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The effect of human cytomegalovirus infection of human monocytes on The expression of monocyte-associated mediator genes with particular reference to interleukin 1

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to resolve IL-1β mRNA expression at the single cell level. For the three different donors analysed (Figure 13), HCMV infection always had the same effect on IL-1β mRNA expression, in that the percentage of positive cells at 1,2 and 4 days post-infection was always higher in infected cells than in uninfected controls (Figure 13). However, the level of intensity of IL-1β expression varied from donor to donor (Figure 13). In each case, a small fraction of the infected population showed chronic expression of IL-1β mRNA which was sustained for up to 96hr (Figures 13 and 14). This fraction constituted 1 to 8% of the total number of positive cells counted, which is in the same range as that reported for expression of HCMV IE antigen after in vitro infection (Rice et al., 1984; Einhorn and Ost, 1984). Further studies using double-labelling in situ hybridization for detection of HCMV IE mRNA and IL-1β mRNA may determine if the cells expressing IE genes are the same as those chronically expressing IL-1β mRNA.

The upregulation of IL-1β expression suggested that HCMV infection could have important influences on the expression of certain cellular genes. The effect of infection on expression of several other monocyte mediator genes was therefore studied. Using the promyelocytic cell line, ML-3, the effect of variable states of differentiation on this response was also investigated. The expression of steady state levels of

IL-1β, TNFα, CSF-1 and three novel monocyte adherence induced genes (MAD 2, 6 and 9) was studied in order to assess the effect of HCMV infection in terms of a more overall response to infection and inflammation such as would be encountered in vivo. In ML-3 cells that had been induced to differentiate along the monocytic pathway by exposure to PMA, significantly higher levels of steady state mRNA for IL-1β, TNFα, MAD-9 and CSF-1 were observed within 24hr of virus exposure and this continued for the next several days of culture. Expression of MAD-2 and MAD-6 was not induced following exposure to PMA and was not further enhanced following HCMV infection (Figure 10). Steady state levels of neither of the constitutively expressed genes-lysozyme nor β-actin-was significantly modulated by HCMV infection (Figure 9). The only genes showing enhanced and continued expression following HCMV infection are those which are induced with PMA prior to infection, demonstrating that HCMV was acting selectively as an enhancer rather than an inducer of these inflammatory related genes. Control studies demonstrated that only a low level of IL-1β induction was detected in undifferentiated HCMV-infected ML-3 cells (Figure 9). These levels of IL-1β mRNA expression took several weeks of exposure of autoradiographs to detect. Infection of monocytes which results in restricted expression of IE viral gene products only is therefore sufficient to modify mRNA expression of selected

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monocyte mediator genes in ML-3 cells induced to differentiate prior to infection. The induction of selected genes therefore has important implications when considering the effect of viral infection in terms of the overall in vivo response to infection and inflammation.

Induction of the granulocyte pathway of differentiation with DMSO did not result in the level of HCMV-dependent upregulation of IL-1β expression that was observed in PMA-differentiated cells (Figure 11) and suggests that the different states of differentiation of mononuclear phagocytic cells have distinct influences on the ability of HCMV to modify expression of a mediator gene such as IL-1β. The low levels of IL-1β mRNA seen at 24hr following exposure to virus had significantly decreased by 72hr, indicating that the enhanced expression was not sustained as was observed with PMA differentiated cells. This apparent requirement for PMA differentiation along the monocytic pathway suggests the need for a host-derived competence factor, one that is apparently present in monocytes but not in granulocytes. In the in vivo situation, cells of the mononuclear phagocyte lineage could therefore respond differently to infection depending on their degree of maturation and the presence of different physiological stimuli at different anatomical sites.

A similar model of selective monokine upregulation as a result of virus infection has been described by Folks et al. (1987) in which cloned promonocyte cell lines were derived from U937 cells chronically infected with HIV. One clone, designated U1, showed minimal constitutive expression of HIV-1 but was latently infected with two integrated copies of provirus per cell. Virus expression could be upregulated by a PHA-induced supernatant containing multiple cytokines, thus monocyte precursors may be noncytopathologically infected , but capable of secreting virus after appropriate induction. HIV infection of the U1 clone resulted in the selective upregulation of IL-1β expression, an indication that HIV infection may have important influences on expression of certain cellular genes, as has observed with HCMV in this study. Further studies on the latently infected promonocyte cell, U1, have shown that differentiation by PMA resulted in HIV replication which occurred concurrently with NF-κB binding activity (Griffin et al., 1988). NF-κB is the same transcription factor that stimulates the HIV enhancer in activated T cells (Nabel et al., 1987). In this study, differentiation in vitro with PMA did not result in increased replication of HCMV, a result confirmed in a study by Weinshenker et al. (1988) who showed that TPA-induced differentiation of the promyelocytic cell line, HL-60, did not result in an increase of HCMV replication beyond expression of IE

gene products. Differentiation of the monocyte cell line, THP-1, however, resulted in productive HCMV infection, showing that initial cell lineage is important.

In this study, a direct relationship between cellular genes that are induced by PMA differentiation and enhancement by the IE gene products that were expressed in ML-3 cells has been shown.

In contrast to the studies which focused on steady-state levels of proIL-1 mRNA, transfection experiments involving putative proIL-1β regulatory region-CAT fusion DNA constructs, demonstrated that HCMV-mediated upregulation was probably at the level of gene transcription. Co-transfection of proIL-1β-CAT plasmids together with plasmids encoding HCMV IE gene region products demonstrated <u>trans</u>-activation of the IL-1β gene only in ML-3 cells induced to differentiate along the monocytic pathway prior to transfection. When plasmid 3M-AT containing 524 bp of proIL-1β genomic DNA was transfected singly into PMA-differentiated ML-3 cells there was no CAT activity (Figure 20). When 3M-AT was co-transfected with the HCMV region 1 and 2 expression plasmid, pHD101SV1, there was stimulation of the IL-1β promoter as judged by CAT activity. Furthermore, the observed <u>trans</u>-activation appeared primarily to require region 2 of the HCMV IE region

DNA. The pHD101SV2 DNA (encoding region 1 sequences only) supported little activation as judged by CAT activity. The observation that 10142, which contains some region 1 sequences in addition to region 2, has some effect on activity, suggests a possible role for IE region 1 gene products perhaps in supporting region 2 gene expression. The HCMV DNAs used in this study have also been shown to be effective at trans-activating HIV LTR transcription in a TAT/TAR independent fashion (Davis et al., 1987). This study has demonstrated that the proIL-1β upstream sequences, like the upstream HIV LTR DNA, can also be trans-activated by HCMV IE region 2. There is no extensive sequence homology between the upstream proIL-1β and HIV LTR responsive sequences. However, some homology does exist, and it is possible that a short factor-binding sequence may be shared between these two DNAs. It is also possible that the HCMV region 2 gene product is indirectly involved in the activation pathway.

Figure 20B shows that co-transfection experiments using proIL-1β -CAT construct DNA together with plasmids encoding HCMV IE gene region products in ML-3 cells that were not induced to differentiate prior to transfection, resulted in no stimulation of proIL-1β promoter activity. This suggested the need for host-derived competence factors as was also observed in steady-state IL-1β mRNA studies.

