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Gamma Interferon Expression during Acute and Latent Nervous System Infection by Herpes Simplex Virus Type 1

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This study was initiated to evaluate a role for gamma interferon (IFN-γ) in herpes simplex virus type 1 (HSV-1) infection. At the acute stage of infection in mice, HSV-1 replication in trigeminal ganglia and brain stem tissue was modestly but consistently enhanced in mice from which IFN-γ was by ablated monoclonal antibody treatment and in mice genetically lacking the IFN-γ receptor (Rgko mice). As determined by reverse transcriptase PCR, IFN-γ and tumor necrosis factor alpha transcripts were present in trigeminal ganglia during both acute and latent HSV-1 infection. CD4$^+$ and CD8$^+$ T cells were detected initially in trigeminal ganglia at day 5 after HSV-1 inoculation, and these cells persisted for 6 months into latency. The T cells were focused around morphologically normal neurons that showed no signs of active infection, but many of which expressed HSV-1 latency-associated transcripts. Secreted IFN-γ was present up to 6 months into latency in areas of the T-cell infiltration. By 9 months into latency, both the T-cell infiltrate and IFN-γ expression had cleared, although there remained a slight increase in macrophage levels in trigeminal ganglia. In HSV-1-infected brain stem tissue, T cells and IFN-γ expression were present at 1 month but were gone by 6 months after infection. Our hypothesis is that the persistence of T cells and the sustained IFN-γ expression occur in response to an HSV-1 antigen(s) in the nervous system. This hypothesis is consistent with a new model of HSV-1 latency which suggests that limited HSV-1 antigen expression occurs during latency (M. Kosz-Vnenchak, J. Jacobson, D. M. Coen, and D. M. Knipe, J. Virol. 67:5383–5393, 1993). We speculate that prolonged secretion of IFN-γ during latency may modulate a reactivated HSV-1 infection.

A primary herpes simplex virus type 1 (HSV-1) infection begins with replication at the cutaneous site of inoculation followed by the rapid spread of virus to the corresponding sensory ganglia (4, 17, 18, 44). The host immune response curtails viral replication in ganglia and the potentially lethal spread to the brain (27, 37). A primary immune response, however, is unable to preclude establishment of latent HSV infection in ganglionic neurons. Latent viral genomes existing in a nonreplicating state can be reactivated by unknown mechanisms, and depending on conditions of local immunity, reactivation may result in recurrent skin lesions (35, 44). The only region of the genome known to be active during latency encodes a family of latency-associated transcripts (LATs) generated by alternative splicing (8, 45). To date no protein product emanating from the LAT locus has been detected in latently infected neurons even though expression of the LAT gene seems necessary for efficient reactivation of HSV in vivo (10, 16).

Recovery from acute HSV infections has been shown to depend critically on T-lymphocyte (T-cell) responses (27, 37). Several studies with the murine model using either adoptive transfer of T cells or in vivo T-cell subset depletion strategies have shown that major histocompatibility complex (MHC) class II-restricted CD4$^+$ T cells are primarily responsible for clearing HSV from the skin, whereas MHC class I-restricted CD8$^+$ cytotoxic T lymphocytes are the predominant effectors that control HSV replication in the nervous system (28, 29). It is likely that T cells limit HSV infection in the nervous system primarily by noncytolytic mechanisms (6, 26, 40), by focusing antiviral cytokines at sites of viral replication (3, 32). There are reports that gamma interferon (IFN-γ) secretion from activated T cells in different viral models controls acute viral infections (14, 21, 24, 25, 33, 34), and recently it was shown that IFN-γ secretion by T cells is critical for clearing HSV-1 skin infections (41).

We report here the results of studies initiated to determine the time course of IFN-γ expression during HSV infection in the nervous system. Most significantly, we found that a T-cell inflammatory response with IFN-γ secretion persists in trigeminal ganglia well into the latent phase of HSV infection, suggesting that IFN-γ may play some role during latency. Results from other studies reported here involving inoculation of mice deficient in IFN-γ or the IFN-γ receptor (IFN-γR) are consistent with a role for IFN-γ in limiting HSV replication at the acute stage.

MATERIALS AND METHODS

Virus stocks and inoculation of mice with HSV. HSV-1 strain F was obtained from the American Type Culture Collection, Rockville, Md. Virus stocks were prepared in CV1 monolayers and stored at −70°C. HSV titers were determined by plaque assay on CV1 or Vero cell monolayers. Adult female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were used at 6 to 8 weeks of age. Breeding pairs of wild-type mice homozygous for the null mutation in the IFN-γR gene (Rgko mice; genotype, 129Sv/Ev/IFN-γR$^{0/0}$) were obtained from Michel Aguet (Institute of Molecular Biology, University of Zurich) (15). Rgko mice were bred under specific pathogen-free (SPF) conditions at the City of Hope Vivarium. Isogenic control mice (129Sv/Ev) were purchased from Taconic Farms Inc., Germantown, N.Y. Anesthetized mice were inoculated with HSV-1 by placement of a 10-μl drop of virus containing 10^6 PFU on the scarified cornea of the left eye. Control mice were sham inoculated with phosphate-buffered saline (PBS) in exactly the same way.

Assay for infectious and latent HSV. Mice were sacrificed at the times indicated below, and trigeminal ganglia, brains, brain stems, and eyes were surgically removed. Tissues to be used for immunostaining or in situ hybridization were snap frozen in isopentane on dry ice and then stored at −70°C until cryostat sections were cut. Infectious virus in trigeminal ganglia, brain stems, and eyes was quantitated by plaque assay of cell-free homogenates using CV1 or Vero cell

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monolayers. Latent HSV in trigeminal ganglia was reactivated by explanting ganglia into culture for 2 days. Reactivated HSV in these ganglia was then detected by plating cell-free ganglionic homogenates onto Vero cell monolayers and monitoring the monolayers for up to 4 days for viral cytopathic effect.

