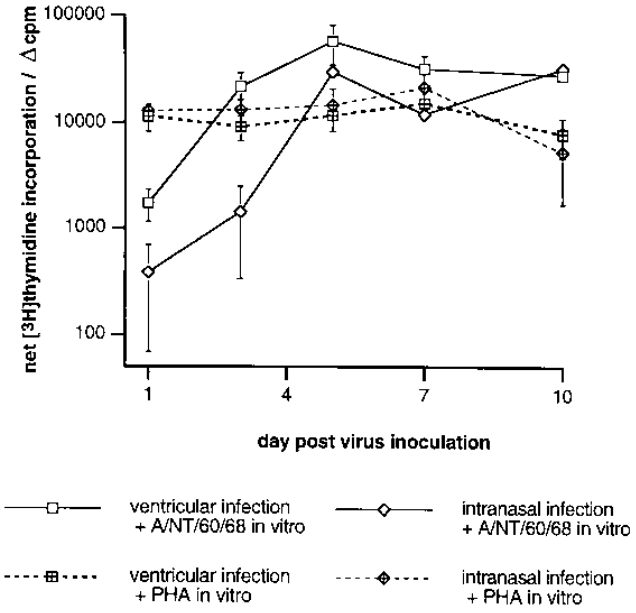

FIG. 4. Time course of proliferative responses from the draining lymph nodes after intranasal or ventricular virus inoculation. Means and standard errors of three trials are shown, each trial including cells pooled from a pair of mice for each time point and for each route of immunization. Δcpm = cpm with the stimulus indicated − cpm with unpulsed feeders. The [^{3}H]thymidine incorporation with unpulsed feeders in these trials did not exceed 1,000 cpm at days 1 to 3, 5,000 cpm at days 5 to 7, or 10,000 cpm at day 10. The response to PHA provided an indicator of the total number of potentially responsive cells in the assay regardless of antigen specificity. For day 1 ventricular and day 1 to 3 intranasal immunizations, the proliferative response with A/NT/60/68-infected feeders was less than three times the response with uninfected feeders and was not considered to be significant. The anti-influenza virus response after parenchymal virus infection did not exceed Δcpm = 500 at any time point (not shown).

nasal virus, corresponding to the earlier onset of serum antibody production after ventricular virus inoculation. Primed virus-specific splenic CTL precursors, however, were detectable at the same time after ventricular or intranasal immunizations (Fig. 5).

Late immune response to intracerebral virus. Immunohistochemistry showed that there was widespread infection of the ependymal cells 1 day after ventricular virus inoculation, but after 10 days, considerable destruction of the ependymal cells had taken place and antiviral staining was minimal (Fig. 6). Virus-infected cells were visible at both 1 and 10 days after parenchymal virus inoculation (Fig. 6) but not in either site after 1 month, when the viral nucleoprotein gene was also undetectable by PCR amplification from total brain cDNA (not shown). Since the nonreplicating virus was apparently lost from the brain by 1 month after inoculation, virus-specific serum antibody was measured by ELISA over 3 months to detect delayed immune responses. Spleen cells from the same mice were restimulated in vitro 3 months after inoculation to detect late CTL priming (Fig. 7). In 7 of 13 (54%) mice given parenchymal virus, there was no detectable antibody at any time. In the remaining 6 (46%) of the 13 mice no antibody was detectable after 10 days but low levels were found from 1 to 3 months, typically maximal at 1 month followed by a slow decline. The presence of primed CTL precursors in the spleen 3 months after parenchymal virus inoculation correlated with the presence of specific serum IgG; mice either had low levels of both or were negative for both. No mice given parenchymal virus had detectable anti-A/NT/60/68 HAI antibody at any time (not shown).

Virus-specific antibody in the CSF. Ventricular infection with influenza virus A/NT/60/68, as with large-volume intracerebral inoculations of other viruses (8, 10), led to a local production of virus-specific immunoglobulin in the CSF (Table 1). Low levels of A/NT/60/68-specific IgG were present in the CSF 10 days after both ventricular and intranasal immunizations, with similar CSF/serum specific IgG ratios, suggesting that the IgG in the CSF was largely derived from that in the serum. But whereas mice given intranasal virus had moderate serum antiviral IgA levels and undetectable CSF levels, mice given ventricular virus had low serum antiviral IgA levels and high CSF levels, consistent with a local synthesis of virus-specific IgA in the CSF. Serum antiviral IgA reached higher levels 90 days after ventricular virus inoculation, accompanied by some diminution in CSF levels. Antiviral IgG or IgA was not detected in the CSF of mice given parenchymal A/NT/60/68 at any time.

