Journal of General Virology (1997), **78**, 195–203. Printed in Great Britain

Herpes simplex virus type 2 synergizes with interferon-γ in the induction of nitric oxide production in mouse macrophages through autocrine secretion of tumour necrosis factor-α

Hüseyin Baskin,† Svend Ellermann-Eriksen, Jette Lovmand and Søren C. Mogensen

Department of Medical Microbiology and Immunology, University of Aarhus, DK-8000 Aarhus C, Denmark

We have analysed the ability of herpes simplex virus type 2 (HSV-2) to induce nitric oxide (NO) production in resting BALB/c mouse peritoneal macrophages. In most experiments, macrophages produced very small amounts of NO upon infection with HSV-2. Mock virus preparations did not induce NO production, and virus inactivation experiments showed that infectious virus was required. Since interferon-γ (IFN-γ) is the prototype cytokine that is able to induce significant NO production in macrophages, we found it of interest to examine the influence of HSV-2 infection on the IFN-γ-induced NO production. The virus exerted a synergistic effect on the IFN-γ-induced NO release, which was accompanied by induction of the *iNOS*-gene as revealed by RT–PCR. This effect was largely dependent on the presence of infectious virus particles, since only a minor effect was seen with mock virus and inactivated virus preparations. From experiments with neutralizing antibodies to tumour necrosis factor-α (TNF-α) and IFN-α/β it was concluded that the synergistic effect is dependent on autocrine secretion of TNF-α, which acts as a second signal and synergizes with IFN-γ in NO production.

Introduction

Macrophages are considered to play an essential role in resistance to virus infections (Mogensen, 1979, 1988; Morahan *et al.*, 1985; Wu & Morahan, 1992). A number of potential antiviral macrophage functions have been described to be at work in a multitude of experimental infections with many different viruses. Both intrinsic and extrinsic antiviral mechanisms of macrophages have been described. *Intrinsic antiviral activity* is defined as the ability of macrophages to restrict virus replication in macrophages *per se* and thus to serve as nonpermissive targets for virus replication. *Extrinsic antiviral activity* is defined as the ability of macrophages to influence extracellular virus and to interfere with virus replication in surrounding permissive cells. Although a number of mechanisms have been suggested to be involved in these antiviral functions of macrophages, their basic nature and relative importance in different virus infections are as yet not clearly defined.

Nitric oxide (NO) is a free radical gaseous molecule that is produced in a number of cell types from molecular oxygen and a guanidino nitrogen from L-arginine, which is converted to L-citrulline (Moncada *et al.*, 1991; Nathan, 1992; Bredt & Snyder, 1994). In activated macrophages this is exerted through induction of expression of the *iNOS* gene, which encodes an inducible isoform of nitric oxide synthase. In recent years, much interest has been paid to NO as an important mediator of vital physiological functions, including host defence against tumours (Hibbs *et al.*, 1987) and a range of bacterial, protozoal and fungal pathogens (James & Claven, 1989; Green *et al.*, 1990; Adams *et al.*, 1990, 1991; Denis, 1991; Alspaugh & Granger, 1991; Boockvar *et al.*, 1994).

In spite of the compelling indications that NO plays a crucial role in the microbiostatic and microbiocidal functions of macrophages, the possible involvement of NO as an antiviral mediator of macrophages has only recently received attention. Thus, Karupiah *et al.* (1993) reported strong evidence that induction of NO synthase in macrophages can be necessary and sufficient for a substantial antiviral effect of interferon-γ (IFN-γ) against ectromelia virus, vaccinia virus and herpes simplex virus type 1 (HSV-1), and Croen (1993) described a direct antiviral effect of an exogenously added NO donor on the replication of HSV-1 in a macrophage cell line. The findings of these initial reports have later been confirmed and extended (Adler *et al.*, 1994; Harris *et al.*, 1995; Karupiah & Harris, 1995; Mělková & Esteban, 1995; Bi & Reiss, 1995; Kreil & Eibl, 1995; Jelachich *et al.*, 1995).

Author for correspondence: Søren C. Mogensen.
Fax +45 8619 6128. e-mail mikrsm@svfcd.aau.dk

† **Present address:** Department of Medical Microbiology, Dokuz Eylül University, Izmir, Turkey.

0001-4295

The above cited studies indicate that NO production in macrophages during their activation by, for instance, IFN-γ in the course of a virus infection might constitute an important antiviral principle. However, most of them do not yield information as to whether NO is actually produced during virus infection of macrophages. It has been found that virus infection of macrophages can either upregulate or down-regulate subsequent NO production in response to a trigger, depending on the virus in question and the triggering signal (Lyon & Hinshaw, 1993; Adler *et al*., 1994; Kreil & Eibl, 1995). Furthermore, recent studies have shown that human monocyte/macrophage cultures infected with human immunodeficiency virus (HIV) (Bukrinsky *et al*., 1995) or treated with recombinant gp120 (Pietraforte *et al*., 1994) produce modest but significant amounts of NO.

In a murine model of generalized infection with herpes simplex virus type 2 (HSV-2) we have previously obtained data which indicate that macrophages play a key role in natural resistance to the infection (for reviews see Mogensen, 1979, 1988). Both the ability of macrophages to intrinsically restrict virus replication and to produce as well as to respond to cytokines like interferon-α/β (IFN-α/β) and tumour necrosis factor-α (TNF-α) seem to be involved in resistance (Mogensen, 1979; Ellermann-Eriksen *et al*., 1986, 1989; Mogensen *et al*., 1989; Ellermann-Eriksen, 1993). In the present study, we have examined whether infection of mouse macrophages with HSV-2 results in production of NO in the cells. We report that HSV-2 in itself has a very limited capacity to directly induce NO production in macrophages. However, the virus can synergize with IFN-γ in the induction of NO release from resting macrophages through autocrine secretion of TNF-α.

Methods

■ **Mice.** Inbred, specific pathogen-free BALB/cABOM mice originally obtained from Bomholtgaard Animal Breeding and Research Center were bred locally behind specific pathogen-free barriers. The mice were regularly checked to be free of mouse hepatitis virus, pneumonia virus of mice, Sendai virus, reovirus type 3 and minute virus of mice. Female mice were used at an age of 8–12 weeks.