In vivo neutralization of IFN-γ. A rat monoclonal antibody (MAb) specific for murine IFN-γ (R4-6A2; obtained from the American Type Culture Collection) (42) and a hamster MAb specific for murine IFN-γ (H22; obtained from Robert Schreiber, Washington University, St. Louis, Mo.) (38) were used for in vivo neutralization of IFN-γ. The rat MAb was prepared as an ascites tumor in nude mice and partially purified from ascites fluid by ammonium sulfate precipitation. The hamster antibody was purified by affinity chromatography on protein A-Sepharose and was shown to be endotoxin free. Mice were injected intraperitoneally (i.p.) with 1 mg of rat immunoglobulin G (IgG) or isotype control IgG in PBS just prior to inoculation with HSV-1 strain F (10^6 to 10^7 PFU), and repeat injections were given on days 1, 3, 5, and 6 after inoculation. Alternatively, mice were given a single injection of 200 μg of hamster anti-IFN-γ MAb or isotype control IgG just prior to inoculation of HSV. The hamster MAb has a half-life of 14 days in the circulation of the mouse (37a). Mice were monitored daily for clinical signs of HSV infection. Eyes, trigeminal ganglia, and brain stems were removed from mice sacrificed at the times indicated below and were stored frozen for immunostaining or were used to assay infectious HSV.

Immunostaining. Tissue sections were cut at 5 μm on a cryostat, air dried overnight, and fixed for 5 min in acetone at room temperature. Sections were then placed in airtight boxes and stored with desiccant at −70°C. Sections were removed from the freezer, air dried for 30 min, and fixed again for 5 min in acetone prior to immunoperoxidase staining. Standard immunohistochemical staining techniques using the streptavidin-biotin immunoperoxidase technique were performed with a kit (Vector Labs, Inc., Burlingame, Calif.). After hydration of sections with PBS (pH 7.4), primary rat MAbs were incubated on sections for 1 h at room temperature. After amplification (as described in the Vector kit) the antibody-antigen reaction was detected by using aminoethylcarbizole as the red chromogen, and sections were lightly counterstained with Meyer's hematoxylin. Controls with the primary antibodies omitted were used to assess the presence of background staining and endogenous peroxidase. Little or no background staining was observed by this method, and few polymorphonuclear cells with endogenous peroxidase were found in these nervous system sections. The rat anti-mouse MAbs used for staining were an IFN-γ MAb (XMG 1.2; PharMingen, San Diego, Calif.) (3); Lyt 1 (CD5), Lyt 2 (CD8), and L3T4 (CD4) (Becton Dickinson, San Jose, Calif.); and F4/80 (macrophages and activated microglia).

For experimental animals treated with systemic hamster immunoglobin, the presence of hamster antibody in nervous tissue was determined by immunoperoxidase staining. Five-micrometer frozen sections of trigeminal ganglia and brain stems were cut and fixed as described above. After rehydration with PBS, sections were incubated with rabbit anti-hamster IgG (Sigma Immunochemicals, St. Louis, Mo.) for 1 h. Amplification was achieved by use of a streptavidin-biotin immunoperoxidase kit (Vector Labs Inc.) with aminoethylcarbizole as the red-colored chromogen.

RNase protection assay. RNase protection assays for LAT transcripts were done according to the instructions in a kit from Invitrogen (Fig. 1). Briefly, a single-stranded ^{32}P-labeled probe complementary to the major LAT in the region of overlap with the 3′ end of ICP0 mRNA was produced by in vitro transcription. The 279-bp probe was designed to protect (from RNase digestion) a fragment of 182 bp when hybridized to HSV-derived LATs. An in vitro-produced LAT used to quantify the assay gave rise to a 190-bp protected fragment (the increased size was due to protected vector-derived sequences). Following hybridization of the probe to test RNA samples and digestion with RNase A, the protected fragments were resolved in a 6% sequencing gel. The gel was dried and exposed to X-ray film. Different amounts of in vitro-transcribed LAT (HSV-1 strain F) complementary to the probe, ranging from 0.1 to 5 ng, were included as a positive control.

RT-PCR. Total RNA was extracted by a modified acid guanidinium thiocyanate-phenol-chloroform RNA extraction method designed to minimize DNA contamination (39). By PCR for ICP0 DNA sequences the RNA was found to be contaminated with low levels of DNA which were subsequently eliminated by DNase I digestion. DNase I-digested RNA gave no PCR signal for ICP0 DNA sequences and was subsequently used for reverse transcriptase PCR (RT-PCR) analysis and for RNase protection assays.

For first-strand cDNA synthesis oligo(dT) primers 12 to 18 bp long were extended with avian myeloblastosis virus RT by using 1 to 2 μg of total RNA as a template in a 20-μl reaction mixture. Human placental RNase inhibitor was included in the reaction to obtain maximum yields. A 2-μl aliquot of the cDNA was then used for PCR with primers specific for the gene of interest. Fifty-microliter PCR mixtures contained, in addition to cDNA template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton X-100, 200 μM each deoxynucleoside triphosphate (dNTP), 20 pmol of each primer, and 1.0 U of *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind., or Perkin-Elmer Applied Biosystems, Foster City, Calif.). All the reaction components excluding the cDNA template were assembled in a DNA-free clean room. The cDNA was added to the reaction mixture in a biosafety hood in another room. The reaction mixture was heated to 95°C for 10 min and then held at 72°C, at which time the *Taq* enzyme was added to produce a "hot start." The reaction was cycled at 55°C

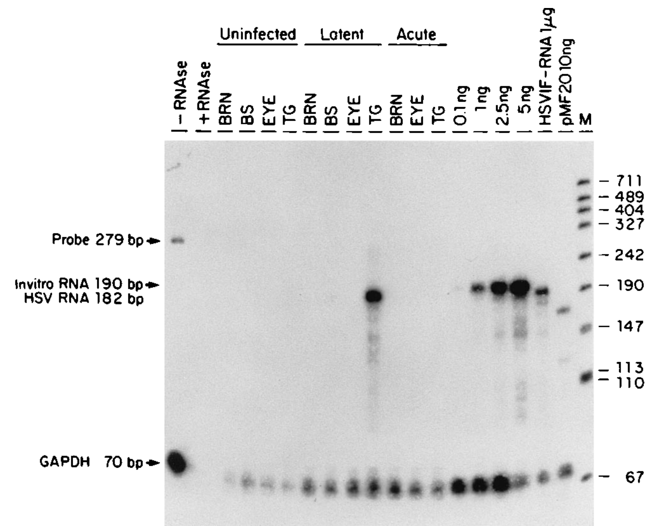

FIG. 1. Detection by RNase protection assay of LATs. Shown is an autoradiogram of RNA extracted from forebrains (BRN), brain stems (BS), eyes, and trigeminal ganglia (TG) of acute- and latent-stage HSV-infected mice and uninfected mice. −RNAse, undigested probe; +RNAse, digested probe; HSV1F-RNA 1μg, 182-bp protected fragmented obtained with total RNA extracted from HSV-1 strain F-infected CV1 cells; pMF20 10ng, protected fragment of about 160 bp obtained with in vitro-transcribed 2-kb LAT (HSV-1 KOS) RNA (the smaller protected fragment is likely due to secondary structure in the 2-kb substrate); 0.1 to 5 ng, different amounts of in vitro-transcribed LAT standard (HSV-1 F strain). The slightly increased size of the protected fragment (190 bp) with these standards is due to incorporation of vector polylinker sequences in the transcript.