DISCUSSION

Viral immunogenicity in the CSF and the brain parenchyma. Large-volume (5- or 30-μl) virus inoculations into the brain parenchyma elicited antibody, proliferation, and cytotoxicity responses equivalent to those elicited by ventricular inoculations (not shown), consistent with a leakage of virus from

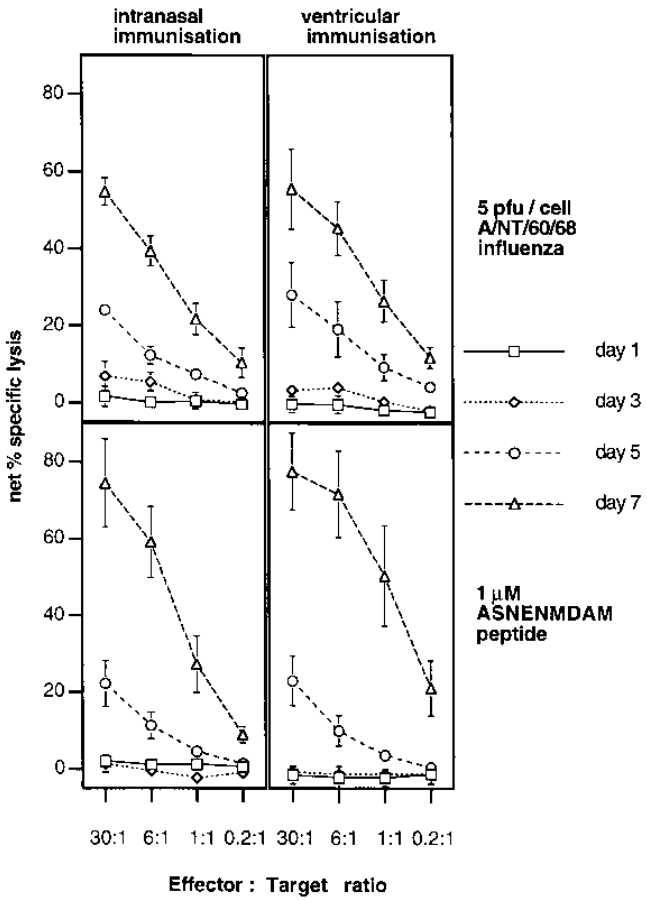

FIG. 5. Cytotoxic activity of restimulated spleen cells with time after intranasal or ventricular virus inoculation. Means and standard errors of three trials are shown, each trial including cells from a pooled pair of mice for each time point and for each route of immunization. Net % specific lysis = % specific lysis of indicated target − % specific lysis of unpulsed targets. Specific lysis of EL-4 cells alone did not exceed 15% at any time point. Net specific lysis after parenchymal virus infection did not exceed 4% at any time point (not shown).