Enhanced IL-1β mRNA expression did not necessarily lead to sustained translational activity as shown by flow cytometric and Western blot analyses. Enhanced levels of intracellular IL-1β protein were observed at 24hr post-infection and not at later time points (72hr), supporting the view that IL-1β protein is either degraded or rapidly exported following expression, in contrast to previous observations with PMA-stimulated THP-1 cells (Fenton et al., 1988). In addition, studies by Haskill et al. (1988) have shown that although IL-1β expression is rapidly induced following adherence of monocytes, little, if any protein is secreted in the absence of a second signal, as is usually produced following exposure to LPS. The data in this study derived from Western blot and ELISA analyses, indicate that HCMV can probably also serve that role.

HCMV infection, although not leading to productive infection in human monocytes, nontheless may contribute to the pathology of the infection through selective enhancement of monocyte-associated mediator genes with susequent stimulation of protein synthesis. The highly variable states of differentiation and/or stimulation that cells of the mononuclear phagocyte series may exhibit appear to profoundly influence the outcome of virus exposure on expression and production of IL-1β at molecular and functional levels.

5.0.0. CONCLUSIONS

1. The laboratory-adapted strains of HCMV, AD169 and Towne, initiated expression of detectable IE antigen in a very low percentage of infected monocytes (0.01% - 0.1%) whereas 2 - 9% of monocytes infected with a clinical isolate expressed IE antigen.

2. The percentage of IE antigen-positive monocytes was not different for stimulated or unstimulated peripheral blood monocytes. Nuclear or perinuclear staining was observed for up to 7 days post-infection.

3. Virus gene expression was also limited to IE gene products in the promyelocytic cell line, ML-3. Induction of differentiation to the monocyte or granulocyte phenotype did not result in a full cycle of replication, suggesting that the initial cell lineage was important for the establishment of productive HCMV infection.

4. At a functional level, peripheral blood monocytes infected *in vitro* with HCMV were suppressive for T lymphocyte blastogenesis. LPS-stimulated monocytes challenged with either strain of HCMV were more suppressive for autologous T lymphocyte responses

than were virus-infected unstimulated monocytes when tested under identical assay conditions.

5. Infection of both unstimulated and stimulated monocytes reduced the production of functional IL-1 bioactivity, as assessed in the conventional bioassay which measures the ability of IL-1 to augment the proliferation of mouse thymocytes in the presence of PHA.

6. Addition of exogenously supplied IL-1 to stimulated HCMV-infected monocytes fully restored the PHA-induced proliferation of autologous T lymphocytes in subsequent culture to that observed in the presence of uninfected, stimulated monocytes. Addition of exogenously supplied IL-1 to T lymphocytes cultured with unstimulated, HCMV-infected monocytes did not reconstitute the PHA-induced proliferative response. The mechanism(s) of HCMV-mediated immunosuppression therefore appear to be different for stimulated and unstimulated monocytes.

7. Quantitative measurement of IL-1β levels in monocyte-conditioned supernatants using an ELISA showed that supernatants derived from unstimulated HCMV-infected monocytes contained substantially more IL-1β than control supernatants from

uninfected monocytes. However, levels of IL-1β in supernatants from LPS-stimulated HCMV-infected monocytes were significantly lower compared with those of uninfected controls.

8. At the level of IL-1β steady-state mRNA expression, both RNA dot-blot and in situ hybridization studies demonstrated that infection of human monocytes with HCMV lead to sustained expression of IL-1β mRNA for up to 96hr post-infection, which contrasted markedly with mock-infected controls. At the single cell level, in situ hybridization studies showed that the sustained IL-1β mRNA expression was due to a small fraction of infected cells chronically expressing IL-1β message rather than the entire population expressing equivalent levels of IL-1β mRNA.

9. Exposure of ML-3 cells to virus prior to induction of differentiation had little influence on mediator gene expression. However, induction of the monocyte phenotype by pretreatment of ML-3 cells with the phorbol ester, PMA, followed by HCMV challenge resulted in a greatly extended period of IL-1β expression, as well as other monocyte-associated mediator genes, TNFα, CSF-1 and MAD-9. Expression of two other inflammatory mediator genes, MAD-2 and MAD-6, were not similarly upregulated, thereby

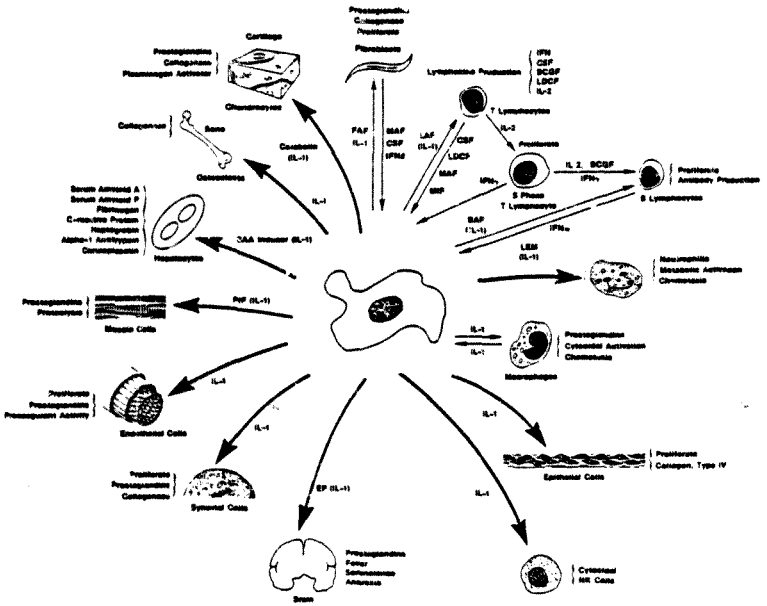
demonstrating that HCMV infection resulted in selective upregulation of monocyte-associated genes in differentiating ML-3 cells.

10. Constitutively expressed genes such as lysozyme and β-actin were not similarly modulated in ML-3 cells induced to differentiate along the monocyte pathway.

11. Induction of the granulocyte phenotype by pretreatment of ML-3 cells with DMSO did not result in upregulation of IL-1β expression.

12. Analyses of the levels of intracellular IL-1β in ML-3 cells and monocytes using flow cytometry and Western blot analyses revealed enhanced levels of IL-1β protein in infected cells over those of controls.

13. Cotransfection experiments using IL-1β-CAT chimeric plasmids together with plasmids encoding HCMV IE gene region products demonstrated trans-activation of the IL-1β promoter in ML-3 cells induced to differentiate along the monocytic pathway prior to transfection.



APPENDIX A

Effects of IL-1 on target cells and tissues. The diagram shows bidirectional interactions between macrophages and lymphoid cells or fibroblasts mediated by IL-1 and other cytokines and the activities of target cells known to be augmented by IL-1. IFN = interferon; CSF = colony stimulating factor; BCGF = B cell growth factor; LDCF = lymphocyte derived chemotactic factor; SAA = serum amgloid A; PIF = proteolysis inducing factor; EP = endogenous pyrogen (after Oppenheim et al., 1986)

APPENDIX 1

AET TREATMENT OF SHEEP RED BLOOD CELLS (SR3C)

(1) MATERIALS

(i) Sterile SRBC in PBS with anticoagulant (not more than 2 weeks old)

(ii) 2-aminoethyl-isothiouronium bromide (AET) (Sigma)

(iii) NaOH, 5N

(iv) Normal saline

(v) Foetal calf serum (FCS)

(2) METHOD

(i) Wash SRBC aseptically 3 times with normal saline.

