J. gen. Virol. (1983), **64**, 1965–1972. *Printed in Great Britain*

Key words: *HSV-1/IgG-mediated/CNS infection/blood–brain barrier*

Delayed IgG-mediated Clearance of Herpes Simplex Virus Type 1 from the CNS but Not Footpad during the Early Stages of Infection: Possible Result of Relative Integrity of the Blood–Brain Barrier

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(*Accepted 11 May 1983*)

SUMMARY

Following footpad inoculation in mice, herpes simplex virus type 1 spreads along nerves to the spinal cord where a myelitis causes hind limb paralysis beginning on day 6. Neutralizing antibody effectively prevents this illness only if given within 72 h. We therefore studied the timing of blood–brain barrier (BBB) disruption relative to the appearance of virus and inflammatory cell infiltrates in the spinal cord. Virus was detectable in dorsal root ganglion and spinal cord explants by 48 h. By 72 h, mononuclear cell infiltrates were evident in the spinal cord. By day 4, high titres of virus were demonstrable in the spinal cord. On day 6 ^{125}I-labelled IgG tracers penetrated the spinal cord BBB. In addition, using a passive transfer model, mice given neutralizing IgG completely cleared footpad virus within 72 h while brain virus titres were unaffected by IgG treatment up to day 7. These observations indicate that the BBB may prevent IgG-mediated virus clearance during the early stages of infection.

INTRODUCTION

Herpes encephalitis has been observed in patients with a previous history of cold sores and in patients proven to be seropositive before onset of encephalitis (Nahmias *et al.*, 1982; Olson *et al.*, 1967). Recurrent herpes labialis and recurrent herpes genitalis by definition occur in seropositive individuals. These facts suggest that antibody is ineffective in control of herpetic infections. However, animal models continue to document an important role for antibody in experimental herpes simplex virus (HSV) infections (McKendall *et al.*, 1979; Oakes & Rosemond-Hornbeak, 1978; Davis *et al.*, 1979; Worthington *et al.*, 1980). Most studies, including ours, have shown that antibody must be given within 48 to 72 h after peripheral inoculation of virus in order to be effective. This requirement was surprising since the clinical disease we observe in our studies does not appear until 6 days after footpad inoculation at which time a myelitis is responsible for the posterior limb paralysis which develops. Therefore, we postulated that the blood–brain barrier (BBB) may prevent IgG access to the spinal cord.

To test this postulate we performed a series of studies using both the footpad model of HSV type 1 (HSV-1) myelitis and an experimental model of HSV-1 encephalitis caused by direct intracerebral (i.c.) inoculation of virus. In the footpad model we studied the kinetics of virus spread to the spinal cord, the appearance of myelitis detected by histopathology and the integrity of the BBB detected by leakage of ^{125}I-labelled IgG into the spinal cord. Correlation of these findings indicated that leakage across the BBB did not occur until several days after virus and histopathological lesions were detectable in the spinal cord. Furthermore, systemic treatment with neutralizing IgG was effective in clearing virus from footpad inoculation sites but ineffective in clearing i.c. inoculated virus from brain tissue. These observations indicate that virus replication proceeds without restriction by antiviral IgG during the early stages of central nervous system (CNS) infection because some integrity of the BBB persists for several days after the initiation of virus infection.

0022-1317/83/0000-5609 $02.00

METHODS

Virus. HSV-1 was isolated from a patient with pharyngitis and was identified as HSV-1 as previously described (McKendall *et al.*, 1974). The virus was passed five times in Hep-2 cells at a multiplicity of infection of 0·1 to 0·001 to produce a stock which contained $1·0 \times 10^7$ p.f.u./ml.

Animals. Thirty-two-day-old BALB/c mice (Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.) were used in all animal studies. New Zealand white rabbits (Simonson, Gilroy, Ca., U.S.A.) were used as a source of hyperimmune HSV-1 serum.

Experimental models

Footpad model. Four-week-old BALB/c mice were inoculated with $10^{5·5}$ p.f.u. HSV-1 into the right rear footpad. Mice were observed daily for the appearance of clinical illness for a period of 30 days. For virus titre studies, groups of three mice were sacrificed at intervals. Footpad, sciatic nerve and spinal cord tissues were removed, weighed and homogenized for later plaque assay using clarified supernatants. In some experiments, lumbosacral dorsal root ganglia from L1 to S1 and spinal cord were removed and explanted as individual tissues from each animal.