■ **Media and reagents.** RPMI 1640 without phenol red and L-glutamine was obtained from Bio-Whittaker. Foetal calf serum (FCS) with an endotoxin content of 0·16 ng/ml was from Gibco BRL. Complete medium consisted of RPMI 1640 supplemented with 5% FCS, 10 mM glutamine, 2 mM HEPES, 200 IU/ml of penicillin and 200 μg/ml of streptomycin (RPMI-medium). Lipopolysaccharide (LPS; Sigma) from *E. coli* was suspended to 1 mg/ml in PBS, pH 7·4, with 0·1% BSA (Sigma) and further diluted in RPMI-medium. N^G-monomethyl-L-arginine acetate (L-NMMA) from Research Biochemicals International was suspended in RPMI-medium and filtered through a 0·22 μm filter before use. Thioglycollate broth was obtained from Difco. The Griess reagent for measuring nitrite levels consisted of equal volumes of 0·1% *N*-(1-naphthyl)ethylenediamine dihydrochloride and 1% *p*-aminobenzenesulfanilamide (both from Sigma) diluted in 2·5% phosphoric acid. Taq polymerase was obtained from Stratagene and deoxynucleotide triphosphates and DNA molecular mass marker VI from Boehringer. Primers for RT–PCR were from DNA Technology.

■ **Cells.** Most experiments were performed with resting mouse peritoneal cells. In a few experiments the mouse macrophage cell line J774A.1 (ATCC TIB 67), grown in DMEM with 10% FCS and antibiotics, or elicited mouse peritoneal exudate cells were used. The elicited cells were obtained by inoculating 2 ml 10% thioglycollate broth intraperitoneally 4 days before cell harvest. Resident and elicited peritoneal cells were harvested from mice by lavage of the peritoneal cavity with cold PBS supplemented with 2% FCS and 20 IU/ml of heparin. Cells were kept on ice, washed once in RPMI-medium and seeded in cultures at the indicated concentrations. In most experiments 2×10^6 cells were seeded in a volume of 0·7–0·9 ml of RPMI-medium in 24-well plates (Multidish wells; 16 mm diam.; Nunc) in order to obtain 1 ml cultures after addition of virus, cytokine and/or antibodies. In some experiments the cultures were down-scaled to 96-well tissue culture plates (Nunc) to give the same number of cells and the same volume per mm^2 culture area ($3{\cdot}5 \times 10^5$ cells in 175 μl final volume). In comparative experiments large and small cultures gave identical results (data not shown).

■ **Virus.** The high-titre stock of the MS strain of HSV-2 used throughout this study was produced as described previously (Ellermann-Eriksen, 1993). Briefly, mycoplasma-free Vero cells in Eagle's MEM with 2% FCS and antibiotics as above were infected at an m.o.i. of 0·01. At nearly complete cytopathic effect (48 h) the tissue culture flasks were subjected to two cycles of freezing (−70 °C) and thawing, and the supernatant was clarified of cellular debris by centrifugation at 3000 ***g*** for 1 h. The virus was pelleted by ultracentrifugation at 45 000 ***g*** for 1 h and resuspended in PBS supplemented with 0·1% BSA. After sonication at 40 W for 3×30 s the virus preparation was aliquoted and stored at −70 °C until use. The virus stock had an infectivity titre of $3{\cdot}8 \times 10^8$ p.f.u./ml as determined by a plaque assay in Vero cells. A mock virus preparation was made in parallel in exactly the same manner, except that the cells were not infected with HSV-2. Inactivated virus preparations were prepared by heating virus for 30 min in a 56 °C water-bath or by subjecting virus to UV irradiation for 15 min at a distance of 10 cm from a 15 W UV source. None of the preparations contained any infectious virus as assessed by subsequent virus titration. UV irradiation of the virus and mock virus preparations diluted in RPMI-medium resulted in the formation of nitrite in amounts which corresponded to addition to the cultures of 2–3 μM nitrite. These values were subtracted from the nitrite values obtained for cultures to which UV-inactivated virus was added.

■ **Cytokine and cytokine antibodies.** Recombinant murine IFN-γ was from Pharmingen and had a specific activity of $> 10^7$ IU/mg protein and a purity of $> 95\%$. Neutralizing polyclonal rabbit anti-mouse TNF-α was obtained from Genzyme; as control antibody we used a normal rabbit serum obtained from our animal house. Neutralizing polyclonal rabbit anti-mouse IFN-α/β and a corresponding control antibody were purchased from Lee BioMolecular. The cytokine and antibodies were aliquoted in PBS with 0·1% BSA, stored at −70 °C and diluted for the experiments in RPMI-medium.

■ **Experimental procedure.** After establishment, cultures were incubated overnight in a humidified atmosphere with 5% CO_2. Infection and stimulation were done by adding 100 μl (or the equivalent smaller volume to down-scaled cultures) of combinations of virus, mock virus, cytokine, antiserum or medium as indicated in individual experiments to give a final volume of 1 ml. In most experiments the cultures were infected with 3×10^6 p.f.u./ml of HSV-2, corresponding to an m.o.i. of 1·5. After continued incubation (usually 24 h) duplicate samples of supernatant were harvested for determination of nitrite concentrations. If supernatants could not be tested immediately they were frozen at −70 °C until assayed. This procedure did not influence the result of the determination (data not shown).

■ **Nitrite determination.** Nitrite is generated by the rapid oxidation of NO. It is stable and its accumulation in the culture medium reflects the amount of NO produced. To assay nitrite we used a modification of a previously published method (Ding *et al.*, 1988). Aliquots of 100 µl culture supernatants were mixed with equal volumes of Griess reagent in a 96-well microtitre plate (Maxisorb Immunoplate, Nunc). After 10 min incubation at room temperature the absorbance at a wavelength of 540 nm was measured in a microplate reader (model 450; Bio-Rad). A range of 2-fold dilutions of sodium nitrite (0·05–100 µM) in RPMI-medium was run in each assay to generate a standard curve. In most experiments, control cultures not infected with virus or stimulated with cytokine gave small nitrite values in the order of 0–1 µM. These figures were subtracted from the experimental values in the data presentation.