(ii) Dissolve 100 mg AET in 5 ml glass distilled water.

(iii) Add 5 ml AET solution to 1 ml packed SRBC.

(iv) Incubate at 37°C for 15 min, mixing the solution every 5 min.

(v) Wash SRBC with normal saline at least 3 times until supernatant is clear.

vi) Resuspend SRBC to a final concentration of 10% in RPMI 164) containing FCS pre-absorbed with fresh SRBC (1h at 37°C followed by 1h at 4°C).

AMMONIUM CHLORIDE LYSING BUFFER

(i) Ammonium chloride	0.829g
(ii) $KHCO_3$	0.1 g
(iii) EDTA	3.7 mg

Dissolve in ice cold distilled water and filter sterilize. Prepare fresh each time.

PBS (10X stock solution /litre)

NaCl	80 g
KCl	2 g
$Na_2HPO_4.7H_2O$	11.5 g
KH_2PO_4	2 g

APPENDIX 2

TITRATION OF HCMV

(1)

(a) HEL fibroblasts were grown to confluency in 35 mm plastic tissue culture dishes in HEL medium ((2)).

(b) Culture supernatant containing virus was serially diluted (10-fold) from a 10^{-1} dilution to a 10^{-8} dilution in 2X HEL medium.

(c) Aliquots (0,2 ml) of each dilution were plated onto three 35 mm tissue culture plates containing confluent HEL fibroblasts. Control plates had 0,2 ml 2X HEL medium plated.

(d) The plates were rocked at 33 C for 2 hrs.

(e) Cells were then overlaid with 2 ml of a 1:1 mixture of overlay medium ((3)) and 0.6% agarose ((5)).

(f) The titration was incubated at 33 C for 14 days, during which cells were fed twice weekly with a 1:1 mixture of overlay medium and 0.6% agarose.

(g)After 14 days, plates were fixed for 4 hrs by addition of 2 ml of 10% buffered formalin ((5)) to each plate.

(h) Following fixation, formalin and agarose were removed and cells stained with 0.5% methylene blue for a minimum of 15 mins.

(i) Plates were rinsed with tap water and plaques counted under a dissecting microscope.

(2) HEL MEDIUM

Dulbecco's Modified Eagle's Medium (DME) containing L-glutamine (2 mM), sodium bicarbonate (0.37%), penicillin (100U/ml), streptomycin (200 µl/ml) and 10% FCS.

2X HEL MEDIUM 1X HEL medium containing sodium bicarbonate (0.74%).

(3) OVERLAY MEDIUM

DME without phenol red and supplemented with 5% FCS.

(4) 10% BUFFERED FORMALIN

$NaH_2PO_4H_2O$ 4g
Distilled H_2O to 500 ml
Stir until dissolved, then add
Na_2HPO_4 6,5g
Distilled H_2O to 400 ml
Stir until dissolved, then add 100 ml formaldehyde.

(5) 0.6% AGAROSE

Agarose, 6g, was dissolved by heating in 1l of distilled water. The solution was then autoclaved and stored at 4°C.

APPENDIX 3

POLYACRYLAMIDE GEL ELECTROPHORESIS

(1) SLAB GEL SOLUTIONS

(a) 4X RUNNING GEL BUFFER (0.4% SDS containing 1.5 M Tris-HCl, pH 8.8)

Tris	18.16 g
conc.HCl (36%)	2.30 ml
SDS	0.40 g
Distilled water	to 100 ml

(b) 4X STACKING GEL BUFFER (0.4% SDS containing 0.5 M Tris-HCl, pH 6.8)

Tris	3.028 g
conc.HCl (36%)	1.50 ml
SDS	0.20 g
Distilled water	to 50 ml

(2) 10X ELECTRODE BUFFER (1.0% SDS containing 0.25 M Tris-glycine, pH 8.6)

Tris	30.28 g
Glycine	144.25 g

SDS	10.00 g
Distilled water	to 1 000 ml

(3) ACRYLAMIDE STOCK SOLUTION (30:0,8)

Acrylamide	150 g
Bis acrylamide (N,N'-methylene diacrylamide)	4 g
Distilled water	to 500 ml

(4) BROMOPHENOL BLUE TRACKING DYE

SDS	5,0 g
2-Mercaptoethanol	5,0 ml
Glycerol	7,5 ml
1 M Tris-HCl pH 6,8	35,0 ml
0.2% bromophenol blue (w/v)	2,5 ml

(5) RUNNING GEL

Acrylamide stock solution	6 ml
1,5 M Tris-HCl pH 8,8	15 ml
Distilled water	6 ml
1,5% Ammonium persulphate	2 ml
TEMED	10 µl

(6) STACKING GEL

Acrylamide stock solution	1 ml
0,5 M Tris-HCl pH 6,8	5 ml
Distilled water	4 ml

1,5% Ammonium persulphate	0.5 ml
TEMED	5 µl

(7) 2X SAMPLE BUFFER

Distilled water	4,00 ml
Tris	0,15 g
pH to 6,8 with 1N HCl	
10% SDS	4,00 ml
Glycerol	1,00 ml
β-mercaptoethanol	0,40 ml

Bring volume to 10 ml with distilled water

(8) MOLECULAR WEIGHT CALIBRATION KITS

Pharmacia high molecular weight kit

Thyroglobulin	669 000
Ferritin	440 000
Catalase	232 000
Lactate dehydrogenase	140 000
Bovine serum albumin	67 000

Dissolve the protein mixture in 100µl of sample buffer and heat it in a boiling water bath for 5 min to dissociate the proteins into their subunits (molecular weights given). Apply 15µl to gel.

Pharmacia low molecular weight calibration kit

Protein	Molecular Weight
Phosphorylase b	94 000
Bovine serum albumin	67 000
Ovalbumin	43 000
Carbonic anhydrase	30 000
Soybean trypsin inhibitor	20 000
α-lactalbumin	14 400

Dissolve the protein mixture in 100 µl of sample buffer and heat it in a boiling water bath for 5 min. Apply 15 µl to gel.

APPENDIX 4

RNA PROCEDURES

(1) Diethylpyrocarbonate (DEPC) treated water

Add 1 ml of DEPC to 1 l of distilled water. Mix thoroughly and autoclave.