for 1 min, 72°C for 2 min, and 95°C for 1 min for 35 cycles. A 10-μl aliquot of the reaction mixture was run in a 1.5% agarose gel together with a size marker, and the DNA was transferred to nylon membrane by vacuum blotting in alkaline solution. The blot was hybridized with a ^{32}P 5′-end-labeled internal probe, and after stringent washing the blot was exposed to X-ray film to visualize the specifically amplified PCR product. In some cases it was necessary to optimize the PCR, and this generally involved altering the Mg^{2+} concentration, the primer annealing temperature, and sometimes the primer and cDNA template concentrations (2). Controls that were done for the RT-PCR assay included omission of the RT step, which resulted in the failure to obtain a PCR signal (data not shown). Several reactions without added cDNA template (primer alone sample) were included in each assay series to monitor for adventitious contamination, and these were routinely negative.

Some PCR primers were designed by using the Oligo Nucleotide Selection computer program obtained from Philip Green and LaDeana Hillier (Washington University School of Medicine, St. Louis, Mo.). The PCR primers for IFN-γ, IFN-γR, tumor necrosis factor alpha (TNF-α) and interleukin 2 (IL-2) were as follows: IFN-γ antisense, 20-mer, 5′-GGACAATCTCTTCCCCACCC-3′; IFN-γ sense, 20-mer, 5′-CATGAAAATCCTGCAGAGCC-3′; IFN-γR sense, 22-mer, 5′-TACCAGAACATGTCACAGACTC-3′; IFN-γR antisense, 18-mer, 5′-AAT ACGAGGACGCAGAGC-3′; TNF-α antisense, 20-mer, 5′-TTGACCTCAGCG CTGAGTTG-3′; TNF-α sense, 20-mer, 5′-CCTGTAGCCCACGTCGTAGC-3′; IL-2 antisense, 21-mer, 5′-AGGGCTTGTTGAGATGATGCT-3′; and IL-2 sense, 21-mer, 5′-ATGTACAGCATGCAGCTCGCA-3′. The IL-2 primers and probe oligonucleotides were from Sivadasan Kanangat (University of Tennessee) (20). Probe sequences for detection of specifically amplified products of the sizes indicated were as follows: IFN-γ, 22-mer, 5′-AGCAACAGCAAGGCGAAAA AGG-3′ (304 bp); IFN-γR, 22-mer, 5′-ATTCCTGCACCAACATTTCTGA-3′ (284 bp); TNF-α, 23-mer, 5′-ATAGCAAATCGGCTGACGGTGTG-3′ (374 bp); and IL-2, 22-mer, 5′-ATTTGAAGGTGAGCATCCTGGG-3′ (500 bp). The IFN-γ and TNF-α PCR products were cloned in the Bluescript vector (Stratagene, San Diego, Calif.).

In situ hybridization. Trigeminal ganglia were snap frozen in a mixture of dry ice and isopentane and stored at −70°C. Frozen sections (5- to 6-μm thickness) were cut and mounted on poly-L-lysine-coated slides. Sections that had been lightly fixed in acetone and stored at −70°C were refixed for 24 h in fresh 4% paraformaldehyde-lysine fixative at 4°C, dehydrated through a series of graded alcohols, and stored in a sealed desiccated container at 4°C. Before hybridization, sections were lightly digested with proteinase K (25 μg/ml) to facilitate probe entry. Riboprobes were labeled with ^{35}S-UTP by in vitro transcription using T7, T3, or SP6 polymerase essentially according to the manufacturer's recommendation (Ambion Inc., Austin, Tex.). The probe size was reduced to an

average of 100 to 200 bp by hydrolysis with a freshly prepared alkaline solution composed of 10 mM dithiothreitol, 80 mM $NaCO_3$, and 120 mM $NaHCO_3$. The digested probe was passed through a spin column (Bio-Rad Laboratories, Richmond, Calif.) to remove small digestion products and stored in 10 mM dithiothreitol at $-20°C$.

In situ hybridization was carried out as described by Deatly and collegues (5). Briefly, sections were hybridized at 50°C in a sealed humidified box with 2×10^5 to 5×10^5 cpm of heat-denatured probe under a baked siliconized coverslip that was sealed with paraffin oil to prevent evaporation during incubation. After overnight hybridization, the sections were washed stringently to remove nonspecifically bound probe and then dehydrated through a series of alcohols, dipped in NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.), and air dried thoroughly. Sections were exposed for 3 to 6 days for LAT detection. After processing with D19 developer and fixer (Eastman Kodak), the sections were counterstained lightly with hematoxylin and eosin and dehydrated through graded alcohols before being mounted with Permount (Pierce, Rockford, Ill.). Sections were photographed under bright-field illumination.

RESULTS

Cytokine expression during the acute infection and effect of IFN-γ on clearance of HSV from the nervous system. Initially brain, brain stem, trigeminal ganglion, and eye tissues were surveyed for expression of IFN-γ and other cytokines and for macrophage and T-cell surface markers by RT-PCR assays (Fig. 2) and/or the immunoperoxidase staining technique (Table 1 and Fig. 3). By RT-PCR, transcripts for IFN-γ were found to be strongly expressed in eye, ganglion, and brain stem samples taken from HSV-infected mice at the acute stage (i.e., 5 days postinfection) but to be absent from the corresponding tissues obtained from uninfected mice (Fig. 2C). IFN-γ receptor mRNAs were expressed in both uninfected and infected mouse tissues (Fig. 2D), reflecting the ubiquity of IFN-γR expression (7). TNF-α transcripts were expressed in eyes, but they were only weakly expressed in ganglia and not at all in brain stems at the acute stage (Fig. 2E). A weak IL-2 PCR signal was detected in eyes and brain stems (data not shown). Neither TNF-α (Fig. 2E) nor IL-2 (data not shown) transcripts were expressed in tissue samples from uninfected mice. Immunoperoxidase staining showed a mild T-cell infiltrate ($CD4^+$ and $CD8^+$) together with low levels of cell-associated IFN-γ in ganglia but not in brain stems (Table 1). No infiltrating lymphocytes were detected in ganglia from sham inoculated mice (Fig. 3g and h). Ganglia, but not brain stem samples, from HSV-inoculated mice showed weak localized immunohistochemical staining for IFN-γ, whereas ganglia from sham inoculated mice were negative for immunoreactive IFN-γ (Fig. 3i and Table 1). Eye samples were not subjected to immunostaining. Macrophages and/or activated microglia expressing the F4/80 marker antigen were slightly increased in ganglia and brain stems from HSV-inoculated mice compared with those from sham inoculated mice (Table 1). Occasionally a few cells in ganglia from sham inoculated mice were positive for the macrophage-activated microglia marker antigen, F4/80 (Table 1). These results demonstrate expression of IFN-γ in all infected tissues of the nervous system during acute HSV infection.