Intracerebral model. Following light ether anaesthesia, the left temporoparietal area of BALB/c mice was inoculated with 10^4 p.f.u. HSV-1 in 10 µl from a 250 µl syringe (Hamilton Co., Reno, Nv., U.S.A.). Animals were then followed for appearance of clinical disease or were sacrificed at intervals at which time the brain was removed in its entirety, weighed and homogenized at 10% (w/v) in minimal essential medium (MEM). Plaque assays were done on individual specimens to determine p.f.u./g of tissue. Four to eight mice were studied at each experimental time point.

Preparation of ^{125}I-labelled neutralizing IgG tracer. Serum IgG from rabbits hyperimmune to HSV-1 was precipitated in 25% saturated solution $(NH_4)_2SO_4$ overnight slowly at 4 °C. Following a second precipitation, the IgG was redissolved in TSV buffer (0·1 M-NaCl, 0·01 M-Tris–HCl, 0·001 M-EDTA). The IgG was then exhaustively dialysed against TSV buffer. The preparation had a protein concentration of 22 mg/ml determined by A_{280} and a neutralization titre of 1:256. By SDS–polyacrylamide gel electrophoresis the IgG preparation was shown to be 90 to 95% IgG. Aliquots of this partially purified neutralizing IgG were labelled with ^{125}I using the chloramine T method (Forghani *et al.*, 1977) to produce a tracer with a specific activity of $7·7 \times 10^5$ ct/min/µg protein.

Blood–brain barrier studies. Mice were inoculated with $10^{5·5}$ p.f.u. HSV-1 in the right rear footpad. Control mice received an inoculation of medium. On days 2 and 3 post-infection, four mice were sacrificed from the control group and four from the infected group. On days 4 and 6 post-infection, six mice were sacrificed from the infected group. Three h prior to sacrifice, each animal received 1×10^8 ct/min ^{125}I-labelled IgG by intraperitoneal (i.p.) inoculation. At sacrifice, the left footpad and spinal cord were removed from each animal, immersed in 10% formalin and counted to determine ct/min/g of tissue. Spinal cord was then further processed with paraffin embedding for histological procedures.

Virus assays

Plaque assay. Vero cells were grown to confluence in multiwell plates (Falcon) in MEM supplemented with 2% foetal calf serum, glutamine and antibiotics. Clarified supernatants from tissue homogenates were added in a volume of 0·1 ml to each of triplicate or quadruplicate wells after the media had been removed. After a 1 h incubation at 37 °C with occasional rotation, each well was overlaid with additional medium in 0·5% agar. After 4 days incubation at 37 °C, a 0·5 ml overlay of medium containing neutral red at a 1:10000 final concentration in 0·5% agar was placed on each well. Plaques were counted on an inverted microscope after an additional incubation at 37 °C overnight.

Isolation of virus by explantation. Lumbosacral dorsal root ganglion tissues were individually explanted in multiwell plates at the time of removal from the animal. Four h later 2×10^5 Vero cells were added to each well. Wells were maintained in MEM containing 2% foetal calf serum with glutamine and antibiotic supplements. Spinal cord was handled similarly except that the tissue was minced at the time of removal from the animal and inoculated into multiwells containing established confluent Vero cell monolayers. All wells were followed daily for the appearance of virus c.p.e. in the Vero monolayer. Medium was replenished weekly.

Histopathology. Appropriate tissues were fixed by immersion in formalin, followed by embedding in paraffin for sectioning. Standard haematoxylin and eosin stain was used throughout. In the BBB studies the severity of the inflammatory reaction in the spinal cord was graded according to the following scheme: +, few small perivascular cuffs; ++, multifocal areas of inflammatory cell infiltrates; +++, large focal areas of myelitis; ++++, necrosis and disruption of cytoarchitecture involving large areas of spinal cord.