■ **RT–PCR.** Total cellular RNA was extracted by the guanidinium thiocyanate procedure as described by Chomczynski & Sacchi (1987). Reverse transcription of RNA was performed using the First Strand cDNA Synthesis Kit from Pharmacia following the manufacturer's instructions. The primer was the same *iNOS*-specific antisense primer as used in the PCR reaction. PCR was performed in an automated thermal cycler (Omn-E; Hybaid) at 94 °C for 3 min followed by 45 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The RT–PCR product was mixed with 0·2 mM of each deoxynucleotide triphosphate, PCR-buffer and 2·5 µM of each primer. The *iNOS*-specific primers were: sense, 5′ CTT CCG AAG TTT CTG GCA GCA GCG 3′; antisense, 5′ GAG CCT CGT GGC TTT GGG CTC CTC 3′. The amplified product spanned 487 bp. The PCR products and a DNA molecular mass marker were subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

■ **Endotoxin content of reagents.** The endotoxin contents of the main reagents in cultures were determined by a chromogenic *Limulus* amoebocyte lysate assay applied as specified by the manufacturer (Bio-Whittaker). Complete RPMI-medium and the virus, mock virus, cytokine and antibodies diluted to give final concentrations similar to those obtained in cultures all contained less than 20 pg/ml of LPS.

■ **Statistical analysis.** The NO production in response to HSV-2 infection alone was tested by the sign test on mean values from all experiments. Statistical testing of a synergistic effect of HSV-2 and IFN-γ on NO production was performed by the paired *t*-test. In this test the amount of nitrite measured in supernatants from cells treated with the combination of HSV-2 and IFN-γ was compared with the sum of the amounts produced in cells treated with either HSV-2 or IFN-γ.

Results

Comparison of NO release from different types of macrophages

Most studies on the induction of NO release from mouse macrophages by different stimuli use established cell lines like RAW264.7 and J774A.1, or the studies are performed with peritoneal macrophages which have been elicited by inoculation with irritants like thioglycollate or mineral oil some days before cell harvest. Since we believe that it is important to work with cells as close to the natural situation as possible, we first examined whether we could establish culture conditions in which we were able to induce NO production in resting peritoneal cells.

In a series of experiments we examined the NO production capability of the mouse macrophage cell line J774A.1, thioglycollate-elicited peritoneal macrophages and resting peritoneal macrophages stimulated for 24 h in culture with IFN-γ and/or LPS, which are well-known inducers of NO production. From Table 1 and Fig. 1 it is seen that resting peritoneal macrophages were able to produce nitrite in amounts comparable to the J774A.1 and thioglycollate-induced macrophages, provided the macrophage culture had a certain density. From microscopic inspection of the cultures it was obvious that significant nitrite production in resting cells was seen preferentially in cultures where individual macrophages had physical contact with neighbouring cells, and nitrite production increased exponentially as a function of the number of cells in the culture. In the following experiments with resting macrophages we normally seeded 2×10^6 cells in 24-well cultures or the corresponding cell number in microtitre wells ($3{\cdot}5 \times 10^5$ cells).

Table 1. NO production by different types of macrophages stimulated with IFN-γ and LPS

Values are mean nitrite (µM) ± SEM in triplicate cultures. Similar results were obtained in two other experiments.

	J774*	Thio PC†	Resting PC‡
IFN-γ (100 IU/ml)	18·5 ± 0·13	0·74 ± 0·16	1·19 ± 0·03
LPS (25 ng/ml)	0·23 ± 0·08	3·20 ± 0·40	11·4 ± 0·69
IFN-γ + LPS	41·4 ± 0·83	36·5 ± 3·71	21·7 ± 0·44

* 10^6 J774A.1 cells/ml.
† 10^6 thioglycollate-induced peritoneal cells/ml.
‡ 2×10^6 resting peritoneal cells/ml.

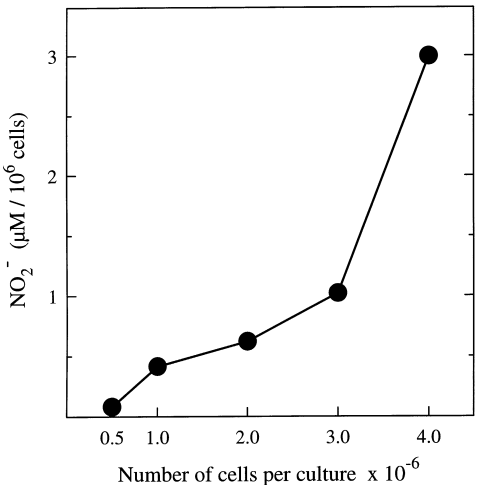

Fig. 1. Influence of cell density on NO production in resting mouse macrophages stimulated with recombinant murine IFN-γ. Increasing numbers of peritoneal cells ($0{\cdot}5–4 \times 10^6$/ml) were stimulated for 24 h with 100 IU/ml of IFN-γ, and the amounts of nitrite in the supernatants were determined by the Griess assay. Results are expressed in µM nitrite produced per 10^6 cells. Essentially similar results were obtained in two other experiments.

Table 2. NO production in resting mouse peritoneal cells infected with various doses of HSV-2

Values are mean nitrite (μM) ± SEM in duplicate cultures (2×10^6 cells/ml).

Dose of HSV-2 (p.f.u./ml)	Expt 1	Expt 2
10^7	1·41 ± 0·04	2·05 ± 0·33
3×10^6	0·34 ± 0·08	1·39 ± 0·23
10^6	0·16 ± 0·06	ND
3×10^5	0 ± 0	ND
10^5	0 ± 0	ND
Mock*	0 ± 0	0 ± 0
Heat-HSV†	ND	0 ± 0
UV-HSV‡	ND	0 ± 0

* Mock virus; all concentrations.
† Heat-inactivated HSV-2 (56 °C, 30 min); all concentrations.
‡ UV-inactivated HSV-2; all concentrations.
ND, Not done.