To ascertain whether there is a role for IFN-γ in clearing HSV in the nervous system, mice were inoculated by corneal scarification with HSV-1, and IFN-γ was ablated in vivo by treatment with two different neutralizing anti-IFN-γ MAbs. Control HSV-inoculated mice were treated with isotype control antibody (IgG). As an alternative approach to circumvent problems of incomplete IFN-γ ablation, mice with a targeted disruption of the IFN-γ receptor gene (7, 15) (Rgko mice) and isogenic control mice (strain 129Sv/Ev) were similarly inoculated with HSV. The results of these experiments are shown in Table 2. The consistent finding in these experiments was that HSV clearance was compromised in the ganglia and brain

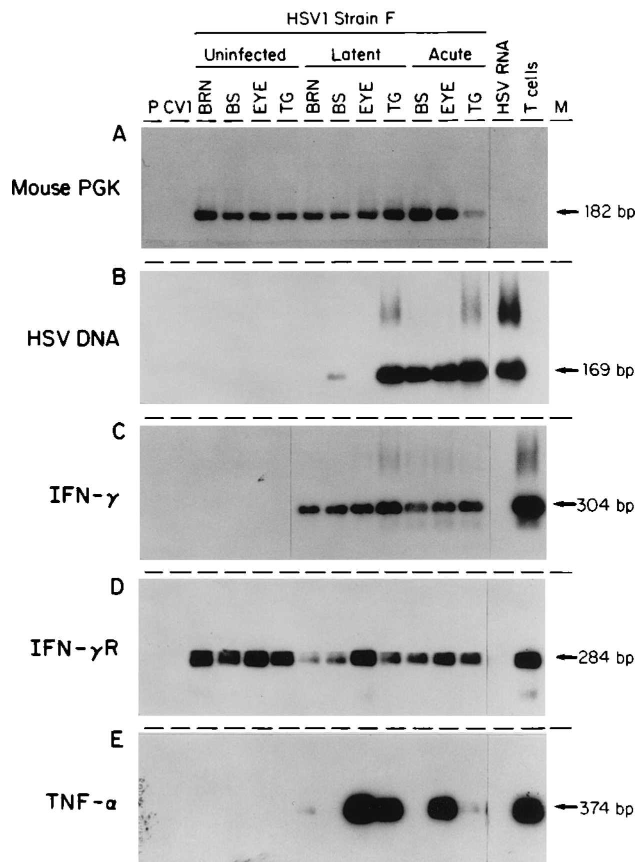

FIG. 2. Detection by RT-PCR of IFN-γ (C), IFN-γR (D), and TNF-α (E) transcripts in nervous system tissues from uninfected and HSV-infected mice during acute and latent infections. Mouse phosphoglycerate kinase transcripts (A) were amplified as internal controls. HSV ICP0 DNA sequences (B) were detected in the RNA samples used for amplification of cytokine transcripts, confirming HSV infection. Latent infection in the trigeminal ganglia and brain stems was confirmed by detection of LATs by RNase protection assay (Fig. 1). PCR products resolved in agarose gels were identified by hybridization with ^{32}P-labeled oligonucleotide probes, and the resulting autoradiogram is shown. P, primer alone, negative control; CV1, total RNA from infected cells; HSV RNA, total RNA from HSV-infected CV1 cells; T cells, total RNA from mitogen-activated T cells; M, sizes of PCR-amplified fragments; BRN, brain RNA; BS, brain stem RNA; TG, trigeminal ganglion RNA; EYE, eye RNA.

stems of mice depleted of INF-γ (experiments I and II) and in those of IFN-γR-deficient (Rgko) mice (experiment III). The greatest differences in HSV titers were generally seen with brain stems rather than ganglia. HSV titers in eyes were also determined in experiment III, but the difference in titers between Rgko and control mice was not significant, suggesting that IFN-γ effects at the site of inoculation do not account for the difference in titers seen in the nervous system (Table 2). Immunoperoxidase staining with a rabbit anti-hamster antibody revealed a diffuse staining pattern for hamster IgG encompassing the entire ganglion from BALB/c mice in experiment II given either hamster anti-IFN-γ MAb or control hamster IgG. For the brain stem, however, only weak localized perivascular staining was seen for the hamster antibodies, indicating an intact blood-brain barrier at 3 days after HSV inoculation (data not shown). The 50% infective doses for ganglionic infection were the same for Rgko and 129Sv/Ev mice. A low mortality rate (14%) was noted in experiment II for mice treated with neutralizing anti-IFN-γ hamster MAb for 6 days. No deaths were noted for Rgko mice in the short term

TABLE 1. Inflammatory cells and IFN-γ in trigeminal ganglia and brain stems of HSV-infected mice[a]

Tissue (day p.i.)[b]	No. of $CD4^+$ T cells[c]	No. of $CD8^+$ T cells[c]	No. of macrophages-microglia[c]	IFN-γ[d]
Sham infected TG (5)	Neg[e]	Neg	5–10	Neg
Sham infected BS (5)	Neg	Neg	0–5	Neg
HSV-infected TG (5)	0–10	0–10	10–30	1^+
HSV-infected BS (5)	Neg	Neg	0–10	Neg
HSV-infected TG (33)	>50	>50	>50	2^+
HSV-infected BS (33)	0–10	0–10	0–20	1^+ in areas of inflammation
HSV-infected TG (~180)	5–>50	5–>50	>50	3^+
HSV-infected BS (~180)	0–3	0–3	0–5	Neg
HSV-infected TG (~270)	0–3	0–3	10–30	Neg
HSV-infected BS (~270)	0–3	0–3	0–10	Neg

[a] Mice (BALB/c; 8 weeks old) were inoculated with HSV-1 F by corneal scarification using a dose of virus that produces virtually 100% latency. Control mice were sham (i.e., scarified) inoculated with PBS. Groups of 3 to 5 mice were sacrificed at each time point, and the trigeminal ganglia and brain stems were snap frozen in isopentane-dry ice before being transferred to a −70°C freezer. Frozen sections (5 μm thick) were cut and subjected to immunoperoxidase staining for $CD4^+$ cells, $CD8^+$ cells, macrophage-microglia marker antigens, and IFN-γ immunoreactivity.