RESULTS

Pathogenesis of HSV-1 myelitis

Following footpad inoculation of $10^{5·5}$ p.f.u. HSV-1, 13/14 (93%) of mice developed hind limb paralysis. Illness reproducibly began on the 6th day following inoculation of virus (Fig. 1*a*). The

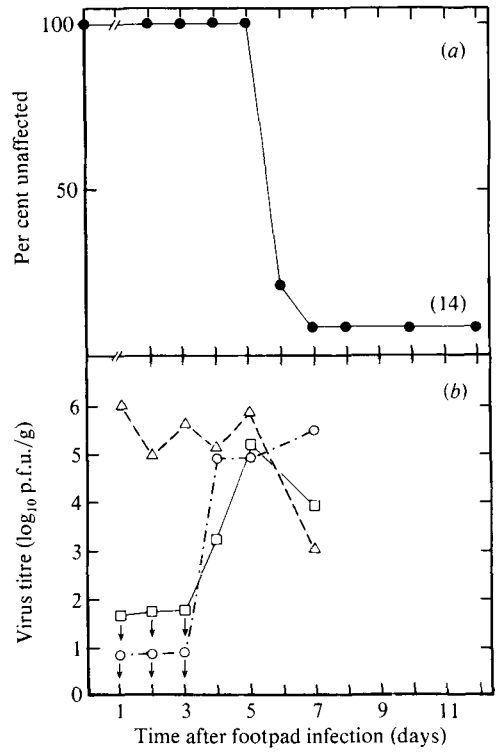

Fig. 1. Pathogenesis of disease in BALB/c mice given $10^{5.5}$ p.f.u. HSV-1 in the footpad. (*a*) Appearance of hind limb paralysis and (*b*) titres of virus in tissue homogenates (○, spinal cord; □, sciatic nerve; △, footpad) at intervals after inoculation. Arrows in Fig. 1 (*b*) indicate the lower limit of plaque assay sensitivity.

illness first developed as a monoplegia on the side of inoculation followed by progressive bilateral hind limb paralysis, ascending myelitis and death from brainstem infection. The virus pathogenesis of the early phases of this infection from days 0 to 7 is shown in Fig. 1 (*b*). Virus titres were determined by plaque assay on tissue homogenates of footpad, sciatic nerve and spinal cord at daily intervals following footpad infection. Footpad titres of virus remained between $10^{5.0}$ and $10^{6.0}$ p.f.u./g for the first 5 days of infection and then dropped. Both sciatic nerve and spinal cord had no virus detectable until the 4th day after infection, when rising titres appeared and persisted until the 7th day of infection. Thus, free virus was present at high titres in spinal cord 2 days prior to the onset of illness.

Virus spread detected by explant technique

Because plaque assay on tissue homogenates may not detect low titres of virus and because virus has been shown to spread very rapidly from footpad to dorsal root ganglion (Cook & Stevens, 1973), we cocultivated spinal cord and dorsal root ganglia explants with Vero cell monolayers to detect the presence of virus at daily intervals following footpad inoculation. Table 1 shows that virus had reached the spinal cord in 3/4 (75%) of mice by 48 h and in all mice by 72 h. Dorsal root ganglion explants of lumbosacral ganglia L1 to S1 became positive about 24 h earlier. Thus, virus was already present in spinal cord 48 h after footpad infection and 4 days prior to the onset of the initial stages of clinical disease.

^{125}I-labelled IgG tracer studies of the blood–brain barrier in HSV-1 myelitis

Several preliminary experiments utilizing various radiolabelled tracer substances indicated that purified ^{125}I-labelled rabbit hyperimmune IgG delivered at high doses provided the most

Table 1. *Virus recovery from spinal ganglia and spinal cord at intervals after footpad infection*

Time after footpad infection (h)	Virus recovery from ganglia explants*	Virus recovery from spinal cord explants*
26	2/3 (66%)	0/3 (0%)
48	4/4 (100%)	3/4 (75%)
72	5/5 (100%)	5/5 (100%)

* Number of animals positive/number studied.

Table 2. *Leakage of ^{125}I-IgG across blood–brain barrier in spinal cord with time after right footpad infection with HSV-1*

	Ct/min/g ± S.E.M.			
		Spinal cord		
Group*	LFP ± S.E.M.	Actual†	Predicted‡	*P* value§
HSV-1 D_2 (N = 4)	556310 ± 50678	78116 ± 10284	70317 ± 6405	NS
D_3 (N = 4)	520860 ± 14175	54049 ± 7000	65881 ± 1792	NS
D_4 (N = 6)	446110 ± 49477	36828 ± 8712	56329 ± 6258	NS
D_6 (N = 6)	548008 ± 81559	116530 ± 28706	69372 ± 10322	<0·01
Control (N = 8)	449476 ± 45740	50391 ± 7458	62426 ± 5127	

* D, Days after footpad inoculation; N, number of animals studied.