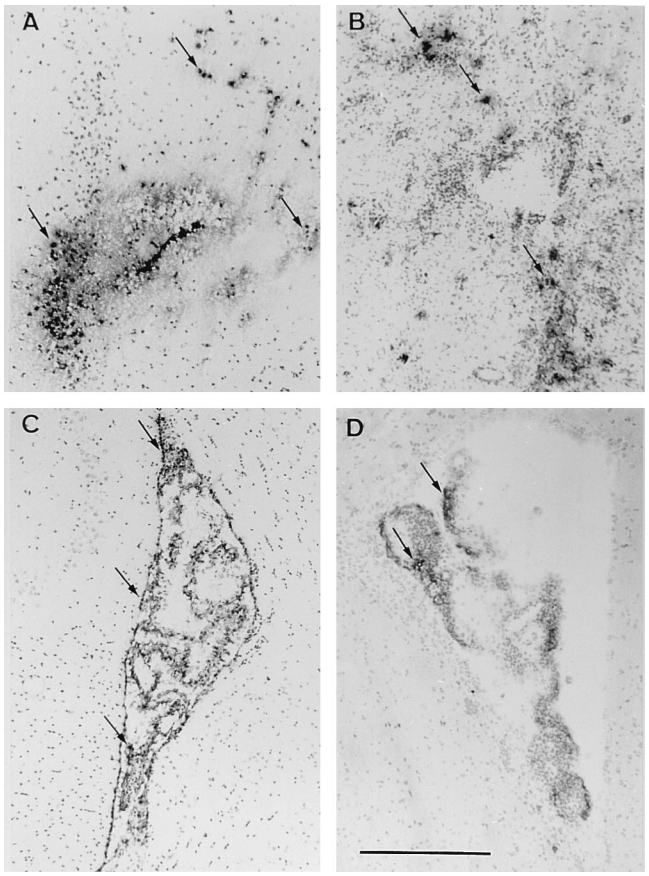

FIG. 6. Intracerebral virus infection 1 and 10 days after parenchymal or ventricular virus inoculation. Anti-influenza virus ribonucleoprotein staining is shown, either 1 day (A and C) or 10 days (B and D) after inoculation of 5 HAU of A/NT/60/68 virus into either the anterior caudoputamen (A and B) or the lateral cerebral ventricle (C and D). Arrows indicate examples of infected cells. After parenchymal virus inoculation, infected intracerebral cells were seen after both 1 day (A) and 10 days (B). After ventricular virus inoculation, almost all the ependymal cells had been infected after 1 day (C), but after 10 days, the virus-infected ependymal lining had largely disappeared. The bar in panel D represents 500 μm for each panel.

the parenchyma into the CSF. Small-volume (0.5-μl) virus inoculations remained confined to the site of inoculation and showed a clear difference in immunogenicity between the lateral cerebral ventricle, where infection primed the immune system to a degree comparable with intranasal immunization, and the brain parenchyma, where infection led only to inconsistent, delayed, low-level responses. Apart from the relative rapidity of the response to ventricular virus infection, there were surprisingly few differences between the immune responses to virus in the CSF and to virus in the lung. The local intracerebral immunoglobulin response to influenza virus infection involved IgA production in the CSF (Table 1), analogous to the mucosal IgA response seen with intranasal virus infection (13).

The fate of influenza virus antigens in the CSF. The deep cervical lymph nodes are the main site of an immune response to antigens in the intracranial CSF (11, 15). Although antigens injected intracerebrally have also been associated with immune responses being initiated in the spleen (26, 28), without the use of small-volume and hence low-pressure injections, direct leakage into the bloodstream by nonphysiological routes cannot be ruled out (4, 16). Small-volume inoculations of influenza virus A/NT/60/68 into the CSF led first to the sensitization of virus-specific cells in the draining lymph nodes (Fig. 4) and then to the appearance of virus-specific antibody in the serum (Fig. 1) and CTL precursors in the spleen (Fig. 5). This was consistent with a primary role for the cervical lymph nodes in the immune response. As with Sindbis virus encephalitis (26), synthesis of virus-specific antibody in the CSF was a relatively late event; A/NT/60/68-specific antibody was not detectable in the CSF 7 days after ventricular virus inoculation (not shown), when specific antibody was abundant in the serum (Fig. 1).