Induction of NO release from macrophages infected with HSV-2

We next examined whether HSV-2 could in itself induce NO production in cultures of resting peritoneal cells. In nine of eleven experiments cultures infected with 3×10^6 p.f.u./ml of HSV-2 for 24 h produced small and varying amounts of nitrite, whereas no significant production was seen in two experiments. The amount of nitrite produced ranged from 0–1·30 μM (mean = 0·36 μM ± 0·38 μM; $P = 0{\cdot}01$) over the amount seen in uninfected cultures. When different virus doses were examined a dose-dependent response was obtained as depicted in Table 2. Mock virus alone did not induce nitrite production, irrespective of the concentrations used. The induction of NO release by HSV-2 required that the virus was infectious, since heat-inactivated virus preparations did not elicit any NO response, and the amount of NO in supernatants from cultures treated with UV-inactivated virus preparations never exceeded the amount generated by the UV-inactivation procedure (Table 2).

Synergistic effect of HSV-2 infection on IFN-γ-induced NO secretion in macrophages

IFN-γ has been shown to be the only cytokine able by itself to induce release of significant amounts of NO in mouse macrophages (Ding *et al.*, 1988). Since IFN-γ is known to be present during virus infections and to participate in the clearance of virus from infectious foci (Karupiah *et al.*, 1990; Kohonen-Corish *et al.*, 1990), we found it of relevance to examine the influence of HSV-2 infection on the IFN-γ-induced NO production in macrophages.

In a total of nine experiments cultures of resting peritoneal cells were infected with 3×10^6 p.f.u./ml of HSV-2 with or without the simultaneous addition of 100 IU/ml of IFN-γ. As seen from Fig. 2(*a*) the virus infection exerted a significant synergistic effect on the capacity of IFN-γ to induce NO release in resting peritoneal cells ($P < 0{\cdot}001$). Production of NO correlated with *iNOS* induction. This was verified by examining expression of the gene at the level of mRNA accumulation in cells stimulated with IFN-γ and infected with HSV-2 (Fig. 2*b*). A similar or even more pronounced synergistic effect between HSV-2 infection and IFN-γ was seen

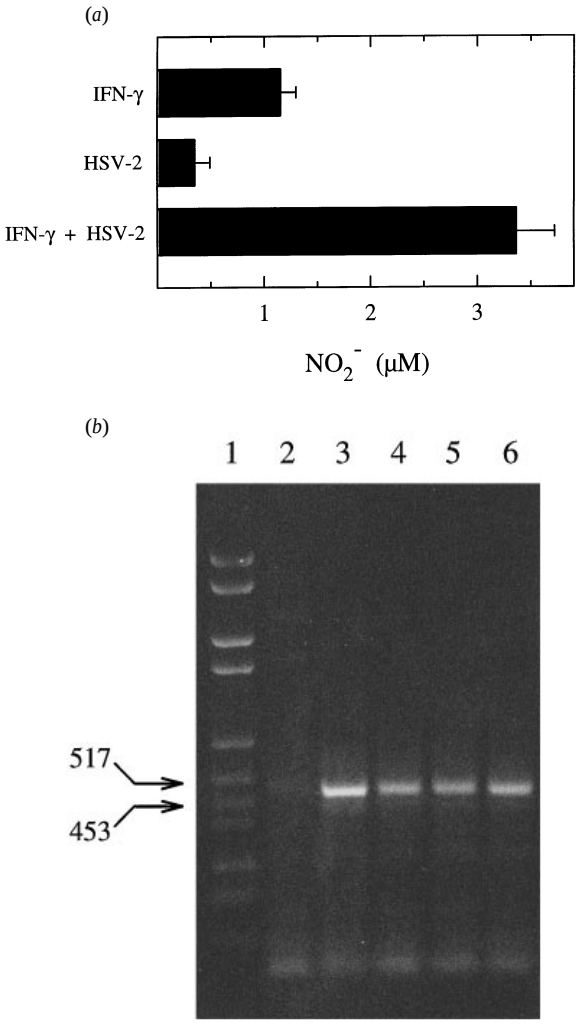

Fig. 2. Synergistic effect of HSV-2 infection on NO production and *iNOS*-expression in resting mouse macrophages stimulated with recombinant murine IFN-γ. (*a*) Peritoneal cells (2×10^6/ml) were stimulated with 100 IU/ml of IFN-γ and/or infected with 3×10^6 p.f.u./ml of HSV-2. The amounts of nitrite in the supernatants after 24 h were determined by the Griess assay. Data are presented as the mean ± SEM of the results from nine experiments. (*b*) RT–PCR with *iNOS*-specific primers was performed on total cellular RNA from 5×10^6 peritoneal cells left unstimulated for 10 h (lane 2), or stimulated with IFN-γ and infected with HSV-2 as above for 10 h (lane 3), 14 h (lane 4), 18 h (lane 5) or 22 h (lane 6). Lane 1, DNA molecular size marker VI. The arrows indicate the positions of the molecular mass size bands of 453 and 517 bp, respectively, situated around the expected position of the RT–PCR products (487 bp).

Table 3. NO production in mouse peritoneal cells stimulated with IFN-γ and challenged with HSV-2 or a mock-virus preparation

Values are mean nitrite (μM) $\pm$ SEM.

	Resting PC*		Thio PC†	
	Expt 1	Expt 2	Expt 1	Expt 2
IFN-γ (100 IU/ml)	1·32 $\pm$ 0·12	1·10 $\pm$ 0·04	0·32 $\pm$ 0·13	0·74 $\pm$ 0·16
HSV-2 (3×10^6 p.f.u./ml)	1·39 $\pm$ 0·23	0·34 $\pm$ 0·08	0·16 $\pm$ 0·14	0·27 $\pm$ 0·16
HSV-2 + IFN-γ	5·30 $\pm$ 0·33	3·77 $\pm$ 0·11	9·19 $\pm$ 0·45	5·12 $\pm$ 0·18
Mock virus	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
Mock virus + IFN-γ	1·41 $\pm$ 0·00	1·52 $\pm$ 0·11	0·87 $\pm$ 0·02	1·94 $\pm$ 0·24

* 2×10^6 resting peritoneal cells/ml.
† 10^6 thioglycollate-induced peritoneal cells/ml.