(2) 4 M GUANIDINE ISOTHIOCYANATE

Guanidine isothiocyanate	94.53 g
3 M sodium acetate pH 6	1.67 ml
β-mercaptoethanol	1.67 ml

Bring volume to 200 ml with DEPC treated water

Filter sterilize (0.8 µm millipore filter)

(3) 5.7 M CsCl

Cesium chloride	95.97 g
3 M sodium acetate (pH 6)	0.83 ml

Bring volume to 100 ml with DEPC treated water

Filter sterilize using a 0.9 µm filter

(4) Morpholinopropane-sulfonic acid (MOPS) buffer (10X)

3-[N-Morpholino]-propane-sulfonic acid	0.2M
Sodium acetate, pH 7.0	0.05M
EDTA	0.01M

(5) LOADING BUFFER

Formamide	7,2 ml
10X MOPS buffer	1,6 ml
Formaldehyde (37%)	2,6 ml
DEPC water	1,8 ml
Glycerol	1,0 ml
Bromophenol blue (saturated solution)	0,8 ml

(6) SSC BUFFER (20X CONCENTRATE)

NaCl	175,3 g
$Na_3Citrate.2H_2O$	88,2 g
H_2O	800 ml
Adjust pH to 7.0 w.	M HCl
Add water	to 1 litre

(6) 100X DENHARDTS

Ficoll 400	10 g
Polyvinylpyrrolidine	10 g
BSA (Pentax Fraction V)	10 g
Distilled water	to 500 ml

Filter and store at -20 °C.

(7) TE (pH 7.4, 7.5, 8.0)

10 mM Tris.HCl pH 7.4, 7.5, 8.0 adjusted with 1N HCl

1 mM EDTA pH 8.0

(8) 10X KINASE BUFFER 1

Tris.HCl (pH 7.6)	0.5 M
$MgCl_2$	0.1 M
Dithiothreitol	50 mM
Spermidine	1 mM
EDTA	1 mM

(9) 10x NICK-TRANSLATION BUFFER

Tris.HCl (pH 7.2)	0.5 M
$MgSO_4$	0.1M
Dithiothreitol	1 mM
BSA	500 µg/ml

APPENDIX 4B

DOT BLOT* PROCEDURE

(1) LYSIS BUFFER

DEPC water	7 ml

10% SDS	1 ml
2 M NaAc pH 5.2	100 µl
5 M NaCl	500 µl
0.2 M EDTA pH 8	500 µl
10 mg/ml heparin	500 µl

Make up to 10 ml with DEPC water. Aliquot into 1 ml amounts and freeze at -20°C. Just before use, add 10 µl of a 10 mg/ml solution of pronase to a 1 ml aliquot of lysis buffer.

APPENDIX 5

PLASMID DNA PREPARATION

(1) MINIMAL BACTERIAL GROWTH MEDIUM

(a) M9 SALTS (10x concentrate)

KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g
Distilled water	900 ml

pH adjusted to 7.4 with 10 N NaOH

(b) Glucose 20 g

Distilled water 100 ml

Filter sterilize using a 0.45 µm filter

(c) Casein-hydrolysed amino acids 100 g

Bring to 1 litre with distilled water

(d) 1 M $MgSO_4$

(e) 1 M $CaCl_2$

Add solutions (a) and (b) together.

To prepare 1 litre of minimal medium, add the following sequentially:-

sterile dH_2O	800 ml
1 M $MgSO_4$	1 ml
1 M $CaCl_2$	0.1 ml
10X M9 salts + 20% glucose	100 ml
10% casamino acids	100 ml
Ampicillin	50ug/ml growth medium

(2) LURIA - BERTANI (LB) MEDIUM

Distilled water	950 ml
Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Adjust pH to 7.4 with 1 N NaOH

Bring volume to 1 litre with distilled water

Autoclave to sterilize

PLASMID PURIFICATION

ALKALINE LYSIS METHOD

SOLUTION A

50 mM glucose

10 mM EDTA

25 mM Tris pH 8

SOLUTION B

Lysozyme, 10 mg/ml made freshly before use

SOLUTION C

0.2 M NaOH

1% SDS

SOLUTION D

Potassium acetate	29.4 g
Glacial acetic acid	11.5 ml

Add water to 100 ml

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CYTOMEGALOVIRUS INFECTION STIMULATES EXPRESSION OF MONOCYTE-ASSOCIATED MEDIATOR GENES[1]

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Monocytes and tissue macrophages play important roles in host defense against virus infections and, in the case of human cytomegalovirus (HCMV) and HIV, may also be the reservoir for latent disease. Because these cells can also rapidly respond to most infections by secretion of inflammatory mediators, we were interested in determining if HCMV infection could have a direct activating effect on macrophage cytokine production. To do this, we primarily investigated the influence of HCMV infection on IL-1β-mRNA expression in peripheral blood monocytes and the promyelocytic cell line, ML-3 as well as the inflammatory response genes TNF-α, MAD-9, MAD-6, and MAD-2 in the promyelocytic ML-3 cell line. Exposure of ML-3 cells to the virus prior to induction of differentiation had little influence on mediator gene expression. However, induction of the macrophage phenotype by pretreatment of ML-3 cells with the phorbol ester, PMA, followed by HCMV challenge, resulted in a greatly extended period of expression of IL-1β, TNF-α, MAD-9, and CSF-1 but not MAD-6 and MAD-2. Constitutively expressed genes such as lysozyme and actin were not similarly modulated. Both RNA dot-blot and in situ hybridization studies demonstrated that infection of human peripheral blood monocytes with HCMV leads to sustained expression of IL-1β mRNA for up to 72 h, which contrasts markedly with mock-infected or LPS-stimulated monocytes. Flow cytometric analysis of the intracellular levels of IL-1β protein in ML-3 cells indicated that not only was there more protein produced in infected cells, but that the majority of the cells had responded. Enhanced levels of the intracellular form of IL-1β in monocytes was confirmed by Western blot analysis. Cotransfection experiments were performed using IL-1β-CAT chimeric plasmids together with plasmid products. Transactivation of the IL-1β gene by region 2 of the immediate-early gene was observed in ML-3 cells that had been induced to differentiate prior to transfection. No stimulation of IL-1β promoter activity was observed in ML-3 cells that were undifferentiated prior to transfection. In summary, HCMV infection, although not leading to productive infection, nonetheless may contribute to the pathology of the infection through enhancement of monocyte inflammatory mediator gene expression with subsequent stimulation of protein synthesis.

HCMV[3] infection of monocytes, albeit restricted to synthesis of the IE and early gene products, appears to be a critical factor in the immunosuppression that accompanies HCMV infections (1-6). A latent infection can be established in human mononuclear cells after in vitro infection with HCMV, with restriction of virus gene expression to IE and early proteins (1, 7). The frequency of cells expressing the IE gene product ranges from 0 to 5% depending upon the strain of virus and the cell type used (1-5, 7). In spite of the low frequency of infection and the lack of a full cycle of replication in monocytes, dramatic influences on monocyte function have been reported (1-5).

Differentiation signals such as phorbol esters have been shown to activate viruses from latency to productive infection, recently, we demonstrated that this occurs in the promyelocytic cell line, THP-1, could be productively infected by HCMV, but only after exposure to PMA (8). Infection with HCMV before induction of differentiation with PMA was ineffective, suggesting that a state of competence had to be established before infection.