† Ct/min/g based on ct/min counted.

‡ Ct/min/g predicted from actual counts in left footpad (LFP). The prediction is calculated from correlation coefficient derived from multiple linear regression analysis of the correlation between LFP and spinal cord.

§ Statistical significance compared to control group of mice; NS, not significant.

sensitive assay of antibody penetration into the spinal cord. We therefore inoculated mice with virus in the footpad and at days 2, 3, 4 and 6 we administered 1×10^8 ct/min of ^{125}I-labelled anti-HSV-1 IgG i.p. Three h later, mice were sacrificed. Spinal cord and left footpad (the uninfected footpad) were collected, individually weighed and ct/min/g of tissue were determined. In addition, spinal cord was processed for haematoxylin and eosin sections. Table 2 shows the results of tracer leakage into the spinal cord. Actual ct/min/g of spinal cord tissue from HSV-1-infected mice sacrificed on days 2, 3 and 4 were not significantly different from one another or from the 50391 ± 7458 ct/min/g present in eight uninfected control mice. In contrast, on day 6 the actual ct/min/g in spinal cord of HSV-1-infected mice were 116530 ± 28706 and markedly higher than that of the control group and that of the HSV-1 groups from earlier days.

Because of variation between animals in uptake of tracer from peritoneum, in blood flow and in tracer clearance, direct measurement of spinal cord ct/min/g might not be the most sensitive indication of BBB leakage. Therefore, ct/min/g in the uninfected left footpad (LFP) was used as a reference value to predict mathematically the ct/min/g expected in the spinal cord of each mouse. The prediction was calculated from the correlation coefficient between LFP and spinal cord using multiple linear regression analysis. The difference between the predicted spinal cord ct/min/g and the actual spinal cord ct/min/g was significant only in mice examined on day 6: 69372 ± 10322 compared to 116530 ± 28706 ($P < 0·01$). Thus, both by actual counts and by statistical analysis of the predicted ct/min and actual ct/min, the BBB was observed to be disrupted on the 6th day following footpad infection.

Histopathology of early HSV-1 myelitis

After determination of tissue radioactivity the spinal cords from the animals used in the tracer studies were processed with longitudinal sections at three levels to survey large areas of tissue. Sections were extensively searched for the presence of early inflammatory lesions. Fig. 2 shows perivascular mononuclear cell infiltration in the white matter of the lumbosacral cord from

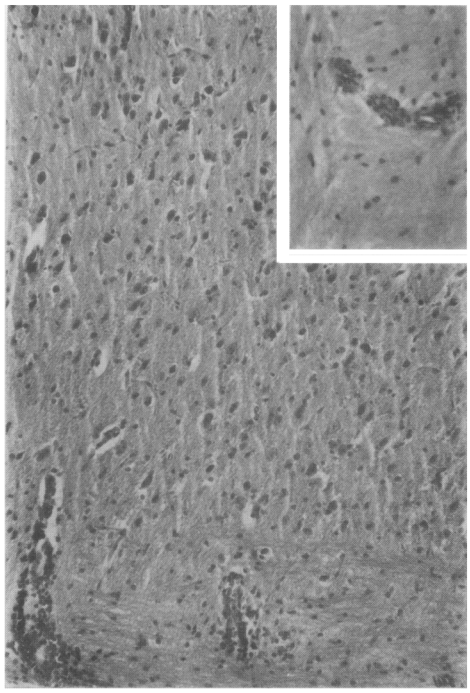

Fig. 2

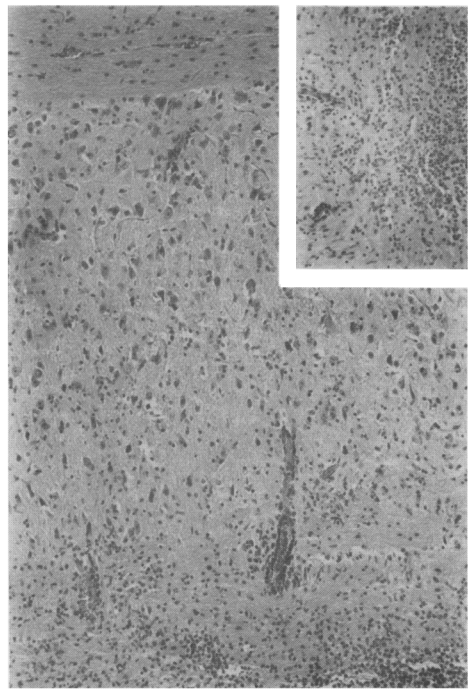

Fig. 3

Fig. 2. Perivascular cuffing in spinal cords from mice with HSV-1 myelitis sacrificed 3 days (inset) or 4 days after footpad infection.