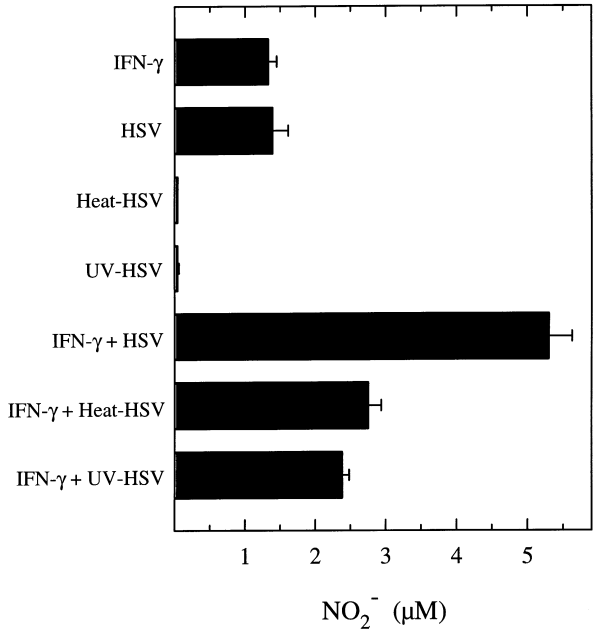

Fig. 3. Effects of infectious and inactivated HSV-2 preparations on NO production in resting mouse peritoneal macrophages stimulated with recombinant murine IFN-γ. Peritoneal cells (2×10^6/ml) were stimulated with 100 IU/ml of IFN-γ and thereafter challenged with 3×10^6 p.f.u./ml of infectious HSV-2 (HSV) or similar amounts of virus inactivated by treatment with UV light (UV-HSV) or heating at 56 °C for 30 min (Heat-HSV). The amounts of nitrite in the supernatants after 24 h were determined by the Griess assay. UV treatment of the virus preparation diluted in RPMI-medium itself resulted in the formation of nitrite (2·6 μM of the measured nitrite in cultures). This value was subtracted from the values obtained in cultures to which UV-inactivated virus was added. Results are expressed as the mean $\pm$ SEM of duplicate cultures. Similar results were obtained in another experiment.

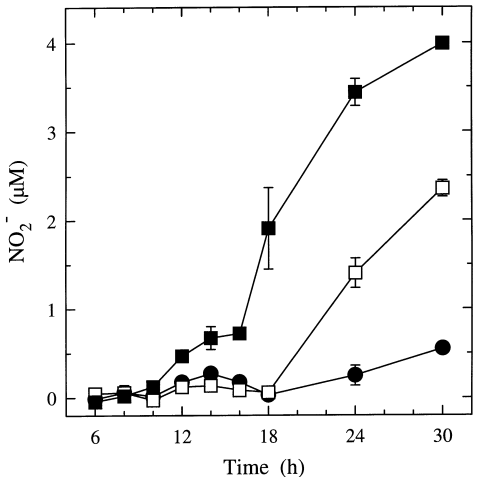

Fig. 4. Kinetics of NO production in resting mouse macrophages stimulated with recombinant murine IFN-γ and infected with HSV-2. Peritoneal cells (2×10^6/ml) received 100 IU/ml of IFN-γ (□), 3×10^6 p.f.u./ml of HSV-2 (●) or a combination of the same concentrations of IFN-γ and HSV-2 (■). At the indicated time intervals duplicate samples of 100 µl supernatant were removed for nitrite determination by the Griess assay, and 200 µl of fresh medium was added to the cultures. The results depicted represent accumulated production of nitrite in a summary of three individual experiments, which showed essentially identical results. The data are presented as the mean $\pm$ SEM of two to seven cultures.

in thioglycollate-induced peritoneal cells (Table 3). In experiments in which mock virus was included, only a minor synergistic effect between mock virus and IFN-γ was seen (Table 3). Heat- and UV-inactivated virus only marginally affected the response of cells to IFN-γ, to a magnitude similar to the effect of mock virus (Fig. 3). Thus, the synergistic effect seemed largely to depend on the presence of infectious virus particles. Addition of the specific substrate-analogue inhibitor L-NMMA, which competes with L-arginine (Hibbs *et al.*, 1987; Moncada *et al.*, 1991), inhibited the production of nitrite by 50–75 % (data not shown).

Examination of the kinetics of the cooperation between HSV-2 infection and IFN-γ treatment revealed that it was evident in most experiments as an additive effect starting between 12 and 18 h after infection and turning into a true synergistic effect from 18 h (Fig. 4).

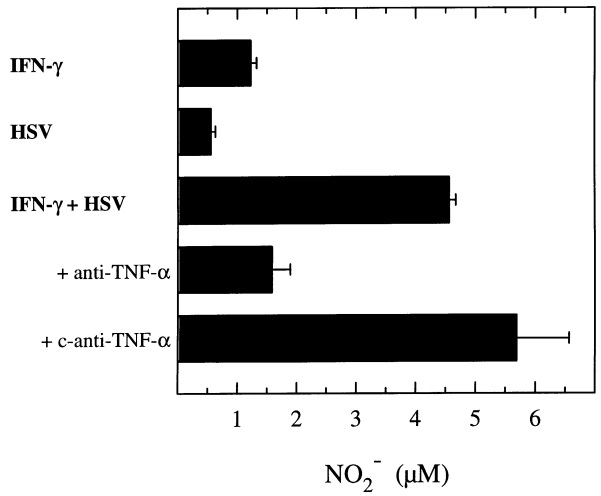

Fig. 5. Effects of neutralizing anti-TNF-α antibodies on the synergistic effect of HSV-2 infection on NO production in resting mouse macrophages stimulated with recombinant murine IFN-γ. Peritoneal cells (2×10^6/ml) were stimulated with 100 IU/ml of IFN-γ and/or infected with 3×10^6 p.f.u./ml of HSV-2. To the indicated cultures were added 1000 neutralizing units of a rabbit polyclonal antibody against murine TNF-α (anti-TNF-α) or an equivalent amount of control rabbit serum (c-anti-TNF-α). After 24 h samples were analysed for nitrite by the Griess assay. The results are represented as the mean ± SEM of two to three cultures. Similar results were obtained in two additional experiments.