[b] p.i., postinfection; TG, trigeminal ganglion; BS, brain stem.

[c] Results are expressed as the relative numbers of cells per square millimeter in the most affected areas.

[d] IFN-γ, IFN-γ extracellular immunoreactivity on a scale of 1^+ to 3^+. 1^+, focal weak positivity associated with sparse antigen-positive lymphocytes; 2^+, confluent areas of positivity associated with moderate numbers of antigen-positive lymphocytes; 3^+, areas of confluent positivity associated with large numbers of antigen-positive lymphocytes.

[e] Neg, no immunoreactivity.

(4 days postinfection; Table 2), but in two separate experiments using the same virus inoculum 9 (50%) of 18 Rgko mice died of encephalitis between days 11 and 12 after inoculation, compared with 2 (12%) of 17 control mice. These differences in mortality are highly significant by Fisher's exact chi-square test ($P < 0.016$). These results indicate that IFN-γ has prominent role in controlling HSV replication in the ganglion and brain stem.

IFN-γ expression in the nervous system during the latent infection. The time course of IFN-γ expression in the nervous system was determined by RT-PCR and by immunoperoxidase staining. Latent HSV infection was confirmed first, by positive HSV cultures in 100% of the explanted trigeminal ganglia tested. Latency was also confirmed by the RNase protection assay detection of LATs in RNA preparations from ganglia and brain stems used for RT-PCR (Fig. 1). Prior to, but not after, DNase I treatment of the RNA preparations, HSV ICP0 DNA sequences were detected by PCR as DNA contaminants of the RNA preparations (Fig. 2B), again confirming latent infection of the tissue. IFN-γ transcripts were highly expressed in forebrains, brain stems, ganglia, and eyes, whereas TNF-α was expressed strongly in ganglia and eyes, very weakly in forebrains, and not at all in brain stems (Fig. 2C and E). By comparison of the PCR signals obtained for IFN-γR transcripts in brains and brain stems of latently infected and uninfected mice with that obtained for mouse phosphoglycerate kinase, it appears that IFN-γR synthesis may be down-regulated in brains and brain stems of latently infected mice (Fig. 2D). IL-2 transcripts were weakly expressed in ganglia and eyes but were not detected in forebrains or brain stems (data not shown).

The immunoperoxidase technique was used to stain macrophage and T-cell markers and IFN-γ in ganglia and brain stems from 3 to 5 mice per time point up to 9 months after inoculation with HSV. The results are summarized in Table 1, and examples of the staining patterns are shown in Fig. 3. In each case the most affected areas of the ganglia on the tissue sections were counted. The most striking result was the strong and sustained chronic inflammatory response with concomitant IFN-γ immunoreactivity lasting up to 6 months in the ganglia (Fig. 3a, d, and f). Control ganglia appeared histologically normal when stained with hematoxylin and eosin, with no sign of a cellular infiltrate (Fig. 3g). The level of macrophage and T-cell ($CD4^+$ and $CD8^+$) infiltrate and the level of IFN-γ secretion reached maximal intensity at 6 months (Table 2). In all cases the T-cell infiltration was patchy and localized predominantly to the region of aggregated neuronal cell bodies; axonal regions were relatively spared (Fig. 3a). It is particularly noteworthy that IFN-γ was present extracellularly and in association with infiltrating T cells focused around neurons that showed no morphological signs of productive viral replication, such as necrosis or inclusion bodies (Fig. 3d and f), consistent with our inability to detect HSV antigens by immunoperoxidase staining (data not shown). In situ hybridization with LAT-specific probes in adjacent step sections detected LAT-expressing neurons in the same general area occupied by infiltrating T cells (Fig. 3c). The level of cellular infiltrate and the production of IFN-γ abated in the ganglia between 6 and 9 months after HSV inoculation, although there was still some increased macrophage and/or microglial activity at 9 months in trigeminal ganglia (Table 1). In contrast to the case with the ganglion, a significant T-cell infiltrate accompanied by IFN-γ secretion was seen at 33 days in the brain stem, but not thereafter, although the number of macrophages and/or activated microglia remained minimally elevated up to 9 months after HSV inoculation (Table 1).

DISCUSSION

The ablation studies whose results are shown in Table 2 demonstrate a modest (four- to fivefold) but consistent effect of IFN-γ in reducing HSV replication in ganglia and brain stems at the acute stage of the infection. In experiment III (Table 2) there was no significant difference in HSV titers in the eyes of Rgko mice and control mice (data not shown), suggesting that local effects of IFN-γ account for the slight increase in HSV titers in the nervous system of Rgko mice compared with control mice. Solely on the basis of the results presented in Table 2 it would be difficult to assert that IFN-γ is important to the outcome of HSV infection. However, results from two separate experiments give a cumulative mortality of 50% (9 of 18) for Rgko mice compared with 12% (2 of