Fig. 3. Perivascular cuffing and diffuse mononuclear cell infiltration in mice with HSV-1 myelitis sacrificed 6 days after footpad infection. Inset shows areas of severe inflammation and necrosis with disruption of normal cytoarchitecture.

animals sacrificed on days 3 and 4. Fig. 3 shows a heavy perivascular accumulation of mononuclear cells as well as diffuse round cell infiltration in the spinal cord of an animal sacrificed on day 6. Table 3 summarizes the histopathology in the study group and shows that there was an increasingly severe myelitis developing between days 3 and 6 and that most animals had evidence of inflammatory lesions. In addition, myelitis was seen primarily in the lumbosacral spinal cords of these animals and less so at thoracic or cervical cord levels, indicating that disease within the spinal cord occurred primarily at a segmental level appropriate for the innervation of the hind limb.

Effect of neutralizing IgG on virus clearance from footpad compared to brain tissue

For footpad studies, mice were inoculated with $10^{5.5}$ p.f.u. by footpad and 2 h later were given neutralizing IgG i.p. by passive transfer at a dose sufficient to provide circulating titres of 1:16. Kinetic studies showed no decrease in serum antibody titre throughout the experimental period. Fig. 4(*a*) shows that high titres of virus were present in the footpad homogenates from animals sacrificed on days 1 and 2, after which there was a phase of rapid virus clearance resulting in undetectable levels from day 3 onward. Homogenates of sciatic nerve and spinal cord showed no virus present at any time (data not shown). Control mice given no neutralizing antibody had persistently elevated titres in footpad for 5 days following infection. These data are shown in Fig. 1(*b*) as part of the basic pathogenesis of HSV-1 myelitis.

To examine IgG-mediated virus clearance from infected CNS tissue, the i.c. model was used. This was done to exclude the possibility that IgG leakage too minor to be detected in tracer studies might be sufficient to cause virus clearance. Preliminary experiments were done to

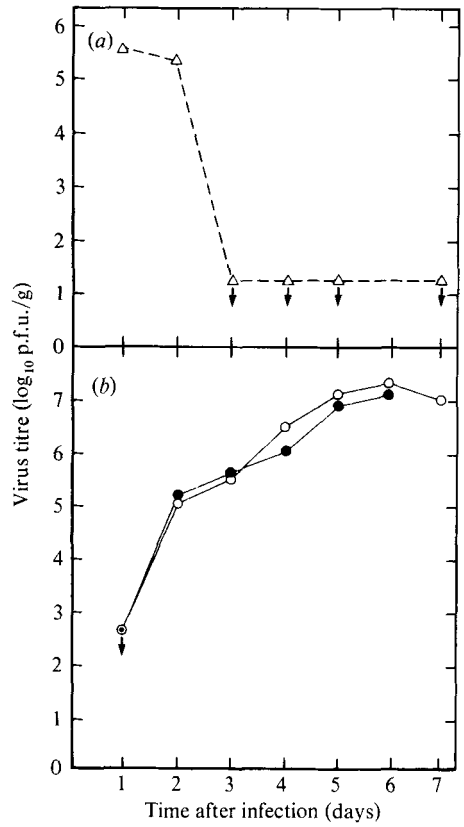

Fig. 4. Effect of neutralizing IgG on virus clearance from (*a*) footpad and (*b*) brain (●, IgG-treated; ○, untreated). Arrows indicate the lower limit of virus detection by the plaque assay employed.