We have been interested in investigating monocyte-associated mediator production in response to HCMV infection and the effect of variable states of differentiation on this response. In this study, we were primarily interested in the effect of HCMV infection on transcription and translation of the important inflammatory mediator gene IL-1β in blood monocytes and in ML-3 cells. However, in order to investigate the effect of HCMV exposure in terms of an overall response to infection and inflammation, we also studied expression of a number of other mediator genes in ML-3 cells, TNF-α, like IL-1β, has a systemic influence on host physiology manifested in rapid and prolonged effects (9, 10). The macrophage-

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[3] Abbreviations used in this paper: HCMV, human cytomegalovirus; IE, immediate-early; CAT, chloramphenicol acetyltransferase.

specific growth and differentiation factor (CSF-1) has been shown to be a sufficient stimulus for monocyte survival and differentiation under serum-free conditions (11). All three genes are similar in that they alter various macrophage functions (autocrine/paracrine) effects (11–13). The three other inflammatory response genes that were studied, MAD-9, MAD-8, and MAD-2, were derived from a series of cDNA clones that were specifically induced in monocytes in a rapid response to adherence. MAD-9 is the same clone as MDNCF/NAF/NAP that has been shown by several investigators to be a chemotactic factor for neutrophils and lymphocytes (14–16). MAD-2 is highly homologous (90% nucleotide and amino acid [illegible] stimulatory growth factor (17, 18). The DNA sequence of MAD-8 shows no homology to known genes.

Interference with the proinflammatory properties of monocytes could contribute to the pathogenesis of HCMV infection and the immunosuppression that accompanies HCMV infection. Indeed, it can be envisaged that even restricted expression of HCMV-IE genes in monocytes might alter transcription of an inflammatory mediator gene such as IL-1δ by virtue of their transactivating properties. Transcription from both viral and cellular promoters has been shown to be transactivated by HCMV-IE gene products (19–22).

We have investigated whether HCMV infection could modify mRNA expression of IL-1δ and other mediators in monocytes and in a promyelocytic cell line. The results indicate that given the appropriate state of differentiation at the time of infection, infection with HCMV may result in enhanced expression of many but not all monocyte mediator genes.

MATERIALS AND METHODS

Preparation and culture of human monocytes. Normal human monocytes were obtained from donors who were seronegative for HCMV, as determined by ELISA. The mononuclear cells were first isolated by Ficoll-Hypaque separation (23). To remove contaminating lymphocytes, Ficoll-isolated cells were suspended in 2.5% AB serum in endotoxin-free RPMI 1640 (Cellgro, Mediatech, Herndon, VA) and plated out in 100 mm petri dishes (Corning 25020, Corning, NY). The cells were allowed to adhere at 37°C for 30 min. After removal of nonadherent cells, the remaining adherent cells which were 98% peroxidase-positive mononuclear cells, were cultured in RPMI 1640 supplemented with 5% AB serum for 18 h at 37°C in the presence or absence of 10 μg/ml LPS (*Escherichia coli*, 0.55: B5; Difco Laboratories, Detroit, MI). After overnight incubation, adherent monocytes were recovered in cell suspension by repeated pipetting with cold Hanks solution (Flow Laboratories, Inc., Rockville, MD). Trypan blue exclusion showed that viability at the time of HCMV infection was greater than 90% for unstimulated and LPS-stimulated monocytes.

Culture of ML-3 cells and induction of cell differentiation. ML-3 cells were grown in endotoxin-free RPMI 1640 (Cellgro, Mediatech) supplemented with 10% FCS (Hyclone, Logan, UT), and were subcultured every 4 days by suspending 2×10^5 cells/ml in fresh media. For induction of cell differentiation, ML-3 cells were cultured in complete RPMI 1640 containing either 10 ng/ml PMA or 10 ng/ml DMSO for 24 h, and then washed once with RPMI 1640. Trypan blue dye exclusion showed that viability at the time of HCMV infection was greater than 90% for control and PMA-treated ML-3 cells. For evaluation of morphological differentiation, 0.1 ml of cell suspension was centrifuged onto glass microscope slides (Shandon Southern Instruments, Sewickley, PA). Cells were stained with Wright-Giemsa dye (Fisher Scientific, Raleigh, NC). Enzymatic activities of DMSO and PMA-treated ML-3 cells were assessed by using Sigma Lymphocyte Enzyme Kits (Procedure No. 180)(Sigma Chemical Co., St. Louis, MO).

Virus. HCMV Towne strain, passages 39 and 40, was used for all experiments. Virus was grown in human embryonic lung fibroblasts (HEL (P-16, ATCC)) and used for infection when the titer was 5×10^6 to 10^7 pfu/ml in fibroblasts.

UV inactivation of virus. [illegible] by adding virus to loose cell pellets at a ratio of 2 to 5 pfu/cell in conical polypropylene centrifuge tubes. Virus was allowed to adsorb to cells at 37°C for 90 min with gentle mixing every 15 min. Nonadsorbed virus was removed by washing the cells twice in RPMI 1640. Cells were mock-infected with supernatant from uninfected fibroblast cultures.

Monocytes were resuspended to a concentration of 1×10^6 cells/ml in RPMI 1640 supplemented with 5% AB serum and [illegible] cells resuspended to the same concentration in RPMI 1640 supplemented with 10% FCS and cultured for varying numbers of days postinfection.

Northern blot analysis of mRNA expression. Total cellular RNA was isolated from ML-3 cells by guanidine isothiocyanate extraction (24) followed by cesium chloride density gradient centrifugation (25). RNA (15μg) was applied per lane of formaldehyde gels for Northern [illegible] at Celsus Corporation from published sequences: IL-1δ (27), TNF-α (2,), CSF-1 (29), lysozyme (synthesized from a consensus sequence), and c-*erbA*-1 (30). All hybridizations were carried out at 42°C. Nitrocellulose filters were washed to a final stringency of 0.2 x SSC at 42°C for oligomer probes and 58°C for nick translated probes (MD-9, MD-8, and MD-2), and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NY) with intensifier screens at -70°C.

"Dot Blot" analysis of monocyte IL-1δ mRNA expression. Dot blot analysis of IL-1δ mRNA levels in monocytes was performed as described by White and Bancroft (31). Total cellular RNA was isolated from 2×10^6 viable monocytes per data point. Cells were lysed in lysis buffer containing 1% SDS, 0.02 M sodium acetate, pH 5.2, 0.35 M NaCl, 10 mM EDTA, 500 μg/ml heparin and 100 μg/ml pronase. Samples were extracted with an equal volume of phenol:chloroform (1:1) and chloroform. RNA was precipitated twice: once ethanol precipitation at -20°C and one $LiCl_2$ precipitation at 4°C. RNA was serially diluted twofold in 15 x SSC and applied to nitrocellulose prewetted with 15 x SSC using a dot blot manifold. The oligonucleotide probe for IL-1δ was the same as that used for Northern blot analysis. Hybridization was carried out at 27°C overnight in 50% formamide and filters washed to a final stringency of 0.2 x SSC at 42°C. Autoradiography was performed for 18 h at -70°C with intensifying screens.