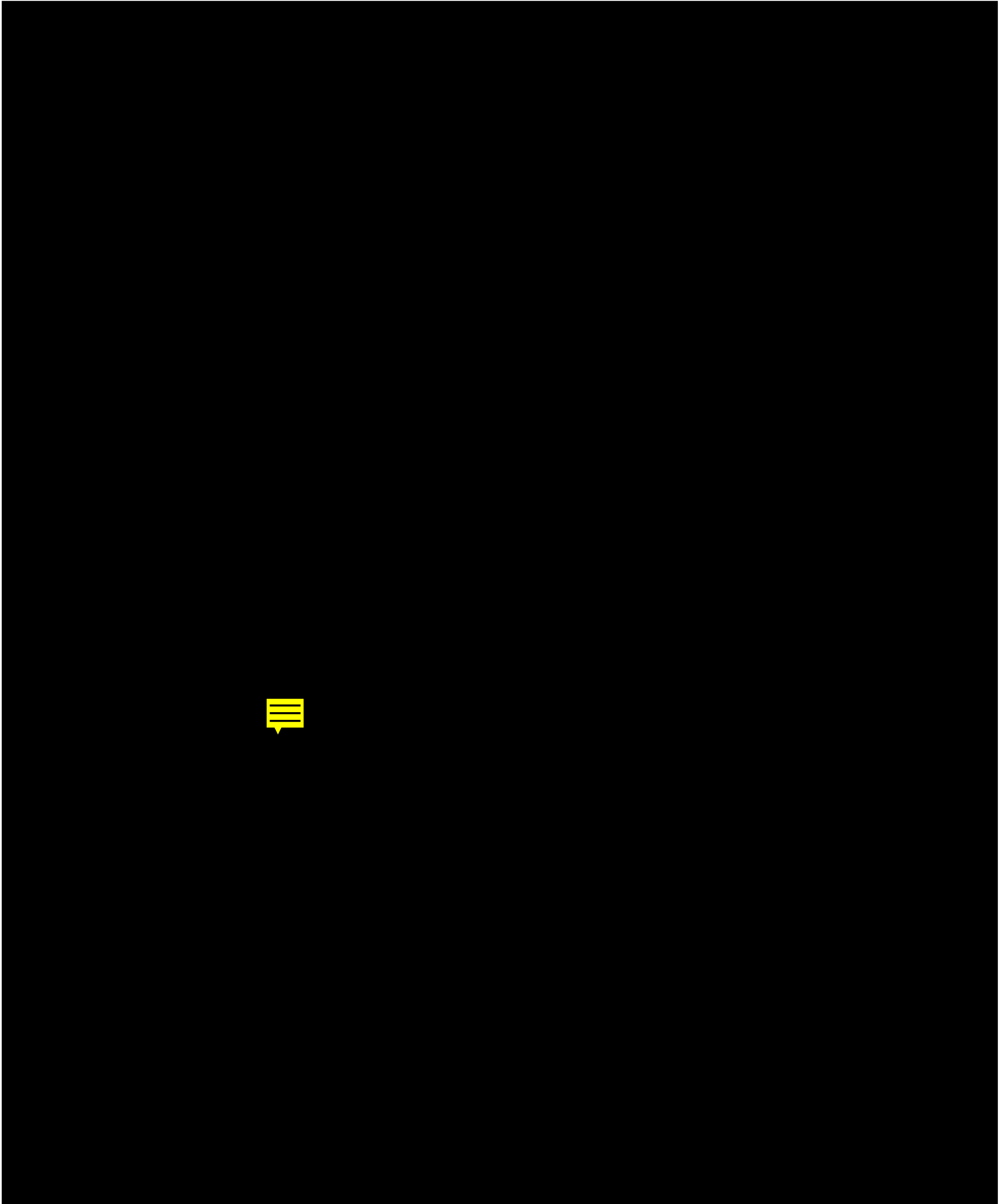

FIG. 3. Detection of T cells, IFN-γ, and HSV LATs in frozen cryostat sections of trigeminal ganglia at 6 months (a to d) and 33 days (e and f) as well as in sections of a 5-day (sham inoculated) control (g to i). Original magnifications, ×90 (a, b, and g to i) and ×360 (c to f). Immunoproxidase stains (b, d to f, h, and i) used aminoethylcarbizole as the red chromogen. (a) Hematoxylin and eosin stain shows prominent lymphoid infiltration in the ganglion between neurons (between arrows). (b) Immunoperoxidase stain for $CD4^{+}$ cells shows widespread infiltration of $CD4^{+}$ lymphocytes in the ganglion. (c) In situ hybridization for HSV LATs reveals several positive neurons with numerous silver grains over the nuclei (arrow). Note the associated lymphoid infiltrate. Since cryostat sections were used for in situ hybridization, some cellular detail has been lost. (d) Immunoperoxidase stain for IFN-γ reveals cell-associated staining in the ganglion in the region of the lymphoid infiltrate. Note the negatively stained neuron (arrow). (e) Immunoperoxidase stain for the pan-T-cell marker reveals infiltration of T cells in the ganglion. (f) Immunoperoxidase stain for IFN-γ reveals positive cells around neurons of the ganglion. (g) A control trigeminal ganglion (d5) stained with hematoxylin and eosin shows normal histology without lymphocytic infiltration. (h) A step section adjacent to that for panel g is stained with a pan-T-cell antibody. No T-cell infiltration is noted. (i) A step section adjacent to that for panel g is stained with an antibody to IFN-γ. Note the lack of immunoreactivity for IFN-γ.

FIG. 3—*Continued.*

17) for control mice, with deaths occurring from 8 to 12 days after inoculation. This difference in mortality is highly significant by Fisher's exact chi-square test ($P < 0.016$) and is unequivocal evidence that IFN-γ is important for controlling HSV replication in vivo.

The modest effects of depleting IFN-γ on HSV replication in the nervous system at the acute stage may be explained by the participation of other cytokines with IFN-γ in mediating the effects of $CD8^+$ T cells. For example, IFN-γ appears to mediate the effects of $CD4^+$ and $CD8^+$ T cells in controlling acute HSV skin infections in conjunction with other cytokines (41, 43). We detected, by RT-PCR, transcripts for TNF-α in eyes and ganglia during both the acute and latent stages of infection (Fig. 2). TNF-α has been shown to inhibit HSV replication both in vitro and in vivo (36, 47), and this activity of TNF-α has been shown to be synergistic with IFN-γ (10). In a published study of the mechanism of virus-induced immunoglobulin class shift, mention is made of an experiment in which all animals from which both TNF-α and IFN-γ were ablated died after HSV inoculation, whereas there were no deaths among HSV-inoculated mice from which either TNF-α alone or IFN-γ alone was ablated (30).