Mechanism of the synergistic effect of HSV-2 infection on the IFN-γ-induced NO production in macrophages

A likely candidate to play a role in the synergistic induction of NO production in macrophage cultures treated with IFN-γ and infected with HSV-2 would be TNF-α (Ding *et al.*, 1988; Drapier *et al.*, 1988), which is produced in such cultures in response to HSV-2 infection (Ellermann-Eriksen, 1993). To test this, resting peritoneal cells were infected with HSV-2 and stimulated with IFN-γ in the presence of 1000 neutralizing units of an antibody against murine TNF-α. From Fig. 5 it is seen that anti-TNF-α almost completely abolished the synergistic effect of the virus infection on the IFN-γ-induced nitrite production. In similar experiments anti-IFN-α/β did not affect nitrite production (data not shown). It thus seems that the synergistic effect of HSV-2 infection on the IFN-γ-induced NO production in macrophages is dependent on endogenous secretion of TNF-α, which in an autocrine fashion synergizes with IFN-γ in NO production.

Discussion

It is now generally agreed that NO production represents a major mechanism by which activated macrophages eliminate intracellular bacteria, protozoa and fungi and thereby resolve the infection (James & Claven, 1989; Green *et al.*, 1990; Adams *et al.*, 1990, 1991; Denis, 1991; Alspaugh & Granger, 1991; Boockvar *et al.*, 1994). The possible involvement of NO in resistance to and recovery from infections with the ultimate intracellular pathogens, the viruses, has received comparatively less attention.

Karupiah *et al.* (1993) presented compelling evidence that induction of NO synthase with subsequent production of NO can be an important antiviral effector mechanism of IFN-γ in macrophages and possibly in other cell types as well. Thus, the ability of IFN-γ to inhibit replication of ectromelia virus, vaccinia virus and HSV-1 in mouse macrophages correlated with the cells' production of NO, and epithelial cells transfected with an iNOS-encoding cDNA or treated with the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) restricted virus replication. Furthermore, treatment of mice with an inhibitor of iNOS interfered with resolution of a generalized mousepox infection. Subsequently, the same authors showed that IFN-γ-induced NO inhibited vaccinia virus late protein synthesis, DNA replication and particle formation in infected macrophages and bystander cells of epithelial origin (Harris *et al.*, 1995). Similar conclusions were obtained independently by Mělková & Esteban (1995) using a different approach. Others have also suggested the involvement of NO as an antiviral mediator of macrophages. Thus, Croen (1993) observed a correlation between nitrite production and antiviral activity in macrophages stimulated with IFN-γ and LPS and infected with HSV-1. The antiviral effect was substantially reduced by inhibitors of NO synthase, and the effect of IFN-γ and LPS could be mimicked by addition of SNAP to the cells.

Although these studies suggest that NO might have important antiviral potential, they do not address the important question as to whether virus infection of macrophages is actually able to induce NO production. Studies by others have yielded equivocal results. Thus, Lyon & Hinshaw (1993) showed that influenza virus infection of avian macrophage cell lines interfered with spontaneous and LPS-induced secretion of NO, whereas Kreil & Eibl (1995) found that infection of thioglycollate-elicited mouse macrophages with a flavivirus primed the cells for LPS-triggered NO production but downregulated NO production in macrophages activated with IFN-γ and TNF-α. Adler *et al.* (1994) found that infection with a noncytopathic strain of bovine viral diarrhoea virus primed bovine macrophages for enhanced NO production in response to *Salmonella dublin*, whereas infection with a cytopathic strain inhibited NO generation. Interestingly, HIV and even recombinant gp120 have been shown without further stimulation to induce NO secretion in human monocyte/macrophages, in which NO secretion is otherwise less readily detected (Bukrinsky *et al.*, 1995; Pietraforte *et al.*, 1994). It thus seems that the outcome of virus infection of macrophages as regards NO production depends on factors like the source of macrophages, their state of differentiation and activation and the virus in question. Presumably the permissiveness of the various macrophage populations for virus replication or expression of virus-induced regulatory proteins might influence the outcome of the virus-macrophage interaction.

In addition to these studies showing that virus infection of

macrophages *in vitro* can modulate NO production following an appropriate triggering signal, a number of studies have shown that macrophages from virus-infected mice are primed during infection for NO secretion, which can be triggered *ex vivo* with virus (Karupiah & Harris, 1995) or IFN-γ (Rowland *et al.*, 1994; Butz *et al.*, 1994).

Our study on the ability of HSV-2 to induce the production of nitrite in cultures of resting peritoneal macrophages from BALB/c mice showed that the virus alone induced secretion of only very low and varying amounts of nitrite. This is in agreement with the generally observed phenomenon that production of higher amounts of NO in macrophages depends on more than one signal being delivered to the cells (Ding *et al.*, 1988; Drapier *et al.*, 1988). The mechanism behind the low but significant production of NO by HSV-2 in peritoneal cell cultures is not apparent. Mock virus preparations did not induce NO secretion and virus inactivation by UV irradiation or heat treatment abolished the effect. It thus seems that the effect was not due to LPS contamination or mere phagocytosis of cellular debris in the virus preparation. Furthermore, the virus preparation was found by *Limulus* amoebocyte lysate assay not to contain appreciable amounts of LPS. Most probably, the NO secretion results from the production of cytokines like IFN-γ by NK cells and TNF-α by macrophages in the unseparated peritoneal cell culture used in our study. We have deliberately chosen to work with cultures of unseparated resting peritoneal cells in order to have the macrophages as close to the natural *in vivo* situation as possible, including the possibility for the macrophages to cooperate with cells with which they are normally found together.

The involvement of cytokines in the HSV-2-induced NO production in mouse peritoneal macrophages was underlined in our examination of the effect of costimulation with IFN-γ. Whereas both HSV-2 and IFN-γ by themselves only induced secretion of marginal or modest amounts of NO over a 24 h period, a clear synergistic effect between the virus and IFN-γ was noticed. Production of nitrite after combined IFN-γ stimulation and HSV-2 infection was accompanied by accumulation of *iNOS* mRNA, indicating that NO production originated from induction of the *iNOS* gene. The ability of HSV-2 to act as a second signal to IFN-γ-treated macrophages also requires infectious virus. However, it does not seem to depend on productive infection, since resting peritoneal macrophages, even from relatively susceptible BALB/c mice, are relatively nonpermissive for HSV-2 replication (Mogensen, 1979). Experiments with an antiserum against TNF-α revealed that the phenomenon was largely dependent on autocrine secretion of endogenous TNF-α, which functioned as a trigger for NO production in macrophages simultaneously primed by IFN-γ.