[illegible] ing to the method described by Harper et al. (32). HCMV-infected and mock-infected monocytes were cultured for varying numbers of days postinfection. Cells were washed twice with PBS and fixed in 4% paraformaldehyde/PBS for 15 min on ice followed by an additional 18 min in 4% paraformaldehyde/PBS at room temperature. Cells were then washed in PBS, dehydrated in 30, 70, and 95% ethanol, and subsequently air dried. For the generation of ^{35}S-labeled sense and anti-sense RNA probes, the full length 1.8-kb IL-1δ cDNA was subcloned into the Bam H1 site of [illegible] plasmid pGEM-blue (Promega Biotech, Madison, WI). Run-on transcripts of both template orientations were synthesized using the T7 RNA polymerase in a reaction mixture containing 0.2 μg of linearized template DNA, 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithrothreitol, 2.5 mM each of ATP, CTP, and GTP, and 0.04 mM ^{35}S UTP (650 Ci/mmol, Amersham, Arlington Heights, IL). After incubation for 1 h at 37°C, the labeled RNA was extracted with phenol/chloroform, ethanol precipitated, and resuspended in hybridization buffer containing 50% deionized formamide, 0.6 M NaCl, 10 mM Tris (pH 7.5), 10 mM [illegible], 10% PEG-6000, 1 x Denhardt's solution, and 0.25 mg/ml tRNA. Fifty μl of this solution containing 3×10^4 cpm/ml RNA probe was applied to each slide and covered with a coverslip. Slides were incubated in petri dishes containing Whatman filter paper (Fisher Scientific) wetted with a solution of 50% v/v deionized formamide, 50% v/v distilled H_2O, and 100 mg/ml PEG-6000. Petri dishes were sealed with plastic tape and slides were incubated at 58°C for 18 to 24 h. The slides were then washed in a solution of 20μg/ml RNAse A in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0 at 37°C to facilitate digestion of nonhybridized ssRNA. Subsequent washes were in 2 x SSC, 50% formamide, 10 mM DTT for 30 min at 50°C, 1 x SSC, 50% formamide, 10 mM DTT for 30 min at 50°C, 1 x SSC, 50% formamide in 10 mM DTT, 0.05% Triton x-100 for 30 min at 37°C and 1 x SSC, 50% formamide, 10 mM DTT, 0.05% Triton X-100 for 30 min at 50°C. Slides were then dehydrated with increasing concentrations of ethanol and air dried. For autoradiography, slides were coated with NTB2 emulsion (Eastman Kodak) and exposed at 4°C for 4 to 6 [illegible] in Kodafix (Eastman Kodak) and counter-stained with hematoxylin and eosin (Sigma).

Western blotting analysis of cell-associated IL-1δ. Approximately 5×10^6 monocytes or ML-3 cells were extracted with 200 μl

of lysis buffer consisting of 0.05 M Tris hydrochloride, 0.15 M NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, and 0.1 mM phenylmethylsulfonylfluoride for 30 min on ice. The lysate was sonicated and centrifuged at 10,000 x g for 20 min to remove nuclei and cell debris. Thirty microliters of each sample were mixed with 2 x SLS sample buffer consisting of 0.125 M Tris hydrochloride, 20% glycerol, 10% 2-ME and 4.6% SDS, pH 6.8. Samples were then boiled for 5 min, applied to 12.5% 0.7 mm thick SDS-polyacrylamide gel, and were electrophoresed for 5 to 6 h at 100 V as described by Laemmli (15). After electrophoresis, the gel was transferred to a nitrocellulose membrane for 16 h at 100 mA using 0.025 M Tris-glycine buffer, pH 8.3. After transferring proteins to the nitrocellulose, IL-1β molecules were visualized by using rabbit anti-human IL-1d (Cistron Biotechnology, Pine Brook, NJ) as the first antibody and goat anti-rabbit Ig conjugated with horseradish peroxidase as the second antibody (34).

Staining of ML-3 cells. The cultured monocytic cell line ML-3 was collected by centrifugation at 1,000 rpm for 5 min. The cell pellet resuspended in 1.5 ml of PBS supplemented with 4% paraformaldehyde. Cells were incubated at 37°C for 25 min with constant rocking. The cell pellet was resuspended in PBS supplemented with 0.2% Triton X-100 and incubated on ice for 10 min. The cells were then resuspended in PBS supplemented with 0.1% Tween 20 and 1% BSA and incubated on ice for 10 min. Cells were then resuspended in 5 μl of anti-human IL-1d (IgG1) mAb (1:4) (Cistron Biotechnology, Pine Brook, NJ) or 5 μl of the T cell-specific Leu 2a, an antibody nonreactive for macrophages of the IgG1 subtype (Becton Dickinson, Mountainview, CA), and incubated for 30 min at 37°C. The cells were then washed twice in PBS supplemented with 0.1% Tween 20 and 1% BSA, resuspended in 10 μl of a 1:20 dilution of phycoerythrin-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL), and incubated at 37°C for 30 min. Cells were then washed twice and resuspended in PBS for flow cytometry analysis. Control cells were stained with secondary antibody only. Flow cytometric analysis was carried out on an Ortho System 50 operating with simultaneous 100 and 514 nm excitation.

Plasmid DNA. Plasmids containing putative human pro-IL-1d gene regulatory regions were derived by insertion of each of two different fragments derived from the BDC-454 clone described by Clark et al. (36). The PvuII/TaqI fragment located between positions -1097 and +12 (Fragment PT) and the AvaI/TaqI fragment located between positions -512 and +12 (Fragment AT) were each treated with the Klenow fragment of *E. coli* DNA polymerase and inserted by blunt-end ligation into the pSVCAT [illegible] plasmid described by Laimins et al. (37). Therefore, the resulting plasmids contained pro-IL-1d genomic DNA corresponding to positions -1097 to +14 for PT and -512 to +14 for AT. These fragments contain a TATA sequence at position -31 as well as other features that have been described elsewhere (38).

Plasmids containing various HCMV immediate-early gene regions, pHD101SV1, pHD101SV2, and 10142 (equivalent to 101-4) (20) were used to study the interaction between HCMV-IE gene products and the IL-1d promoter. In pHD101SV1, the region 1 and region 2 of the HCMV-IE region are under control of the HCMV-IE promoter with the SV40 enhancer located further upstream. Plasmid pHD101SV2 [illegible] containing region 2 has been deleted. Plasmid 10142 has the exon sequence of region 1 deleted. Plasmids were purified from chloramphenicol-amplified cultures by isopycnic banding on CsCl ethidium bromide gradients.

Gene transfer into ML-3 cells. DNA was introduced into PMA-stimulated or unstimulated ML-3 cells by electroporation with a Zapper electroporation unit at 1300 V (University of Wisconsin Medical Electronics Lab, Madison, WI). Approximately 10^7 cells were transfected in 0.5 ml of growth medium with 10 μg of either pSVCAT or pME-AT, and varying amounts of HCMV-IE region plasmid DNA. After voltage was applied, the cells were diluted into 10 ml medium and incubated at 37°C in an atmosphere of 5% CO_2/95% air for 48 h.

Assay of CAT. Cell harvesting and CAT assays were performed 48 h after transfections as described by Gorman et al. (39). ^{14}C chloramphenicol (0.2 μCi; 1 Ci = 37 GBq; New England Nuclear, Boston, MA) was incubated with 0.7 mM acetyl coenzyme A (Sigma) and 100 μl of cell extract in a final volume of 120 μl of 0.25 M Tris-HCl, pH 7.8, for 90 min at 37°C. Radioactive materials were extracted with ethyl acetate and the nonacetylated chloramphenicol and acetylated reaction products separated by chromatography on thin layer plates.