We show in the present study that chronic inflammatory cells persist in mouse trigeminal ganglia for up to 6 months after HSV inoculation. Previously, Gebhardt and Hill reported the persistence of T cells in rabbit trigeminal ganglia, but these cells were cleared much earlier (by day 45 after HSV inoculation) (11, 12). Our finding of IFN-γ expression for the first 6 months of latency (Fig. 3d) argues further for the presence of continual or intermittent antigenic stimulation during latency,

TABLE 2. Effect of IFN-γ ablation on HSV viral titer and mortality[a]

Experimental paradigm	HSV titer[b]				Mortality (%)[c]
	Trigeminal ganglia		Brain stems		
	Days 3 and 4	Day 6	Days 3 and 4	Day 6	
Expt I					
Rat anti-IFN-γ MAb (ascites), i.p. injection	ND[d]	1.71 ± 0.07	ND	1.96 ± 0.13[e]	0
Control rat IgG, i.p. injection	ND	1.69 ± 0.22	ND	1.44 ± 0.10	0
Expt II					
Hamster anti-IFN-γ MAb, i.p. injection	4.31 ± 0.07[e]	2.10 ± 0.18[f]	1.24 ± 0.21[g]	4.74 ± 0.02[h]	14
Control hamster IgG, i.p. injection	3.76 ± 0.18	1.62 ± 0.16	0.63 ± 0.13	4.40 ± 0.02	0
Expt III					
Rgko mice (IFN-γR knockout)	4.57 ± 0.04[g]	ND	3.79 ± 0.06[h]	ND	0
129Sv/Ev control (isogenic) mice	4.31 ± 0.11	ND	3.46 ± 0.08	ND	0

[a] BALB/c mice (experiments I and II) were injected with neutralizing anti-IFN-γ MAbs and immediately inoculated with HSV-1 as described in Materials and Methods. IFN-γR knockout mice and control isogenic 129Sv/Ev mice were similarly inoculated with HSV-1. Mice were sacrificed and cell-free homogenate titers were obtained on day 3 (day 4 in experiment III) and day 6.
[b] Mean HSV titer as $\log_{10}$ ± standard error of the mean for five mice per group (seven mice per group for experiment III).
[c] Mortality in experiment II was based on a group of seven mice, five of which were used for virus titers and two of which were used for immunohistochemistry. One of the latter two mice died on day 6.
[d] ND, not determined.
[e] $P < 0.01$ by one-tailed t test.
[f] $P < 0.05$ by one-tailed t test.
[g] $P < 0.025$ by one-tailed t test.
[h] $P < 0.005$ by one-tailed t test.

and the location of inflammatory cells surrounding ganglionic neurons (Fig. 3a) suggests that the antigenic stimulus emanates from neurons. These neurons show no obvious signs of viral replication, consistent with our failure to detect HSV antigens by immunoperoxidase staining. The increased T-cell infiltrate from 1 to 6 months of latency suggests that T cells traffic into ganglia well after infectious virus has been cleared.

Although autoimmunity induced by an HSV infection cannot be excluded, our working hypothesis is that the persistence of these T cells and the sustained expression of IFN-γ occur in response to HSV antigen. During latency, only one HSV transcript has been consistently detected, the LAT, but no protein corresponding to LAT has been detected (10). However, a new model of HSV latency envisions low-level expression of immediate-early (IE) HSV proteins during latency (22), and in at least one study, the ICP4 IE protein has been detected during latency (13). More recently, replication-associated transcripts for ICP4 and thymidine kinase have been detected in latently infected ganglia by quantitative RNA PCR in the absence of detectable reactivation (23). This new model (22), based on evidence that IE promoters function relatively inefficiently in ganglionic neurons compared with cultured cells, suggests that this suboptimal IE expression leads to latency and LAT expression rather than to early and late gene expression and virus replication (35, 44). It is only with limited HSV DNA replication (and/or late gene expression, e.g., VP16 expression) that IE expression is augmented to levels surpassing a critical threshold required for initiation of the normal cascade of early and late gene expression and HSV replication. Our hypothesis, consistent with this model, is that suboptimal levels of IE gene expression occur as HSV senses the fitness of the neuronal environment for reactivation and this provides the antigenic stimulus to maintain the inflammatory cell response and trigger IFN-γ secretion in ganglia.

A potential difficulty with this hypothesis is that neurons—the only cells expressing LATs and presumably the only cell type latently infected—are deficient in MHC expression (19), and such expression is necessary to present antigen to activated T cells to provoke IFN-γ secretion (7). There are several possible scenarios to explain this discrepancy. (i) HSV antigens may be synthesized by ganglion cells other than neurons, (ii) HSV antigens synthesized in neurons may be transported to surrounding satellite cells, (iii) IFN-γ secretion may be induced independently of antigen recognition by activated T cells, or (iv) MHC class I synthesis may be induced on neurons under special circumstances. Pertinent to scenario iv, expression of transcripts related to the classical MHC class I transcript has recently been demonstrated to occur in neurons during acute ganglionic infection with HSV, although expression of MHC antigen was not detected. Since an introduced MHC class I gene was shown to be expressed on the neuronal cell surface, it is possible that the MHC transcript detected encodes a nonclassical class I allele which facilitates antigen presentation to T cells (31).

The observation of prolonged secretion of IFN-γ during latency raises the possibility that IFN-γ and other cytokines modulate a reactivated HSV infection. The actual process of reactivation (i.e., the process leading to expression of the full repertoire of viral proteins and the initiation of viral replication) is probably independent of immune factors. However, once reactivation occurs, local immune factors in ganglia may be critical in determining whether reactivation is abortive in the neuron or progresses to become clinically apparent. Our hypothesis is that IFN-γ and TNF-α (or other cytokines) when present locally reduce or block HSV replication when a reactivation event occurs in ganglionic neurons (34). This hypothesis would predict a greater frequency of detectable reactivation (experimentally induced and spontaneous) in mice from which both TNF-α and IFN-γ have been ablated. Clinical studies which show that the interval between recurrent infections is greater in individuals with higher levels of IFN-γ in recurrent lesions or in in vitro-stimulated peripheral blood mononuclear cells support the contention that IFN-γ plays a role in reactivation from latency (4, 46). Additionally, there are clinical reports of a prolonged inflammatory response that lasts for years in patients following the acute stage of HSV encephalitis, and the conclusion drawn from these studies was that HSV antigen expression was ongoing in the central nervous system (1). Clearly, the studies that we have described here have parallels to events in the natural host, suggesting that future studies with the mouse model will aid in clarifying the roles of IFN-γ and other cytokines in the pathogenesis of HSV brain infections.