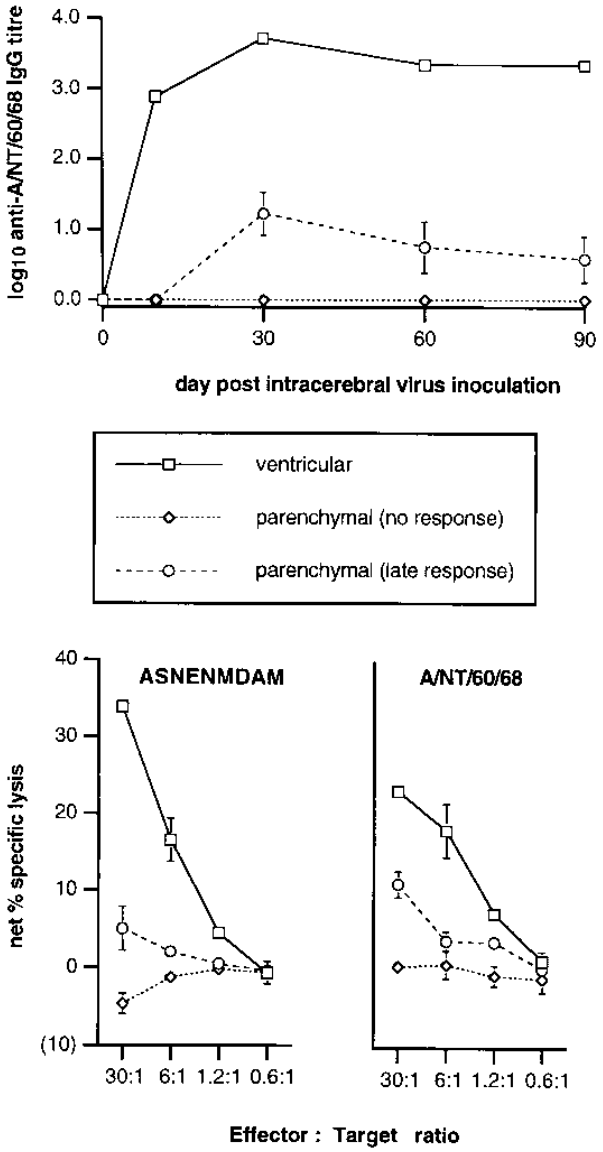

FIG. 7. Late immune response after parenchymal or ventricular virus inoculation. The upper diagram shows A/NT/60/68-specific serum IgG responses over 3 months following virus inoculation, with means and standard errors of six to seven mice per arm pooled from two trials. The mice given parenchymal virus are shown as two separate groups, those which developed late antibody and those which did not. The lower diagram shows the cytotoxic activity of spleen cells from the mice in one representative trial, restimulated in vitro with A/NT/60/68-infected syngeneic spleen cells 3 months after intracerebral virus inoculation. Net % specific lysis = % specific lysis of indicated target − % specific lysis of EL-4 cells alone. The specific lysis of EL-4 cells alone did not exceed 15%. Spleen cells from each mouse were restimulated and tested separately; means and standard errors of three mice in each arm are shown.

TABLE 1. Comparison of serum and CSF A/NT/60/68-specific antibody titers at 10 and 90 days after virus inoculation

Route of immunization	Source of antibody	Titer[a]			
		Anti-A/NT/60/68 IgG		Anti-A/NT/60/68 IgA	
		Day 10	Day 90	Day 10	Day 90
Ventricular	Serum	3.32 ± 0.08	3.47 ± 0.15	0.68 ± 0.14	2.69 ± 0.10
	CSF	1.49 ± 0.22	1.91 ± 0.08	2.63 ± 0.19	1.96 ± 0.19
Intranasal	Serum	3.08 ± 0.19	ND	1.96 ± 0.13	ND
	CSF	1.41 ± 0.26	ND	0.00 ± 0.00	ND
Parenchymal	Serum	0.00 ± 0.00	0.48 ± 0.23	0.00 ± 0.00	0.07 ± 0.07
	CSF	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a] Mean ± standard error $\log_{10}$ anti-A/NT/60/68 antibody titer from 12 to 15 mice. For each route of immunization, the same mice were used for serum and CSF titers. The results for day 90 parenchymal inoculation were pooled from all such mice, the majority of which had no detectable antiviral antibody. ND, not done.

However, we were unable to demonstrate directly viral antigens in the deep cervical lymph nodes after inoculation into the CSF. The viral nucleoprotein gene was undetectable by PCR amplification from cervical lymph node cDNA 1 to 3 days after ventricular virus inoculation, despite being readily amplified from whole brain cDNA at the same time (not shown). Immunohistochemistry also failed to detect the viral nucleoprotein in the deep cervical lymph nodes (not shown), despite its presence in the brain (Fig. 6). These negative results suggested that the antigen reaching the draining lymph nodes either was only a small proportion of the initial inoculum or was present in a different form to the infected cells in the brain. Indirect evidence of immunogenic viral antigens reaching the draining lymph nodes soon after ventricular inoculation was provided by the antiviral proliferative responses (Fig. 4).

The more rapid response to ventricular than to intranasal virus infection was probably due to viral antigens reaching the draining lymphatics directly from CSF (2), whereas intranasal influenza virus first had to cross the respiratory epithelium. A direct entry into lymphatics, avoiding degradation by tissue macrophages and proteases, could also explain the increased intracerebral immunogenicity of a variety of inert antigens (9, 11, 17, 18, 28). An exact quantitative comparison between intranasal and intracerebral virus infections was not possible, since influenza virus A/NT/60/68 replicated productively in the mouse lung (not shown) but not in the mouse brain (21).