Induction of ML-3 cell differentiation. ML-3 cells acquire morphological characteristics of monocytes/macrophages after treatment with PMA and neutrophil characteristics after treatment with DMSO. Predifferentiated ML-3 cells were greater than 90% adherent and showed significantly decreased proliferation. Acid phosphatase and α-naphthyl-butyrate esterase were increased markedly after PMA treatment and little or not at all after DMSO treatment. Kinetic and functional studies have previously been described (20, 21).

HCMV enhances expression of many but not all monocyte-associated mediator genes. In order to study the influence of HCMV infection on expression of monocyte mediator genes, ML-3 cells were induced to differentiate along the monocytic pathway by overnight exposure to 10 ng/ml PMA (Sigma Chemical Co., St. Louis, MO). Cells were washed and exposed to virus or mock-infected with culture supernatant from uninfected fibroblasts (Fig. 1 and 2). Within 24 h of virus exposure, significantly higher levels of steady-state mRNA for IL-1β, TNF-α, MAD-9, and CSF-1 were observed, and this continued through the next

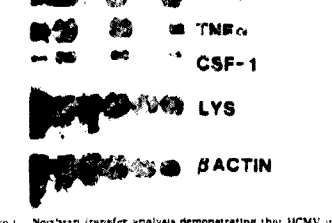

Figure 1. Northern transfer analysis demonstrating that HCMV infection modulates expression of IL-1β, TNF-α, and CSF-1 in ML-3 cells induced to differentiate along the monocytic pathway before infection. Autoradiographs were exposed overnight for IL-1d, TNF-α, and CSF-1, for 2 days for lysozyme (LYS), and for actin (β, HCMV-infected (+), uninfected (-).

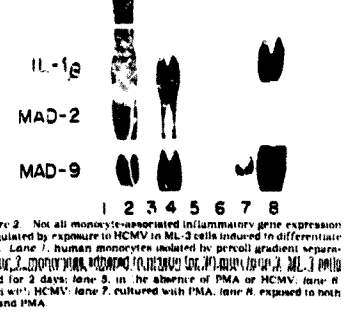

Figure 2. Not all monocyte-associated inflammatory gene expression is upregulated by exposure to HCMV in ML-3 cells induced to differentiate by PMA. *Lane 1*, human monocytes isolated by percoll gradient separation; [illegible] cultured for 2 days; *lane 3*, ML-3 cells cultured for 2 days; *lane 5*, in the absence of PMA or HCMV; *lane 6*, cultured with HCMV; *lane 7*, cultured with PMA; *lane 8*, exposed to both HCMV and PMA.

several days of culture. Expression of the adherence-induced monocyte genes, MAD-6 and MAD-2, was not induced following exposure to PMA, and steady-state mRNA levels of either of the constitutively expressed genes, lysozyme, or β-actin, were not significantly modulated by HCMV infection (Fig. 1). The only genes showing enhanced and continued expression following HCMV infection are those which are induced with PMA before infection, demonstrating that HCMV was probably acting selectively as an enhancer rather than an inducer of these inflammatory related genes. Control studies demonstrated that only a low level of IL-1β induction was detected in undifferentiated HCMV-infected ML-3 cells after several weeks of exposure, and infection with HCMV before induction of differentiation by PMA failed to induce enhanced mRNA levels over those observed in the continued presence of PMA (data not shown).

HCMV-dependent enhancement requires induction of the macrophage phenotype. A major fraction of ML-3 cells (like the promyelocytic HL-60 line [41]) can be induced by DMSO to differentiate along the granulocytic pathway. We used this treatment to examine the possibility that different states of differentiation would have distinct influences on the ability of HCMV to modify expression of a mediator gene such as IL-1β. Results shown in Figure 3 indicate that stimulation with DMSO before HCMV infection failed to significantly induce IL-1β mRNA. Low levels of mRNA seen at 24 h after exposure to virus were markedly less than were observed in PMA-differentiated cells. By 72 h postinfection these levels had significantly decreased, indicating that enhanced IL-1β mRNA expression was not sustained as was observed in PMA-differentiated cells challenged with HCMV.

HCMV infection of monocytes results in enhanced and long-term expression of IL-1β mRNA. Because the previous studies had been carried out with a transformed line that required exposure to the pleiotropic effects of PMA in order to demonstrate virus-dependent modulation of IL-1β, we carried out a series of studies with normal blood monocytes, an example of which is given in Figure 4. Monocytes were collected and cultured under endotoxin-free conditions and cultured either alone, in the presence of LPS, HCMV only, or LPS and HCMV. Cytoplasmic dot-blot analysis of the experiment is shown in Figure 4B and the densitometric representation shown in Figure 4A. Monocytes cultured in the absence of LPS gave only a weak transient signal, whereas LPS stimulated a strong transient response that was weakly detectable at 24 to 48 h. In contrast, HCMV exposure resulted in a more sustained response that continued for 96 h. The combination of HCMV and LPS gave a transient response not unlike LPS alone, except that the plateau phase was significantly elevated. These data confirm that expression of a mediator gene such as IL-1β can be enhanced by exposure to HCMV and demonstrate that the observations are not related to contaminating endotoxin. Cytoplasmic dot-blot analysis was performed on three different donors, all of whom gave a similar response.

In order to determine if the enhanced levels of steady state IL-1β mRNA were due to increased expression by the entire population of monocytes or only a certain percentage of cells, we used in situ hybridization to resolve IL-1β mRNA expression at the single cell level. In view of the fact that we observed donor variation in levels of IL-1β expression at the more sensitive single cell level, we have shown data from three different donors (Fig. 5). For each donor, however, we observed that HCMV infection always had the same effect on IL-1β mRNA expression, as is shown in data from 4 days postinfection for each donor. The percentage of positive cells at 1, 2, and 4 days was always consistently higher in infected monocytes than in uninfected controls, as is shown for donor 1. We also observed that, for all three donors, HCMV exposure resulted in a small percentage of monocytes (1 to 8%) showing chronic expression of IL-1β mRNA (>100 grains/cell for donor 2, and 200 grains/cell for donor 1 and 3) which was sustained for up to 4 days postinfection (Fig. 6). IL-1β-mRNA expression in HCMV-infected and

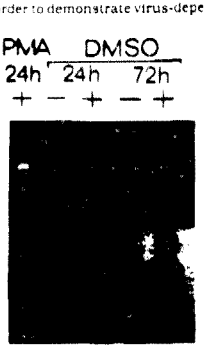

Figure 3. ML-3 cells require differentiation with PMA rather than DMSO in order to express competence for HCMV modulation of the IL-1 response. Cells were cultured overnight to induce differentiation and then cultured with (+) or without (−) infection with HCMV. Northern transfer analysis and quantitation of IL-1β mRNA levels were carried out as in Figure 1. PMA-treated, HCMV-infected cells cultured as in Figure 1A were used for reference.