We have previously shown that 10–20 units/ml of TNF-α are produced within 10 h in mouse peritoneal cell cultures infected with a dose of HSV-2 similar to that used in this study, and that TNF-α in an autocrine manner synergizes with endogenously produced IFN-α/β in priming of macrophages for a respiratory burst upon infection with HSV-2 (Ellermann-Eriksen, 1993). It thus seems that the second signal function in NO production in virus-infected macrophages is yet another of the multifarious accessory functions of TNF-α in inflammation. In the study by Kreil & Eibl (1995) the effect of flavivirus infection of macrophages on subsequent induction of NO by LPS or IFN-γ/TNF-α could be mimicked by addition of IFN-α/β and neutralized by anti-IFN-α/β antibodies, indicating that endogenously produced IFN-α/β is responsible for the effects of the virus infection. In our study IFN-α/β did not seem to be involved, since we did not observe an effect of a neutralizing anti-IFN-α/β antibody on the synergistic effect of HSV-2 infection on IFN-γ-induced NO production.

The mechanism described in this study with TNF-α as an intermediary, endogenously produced trigger of NO production in IFN-γ-primed and HSV-2-infected macrophages is similar to the one by which *Leishmania major* amastigotes induce NO secretion in IFN-γ-stimulated macrophages, which eventually leads to killing of the intracellular parasite (Green *et al.*, 1990). Our results, together with the above-mentioned studies on the implication of NO production in antiviral activity of macrophages against a number of viruses (Karupiah *et al.*, 1993; Croen, 1993; Adler *et al.*, 1994; Harris *et al.*, 1995; Karupiah & Harris, 1995; Mělková & Esteban, 1995; Bi & Reiss, 1995; Kreil & Eibl, 1995; Jelachich *et al.*, 1995), and among these notably HSV (Karupiah *et al.*, 1993; Croen, 1993; Karupiah & Harris, 1995), suggest that NO production by macrophages recruited into an infectious focus, where the cells might be primed by IFN-γ *and* infected with the virus, could constitute a localized and targeted antiviral mechanism working both at the level of the macrophage itself, making it nonpermissive for virus replication (intrinsic restriction) as well as on surrounding stromal cells (extrinsic restriction). An antiviral mechanism implicating depletion of arginine (which is essential for virus replication) by arginase secreted by activated macrophages in both intrinsic (Sethi, 1983) and extrinsic (Wildy *et al.*, 1982) restriction of HSV replication was suggested in the early 1980s. However, a direct link between this antiviral mechanism and one involving arginine-derived toxic nitrogen intermediates does not seem clear at the moment (Karupiah *et al.*, 1993; Croen, 1993).

In our murine model of genetically determined resistance to infection with HSV-2, which has its human counterpart in the generalized herpesvirus infection of the neonate, we have previously shown that resistance to the infection *in vivo*, restriction of virus replication in macrophages *in vitro* and the production of IFN-α/β and TNF-α by macrophages, as well as the sensitivity of macrophages to activation by these cytokines, cosegregate between susceptible and resistant strains of mice (Mogensen, 1979, 1988; Ellermann-Eriksen *et al.*, 1986, 1989; Mogensen *et al.*, 1989; Ellermann-Eriksen, 1993). We are currently investigating the possible role of NO in antiviral resistance in this model.

This study was supported by grants from the Danish Health Science Research Council (grant number 12–1622) and the Aarhus University Research Foundation (grant number F-1993-Sun-1–53). H.B. was supported by a fellowship from the Turkish Research Council [grant number B.02.1.Bak.0.09.00.00(94)/3680]. The excellent technical assistance of Ms Birthe Søby is greatly appreciated.

References

Adams, L. B., Hibbs, J. B., Jr, Taintor, R. R. & Krahenbuhl, J. L. (1990). Microbiostatic effect of murine activated macrophages for *Toxoplasma gondii*: role of synthesis of inorganic nitrogen oxides from L-arginine. *Journal of Immunology* **144**, 2725–2729.

Adams, L. B., Franzblau, S. G., Vavrin, Z., Hibbs, J. B., Jr & Krahenbuhl, J. L. (1991). L-Arginine-dependent macrophage effector functions inhibit metabolic activity of *Mycobacterium leprae*. *Journal of Immunology* **147**, 1642–1646.

Adler, H., Frech, B., Meier, P., Jungi, T. W. & Peterhans, E. (1994). Noncytopathic strains of bovine viral diarrhea virus prime bovine bone marrow-derived macrophages for enhanced generation of nitric oxide. *Biochemical and Biophysical Research Communications* **202**, 1562–1568.

Alspaugh, J. A. & Granger, D. L. (1991). Inhibition of *Cryptococcus neoformans* replication by nitrogen oxides supports the role of these molecules as effectors of macrophage-mediated cytostasis. *Infection and Immunity* **59**, 2291–2296.

Bi, Z. & Reiss, C. S. (1995). Inhibition of vesicular stomatitis virus infection by nitric oxide. *Journal of Virology* **69**, 2208–2213.

Boockvar, K. S., Granger, D. L., Poston, R. M., Maybodi, M., Washington, M. K., Hibbs, J. B., Jr & Kurlander, R. L. (1994). Nitric oxide produced during murine listeriosis is protective. *Infection and Immunity* **62**, 1089–1100.

Bredt, D. S. & Snyder, S. H. (1994). Nitric oxide: a physiologic messenger molecule. *Annual Review of Biochemistry* **63**, 175–195.

Bukrinsky, M. I., Nottet, H. S. L. M., Schmidtmayerova, H., Dubrovsky, L., Flanagan, C. R., Mullins, M. E., Lipton, S. A. & Gendelman, H. E. (1995). Regulation of nitric oxide synthase activity in human immunodeficiency virus type 1 (HIV-1)-infected monocytes: implications for HIV-associated neurological disease. *Journal of Experimental Medicine* **181**, 735–745.

Butz, E. A., Hostager, B. S. & Southern, P. J. (1994). Macrophages in mice acutely infected with lymphocytic choriomeningitis virus are primed for nitric oxide synthesis. *Microbial Pathogenesis* **16**, 283–295.