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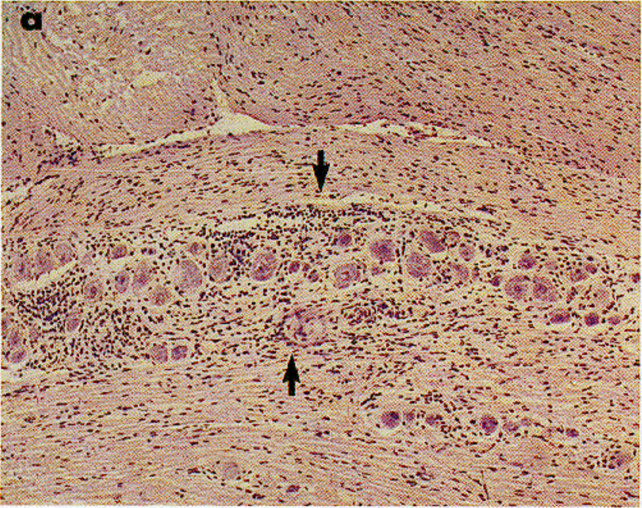
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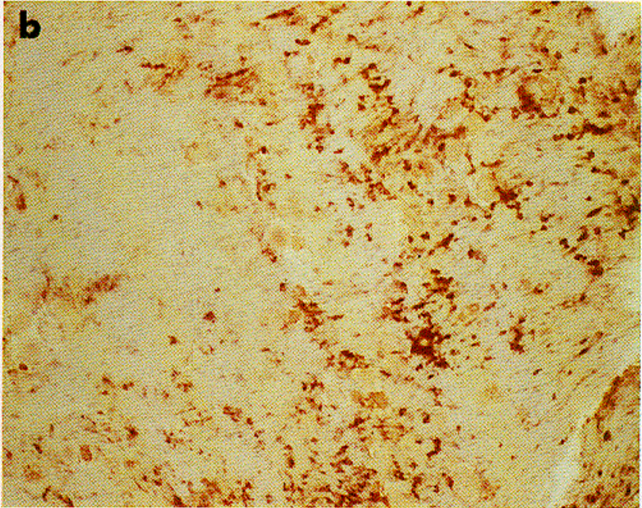
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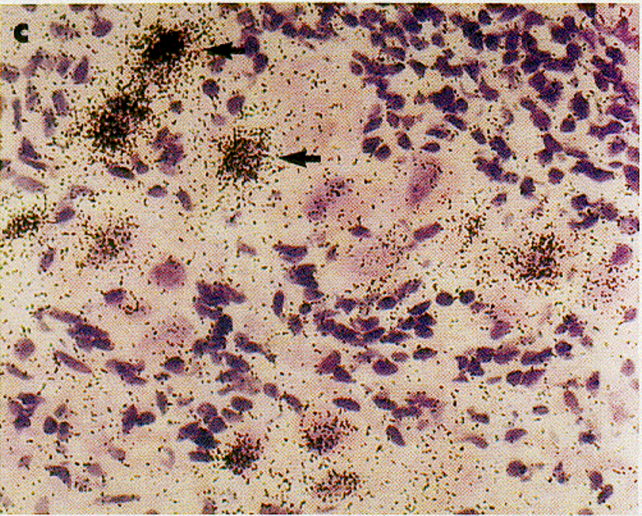
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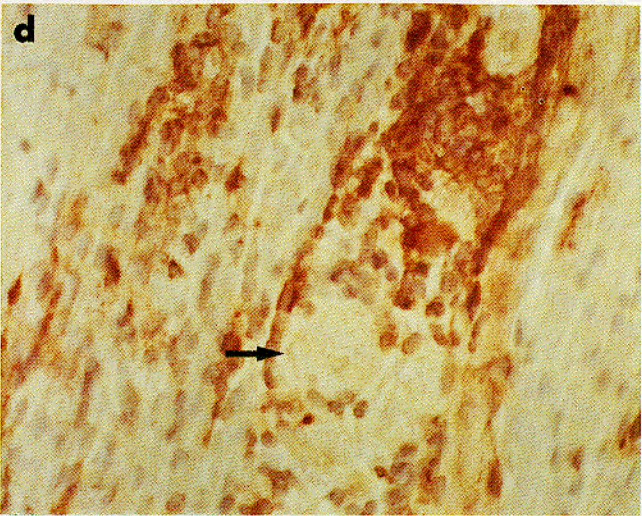
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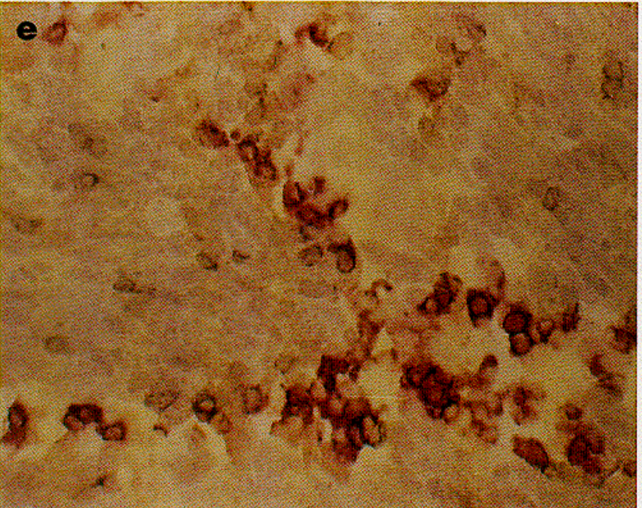
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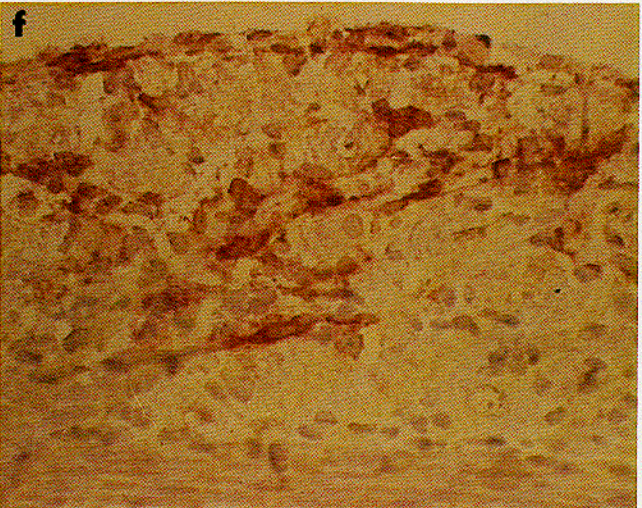
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