After ventricular or larger-volume virus inoculations, influenza virus-specific proliferation was frequently seen (six of nine pairs of mice tested) with cells from the abdominal paraaortic lymph nodes and less often (two of nine pairs of mice tested) with cells from the mediastinal lymph nodes (not shown). These responses were never present without a response in the deep cervical lymph nodes and suggested that virus in the lateral cerebral ventricle may also be carried by CSF flow to more caudal sites with separate lymphatic drainage pathways.

The fate of influenza virus antigens in the brain parenchyma. The simplest interpretation of the lack of response to parenchymal virus infection was that antigens failed to reach the draining lymph nodes. Protein antigens drain efficiently from the brain parenchyma to the cervical lymph nodes (6) and initiate immune responses (9). Influenza virions, however, bind to the abundant sialic acid on cell surfaces and were probably endocytosed before leaving the extracellular spaces of the brain parenchyma. Without virus-infected dendritic cells reaching the lymph nodes, the intracellular viral antigens remained immunologically unseen.

The late low-grade immune responses to parenchymal influenza virus may have resulted from the eventual breakdown of infected cells (when virus was no longer detectable by immunohistochemistry), with viral protein debris being released into the extracellular spaces. Since a late immune response was detected in fewer than half of the mice tested, such debris may often have been degraded before leaving the brain. Nevertheless, persistent immune evasion by intracerebral viruses, whether natural pathogens or used as vehicles for gene therapy, probably depends upon the infected cells not undergoing apoptosis.

Implications for intracerebral virus infection. Although viruses often replicate in extracerebral sites before reaching the brain, congenital transmission, infection of circulating lymphocytes, and retrograde transport along peripheral neurons are all ways by which viruses may reach the brain without first initiating an immune response. These viruses could then remain in the brain parenchyma without priming the immune system. In addition, viruses are able to vary their gene expression according to the cell type infected, and viral proteins expressed specifically in the brain would be unrecognized by the immune system. When immune privilege does contribute to viral persistence, specific extracerebral immune priming might be one way of eliminating infection from the central nervous system.

ACKNOWLEDGMENTS

P. G. Stevenson is a Medical Research Council clinical training fellow. This work was supported by grants from the Medical Research Council (United Kingdom) and the Multiple Sclerosis Society of Great Britain and Ireland.

Thanks are due to A. R. Douglas for advice on growth, purification, and titration of influenza viruses.

REFERENCES

1. **Barrett, T., and S. C. Inglis.** 1985. Growth and purification of influenza virus, p. 119–150. *In* B. W. J. Mahy (ed.), Virology—a practical approach. IRL Press, Oxford, England.
2. **Bradbury, M. W. B.** 1990. Overview of passage routes of interstitial fluid to the lymphatics: history and current concepts, p. 403–412. *In* B. B. Johansson, C. Owman, and H. Widner (ed.), Pathophysiology of the blood-brain barrier. Elsevier, Oxford, England.
3. **Brent, L.** 1990. Immunologically privileged sites, p. 383–402. *In* B. B. Johansson, C. Owman, and H. Widner (ed.), Pathophysiology of the blood-brain barrier. Elsevier, Oxford, England.
4. **Cairns, H. G. F.** 1950. Intracerebral innoculation of mice: fate of the inoculum. Nature **166:**910–911.
5. **Cserr, H. F., C. Harling-Berg, T. Ichimura, P. M. Knopf, and S. Yamada.** 1990. Drainage of cerebral extracellular fluids into cervical lymph: an afferent limb in brain/immune system interactions, p. 413–420. *In* B. B. Johansson, C. Owman, and H. Widner (ed.), Pathophysiology of the blood-brain barrier. Elsevier, Oxford, England.
6. **Cserr, H. F., and P. M. Knopf.** 1992. Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. Immunol. Today **13:**507–512.
7. **Fraser, K. B., R. C. Nairn, M. G. McEntegart, and C. S. Chadwick.** 1957. Neurotropic and non-neurotropic influenza-A infection of mouse brain stud-