Table 3. *Appearance of inflammatory infiltrates in spinal cord at intervals after footpad infection*

Day after infection	Spinal cord infiltrates*	Degree of inflammation
2	0/4 (0%)	0
3	2/4 (50%)	+
4	4/6 (66%)	++
6	5/6 (85%)	++++

* Number of animals positive/number studied.

determine a dose of virus compatible with a 5 or more day survival since the data on virus clearance from footpad indicated that 3 days was enough time to achieve IgG-mediated virus clearance. Fig. 4(*b*) shows virus titres in homogenates of brain from mice inoculated with 10^4 p.f.u. of virus at day 0. There was no difference between mean virus titres in IgG-treated and control animals. Furthermore, there was no evidence for a rapid phase of virus clearance 2 to 3 days after inoculation despite the fact that no illness appeared until the 4th or 5th day following infection.

DISCUSSION

When, in the natural course of HSV-1 CNS infection, the BBB is first disrupted is not known from the reports in the literature. McClellan & Goodpasture (1923) showed leakage of Evans blue dye in rabbit spinal cord on day 10 after footpad inoculation and in brain on day 6 after i.c.

inoculation. Kristensson & Sourander (1969) showed dye leakage in mouse brain 4 days after i.c. inoculation using a high dose of virus which killed all mice by day 5. Histopathology in the latter study showed a necrotic encephalitis at the time of BBB disruption. This finding is similar to the pathological findings seen in spinal cords of the 6th-day animals in this report. Neither of the published reports investigated the integrity of the BBB during earlier stages of the inflammatory process.

In the footpad model the following series of pathogenetic events occurs during the early stages of infection. After inoculation, virus replication in footpad produces persistently high titres for several days. Explants of dorsal root ganglia and spinal cord become positive at 26 and 48 h respectively. At day 3, early myelitis is detected as small perivascular mononuclear infiltrates. Following this, by day 4, virus replication bursts forth in spinal cord and sciatic nerve to high and rising titres which persist for the next 2 days. On the 6th day following infection, in the face of progressive myelitis, the BBB is shown to be disrupted by leakage of ^{125}I-labelled IgG. On the same day clinical illness is first evident. This time course indicates that disruption of the BBB is a relatively late event in the pathogenesis of HSV-1 myelitis.

The demonstration of a late break in the integrity of the BBB, coupled with the failure of antibody administered later than 72 h to be protective (McKendall *et al.*, 1979; Oakes & Rosemond-Hornbeak, 1978; Davis *et al.*, 1979), suggests that antibody access to infected spinal cord during the early stages of myelitis might be restricted by the BBB or at least reduced to a level not detected by the ^{125}I-labelled IgG tracer studies. The studies on IgG-mediated virus clearance support this hypothesis. Virus clearance from footpad is remarkably rapid and complete within 72 h after IgG administration. In sharp contrast, no clearance was seen for 7 days following i.c. inoculation. Thus, we conclude that during the early stages of CNS infection, when small histological lesions are easily visible, there remains sufficient integrity of the BBB to prevent IgG-mediated virus clearance. The BBB-caused delay in IgG-mediated virus clearance may or may not be completely accounted for by decreased access of IgG to the CNS. The relative integrity of the BBB may influence other factors which are involved in virus clearance; for example, the BBB may also retard entry of cellular components, either sensitized or non-sensitized. It is noteworthy that a cellular contribution to IgG-mediated immunity has been described in some systems (Oakes *et al.*, 1980). The data presented here can not determine how much of the failed virus clearance is attributable to insufficient IgG entry into the CNS rather than to insufficiency of other immune system components.

Regardless of the mechanism it is clear that the relative integrity of the BBB during the early days of infection plays an important role in delaying IgG-mediated virus clearance. Virus is therefore able to replicate for a significantly longer period of time in CNS tissue than in peripheral tissues without restriction by IgG. This phenomenon may be one factor in the appearance of HSV-1 encephalitis in patients with pre-existent serum antibodies where a focus of reactivated latent virus may replicate and spread for several days before the BBB is significantly disrupted. A detrimental effect of the BBB may therefore operate in a majority of patients with HSV-1 encephalitis, since 70% of biopsy-verified encephalitis is thought to be due to reactivated infection (Nahmias *et al.*, 1982). Additionally, this effect of the BBB must be recognized in the development of future immunotherapeutic strategies.

The excellent technical assistance of Mr W. T. Monte and the secretarial skills of Ms J. Rohrer and Ms D. Jarest are greatly appreciated. The statistical analyses performed by George Fein, Ph.D., are gratefully acknowledged. This work was supported in part by Young Investigator Award No. AI 14564 from NIAID, the Research Service of the Veterans Administration and the Kroc Foundation.

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(Received 27 January 1983)