Chomczynski, P. & Sacchi N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

Croen, K. D. (1993). Evidence for an antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. *Journal of Clinical Investigation* **91**, 2446–2452.

Denis, M. (1991). Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. *Cellular Immunology* **132**, 150–157.

Ding, A. H., Nathan, C. & Stuehr, D. J. (1988). Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *Journal of Immunology* **141**, 2407–2412.

Drapier, J.-C., Wietzerbin, J. & Hibbs, J. B., Jr (1988). Interferon-γ and tumour necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. *European Journal of Immunology* **18**, 1587–1592.

Ellermann-Eriksen, S. (1993). Autocrine secretion of interferon-α/β and tumour necrosis factor-α synergistically activates mouse macrophages after infection with herpes simplex virus type 2. *Journal of General Virology* **74**, 2191–2199.

Ellermann-Eriksen, S., Liberto, M. C., Iannello, D. & Mogensen, S. C. (1986). X-linkage of the early *in vitro* α/β-interferon response of mouse peritoneal macrophages to herpes simplex virus type 2. *Journal of General Virology* **67**, 1025–1033.

Ellermann-Eriksen, S., Sommerlund, M. & Mogensen, S. C. (1989). Differential sensitivity of macrophages from herpes simplex virus-resistant and -susceptible mice to respiratory burst priming by interferon-α/β. *Journal of General Virology* **70**, 2139–2147.

Green, S. J., Crawford, R. M., Hockmeyer, J. T., Meltzer, M. S. & Nacy, C. A. (1990). *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism of IFN-γ-stimulated macrophages by induction of tumor necrosis factor-α. *Journal of Immunology* **145**, 4290–4297.

Harris, N., Buller, R. M. L. & Karupiah, G. (1995). Gamma interferon-induced, nitric oxide-mediated inhibition of vaccinia virus replication. *Journal of Virology* **69**, 910–915.

Hibbs, J. B., Jr, Taintor, R. R. & Vavrin, Z. (1987). Macrophage cytotoxicity: role for L-arginine deiminase and iminonitrogen oxidation to nitrite. *Science* **235**, 473–476.

James, S. & Claven, J. (1989). Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *Journal of Immunology* **143**, 4208–4212.

Jelachich, M. L., Bandyopadhyay, P., Blum, K. & Lipton, H. L. (1995). Theiler's virus growth in murine macrophage cell lines depends on the state of differentiation. *Virology* **299**, 437–444.

Karupiah, G. & Harris, N. (1995). Inhibition of viral replication by nitric oxide and its reversal by ferrous sulfate and tricarboxylic acid cycle metabolites. *Journal of Experimental Medicine* **181**, 2171–2179.

Karupiah, G., Blanden, R. V. & Ramshaw, I. A. (1990). Interferon γ is involved in recovery of athymic nude mice from recombinant vaccinia virus/interleukin 2 infection. *Journal of Experimental Medicine* **172**, 1495–1503.

Karupiah, G., Xie, Q., Buller, R. M. L., Nathan, C., Duarte, C. & MacMicking, J. D. (1993). Inhibition of viral replication by interferon-γ-induced nitric oxide synthase. *Science* **261**, 1445–1448.

Kohonen-Corish, M. R. J., King, N. J. C., Woodhams, C. E. & Ramshaw, I. A. (1990). Immunodeficient mice recover from infection with vaccinia virus expressing interferon-γ. *European Journal of Immunology* **20**, 157–161.

Kreil, T. R. & Eibl, M. M. (1995). Viral infection of macrophages profoundly alters requirements for induction of nitric oxide synthesis. *Virology* **212**, 174–178.

Lyon, J. A. & Hinshaw, V. S. (1993). Inhibition of nitric oxide induction from avian macrophage cell lines by influenza virus. *Avian Diseases* **37**, 868–873.

Mělková, Z. & Esteban, M. (1995). Inhibition of vaccinia virus DNA replication by inducible expression of nitric oxide synthase. *Journal of Immunology* **155**, 5711–5718.

Mogensen, S. C. (1979). Role of macrophages in natural resistance to virus infections. *Microbiological Reviews* **43**, 1–26.

Mogensen, S. C. (1988). Macrophages and natural resistance to virus infections. In *The Reticuloendothelial System: A Comprehensive Treatise*, vol. 10, pp. 207–223. Edited by J. P. Utz & M. R. Escobar. New York: Plenum Publishing Corporation.

Mogensen, S. C., Ellermann-Eriksen, S. & Sommerlund, M. (1989). Herpes simplex virus type 2 primes mouse macrophages for an early and

genetically determined respiratory burst mediated by interferon-α/β. *Journal of General Virology* **70**, 1371–1379.

Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* **43**, 109–142.

Morahan, P. S., Connor, J. R. & Leary, K. R. (1985). Viruses and the versatile macrophage. *British Medical Bulletin* **41**, 15–21.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB Journal* **6**, 3051–3064.

Pietraforte, D., Tritarelli, E., Testa, U. & Minetti, M. (1994). gp120 HIV envelope glycoprotein increases the production of nitric oxide in human monocyte-derived macrophages. *Journal of Leukocyte Biology* **55**, 175–182.

Rowland, R. R. R., Butz, E. A. & Plagemann, P. G. W. (1994). Nitric oxide production by splenic macrophages is not responsible for T cell suppression during acute infection with lactate dehydrogenase-elevating virus. *Journal of Immunology* **152**, 5785–5795.

Sethi, K. K. (1983). Contribution of macrophage arginase in the intrinsic restriction of herpes simplex virus replication in permissive macrophage cultures induced by gamma-interferon containing products of activated spleen cells. *Immunobiology* **165**, 459–474.

Wildy, P., Gell, P. G., Rhodes, J. & Newton, A. (1982). Inhibition of herpes simplex virus multiplication by activated macrophages: a role for arginase. *Infection and Immunity* **37**, 40–45.

Wu, L. & Morahan, P. S. (1992). Macrophages and other nonspecific defenses: role in modulating resistance against herpes simplex virus. *Current Topics in Microbiology and Immunology* **179**, 89–110.

Received 24 July 1996; Accepted 27 September 1996