ied with fluorescent antibody. J. Pathol. Bacteriol. **78:**423–433.
8. **Gerhard, W., Y. Iwasaki, and H. Koprowski.** 1978. The central nervous system-associated immune response to parainfluenza type I virus in mice. J. Immunol. **120:**1256–1260.
9. **Gordon, L. B., P. M. Knopf, and H. F. Cserr.** 1992. Ovalbumin is more immunogenic when introduced into brain or cerebrospinal fluid than into extracerebral sites. J. Neuroimmunol. **40:**81–88.
10. **Griffin, D. E.** 1981. Immunoglobulins in the cerebrospinal fluid: changes during acute viral encephalitis in mice. J. Immunol. **126:**27–31.
11. **Harling-Berg, C., P. M. Knopf, J. Merriam, and H. F. Cserr.** 1989. Role of cervical lymph nodes in the systemic humoral immune response to human serum albumin microinfused into rat cerebrospinal fluid. J. Neuroimmunol. **25:**183–193.
12. **Hart, D. N. J., and J. W. Fabre.** 1981. Demonstration and characterisation of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. J. Exp. Med. **153:**347–361.
13. **Jones, P. D., and G. L. Ada.** 1986. Influenza virus-specific antibody-secreting cells in the murine lung during primary influenza virus infection. J. Virol. **60:**614–619.
14. **Kawaoka, Y.** 1991. Equine H7N7 influenza A viruses are highly pathogenic in mice without adaptation: potential use as an animal model. J. Virol. **65:**3891–3894.
15. **Lynch, F., P. C. Doherty, and R. Ceredig.** 1989. Phenotypic and functional analysis of the cellular response in regional lymphoid tissue during an acute virus infection. J. Immunol. **142:**3592–3598.
16. **Mims, C. A.** 1959. Intracerebral injections and the growth of viruses in the mouse brain. Br. J. Exp. Pathol. **41:**52–59.
17. **Panda, J. N., H. E. Dale, R. W. Loan, and L. E. Davis.** 1965. Immunologic response to subarachnoid and intracerebral injection of antigens. J. Immunol. **94:**760–764.
18. **Quirico Santos, T., and H. Valdimarsson.** 1982. T-dependent antigens are more immunogenic in the subanachnoid space than in other sites. J. Neuroimmunol. **2:**215–222.
19. **Ravenholt, R. T., and W. H. Foege.** 1982. 1918 influenza, encephalitis lethargica, parkinsonism. Lancet **ii:**860–864.
20. **Reinacher, M., J. Bonin, O. Narayan, and C. Scholtissek.** 1983. Pathogenesis of neurovirulent influenza A virus infection in mice. Lab. Invest. **49:**686–692.
21. **Schlesinger, R. W.** 1950. Incomplete growth cycle of influenza virus in mouse brain. Proc. Soc. Exp. Biol. Med. **74:**541–548.
22. **Sloan, D. J., M. J. Wood, and H. M. Charlton.** 1991. The immune response to intracerebral neural grafts. Trends Neurosci. **14:**341–346.
23. **Steinman, R. M.** 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. **9:**271–296.
24. **Taylor, P. M., D. B. Thomas, and K. H. G. Mills.** 1987. In vitro culture of T cell lines and clones, p. 133–147. *In* G. G. B. Klaus (ed.), Lymphocytes—a practical approach. IRL Press, Oxford, England.
25. **Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael.** 1986. The epitopes of influenza nucleoprotein recognised by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell **44:**959–968.
26. **Tyor, W. R., and D. E. Griffin.** 1993. Virus specificity and isotype expression of intraparenchymal antibody-secreting cells during Sindbis virus encephalitis in mice. J. Neuroimmunol. **48:**37–44.
27. **Webster, R. G., W. G. Laver, G. M. Air, and G. C. Schild.** 1982. Molecular mechanisms of variation in influenza viruses. Nature **296:**115–121.
28. **Widner, H., G. Moller, and B. B. Johansson.** 1988. Immune response in deep cervical lymph nodes and spleen in the mouse after antigen deposition in different intracerebral sites. Scand. J. Immunol. **28:**563–571.
29. **Yolken, R. H., and E. F. Torrey.** 1995. Viruses, schizophrenia and bipolar disorder. Clin. Microbiol. Rev. **8:**131–145.