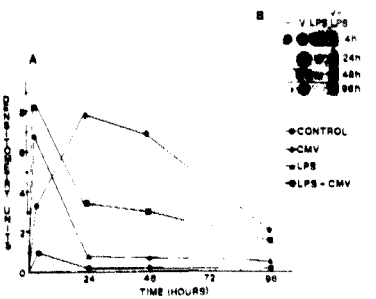

Figure 4. Total cytoplasmic dot-blot analysis demonstrating that IL-1β expression in monocytes is enhanced and prolonged by infection with HCMV more than is obtained under conditions of LPS stimulation. Densitometric analysis (A) was derived from analysis of the dot-blots in panel B. It is intended as an approximation of the kinetics of the response rather than an exact quantitation.

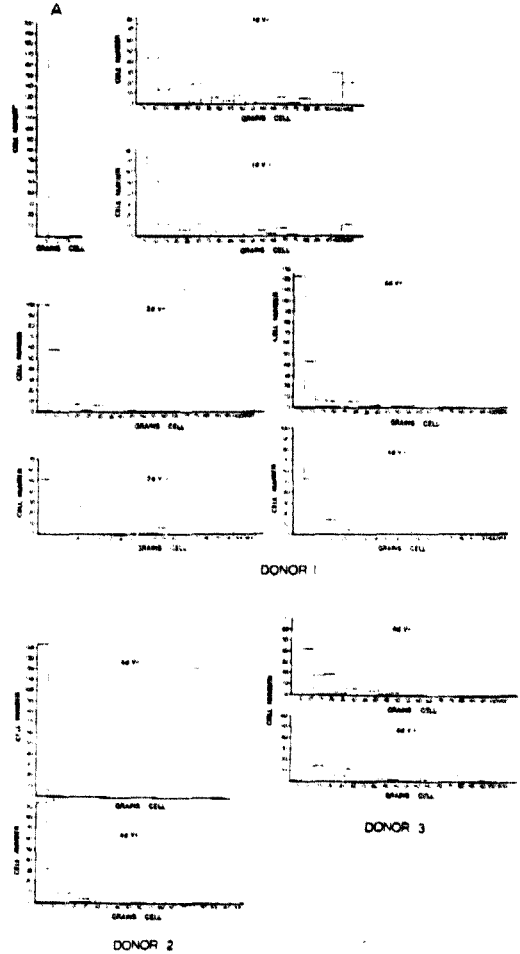

Figure 5. IL-1*β*-mRNA expression in HCMV-infected and uninfected peripheral blood monocytes analyzed by in situ hybridization using ^{35}S-labeled anti-sense RNA probes specific for IL-1*β*. Background staining was revealed by hybridizing cells to ^{35}S-labeled IL-1*β* sense RNA sequences (*A*). Grain counts and numbers of positive cells were determined by counting approximately 200 cells/slide. IL-1*β*-mRNA expression at 4 days postinfection is shown for donors 1, 2, and 3 together with data from 1 day and 2 days postinfection for donor 1.

uninfected peripheral blood monocytes from donor 1, at 1, 2 and 4 days postinfection, is shown in Figure 6.

HCMV-dependent enhancement of mRNA levels leads to transiently enhanced translation of IL-1β. We wished to determine if the enhanced levels of steady-state mRNA were a reflection of enhanced translation of this important mediator gene, inasmuch as we had previously shown that adhered monocytes rapidly expressed mRNA for IL-1*β*, TNF-*α*, and CSF-1 in vitro, but failed to secrete protein in the absence of a second signal, which could be derived from LPS (42). To investigate this we carried out two studies. In the first, we used flow cyto-

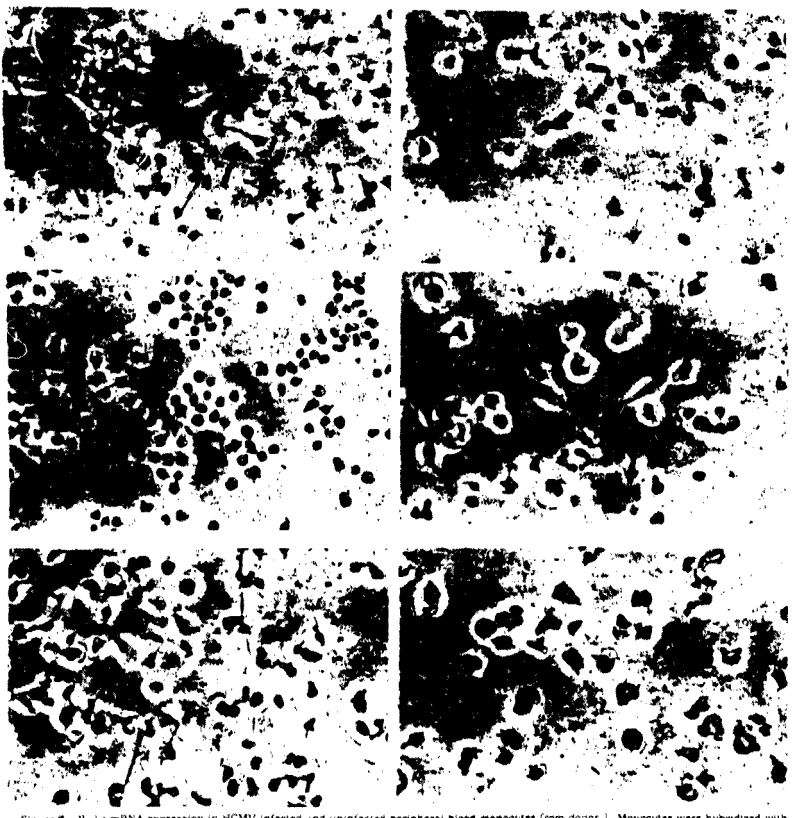

Figure 6. IL-1β-mRNA expression in HCMV-infected and uninfected peripheral blood monocytes from donor 1. Monocytes were hybridized with ^{35}S-labeled anti-sense RNA probes specific for IL-1β or sense IL-1β RNA probes for determination of background staining as described in *Materials and Methods*. IL-1β-mRNA expression was analyzed at 1 day and 4 days postinfection in uninfected monocytes (*a* and *d*) and in infected monocytes (*b* and *e*). Control hybridization at 1 day and 4 days respectively is shown in *c* and *f*.

metric analysis. These experiments demonstrated that although PMA treatment alone led to the expression of protein, the combined exposure to HCMV following induction of differentiation (as in Fig. 1*A*) led to a marked increase in the amount of intracellular IL-1β (Fig. 7). The isotype control mAb we used (Leu 2A) gave similar levels of fluorescence on both cell preparations. HCMV-exposed cells had control levels of fluorescence (data not shown).

To confirm the presence of intracellular protein and to compare responses in ML-3 cells and monocytes, we also carried out an analysis by Western blotting, the results of which are given in Figure 8. In both cell types activity was only detected at the earliest time points, indicating that sustained steady-state mRNA expression (Fig. 4) did not necessarily lead to continued translation. Monocytes exposed only to culture for 24 h showed no pro-IL-1β protein. Monocytes challenged with heat-killed virus (which still contains intact DNA) exhibited a significant level of the intracellular 31-kDa form of the pro-IL-1β protein. Exposure to live HCMV, however, gave a marked enhancement of the intracellular form of IL-1β. No activity was detected in cells cultured for 72 h. ML-3 cells

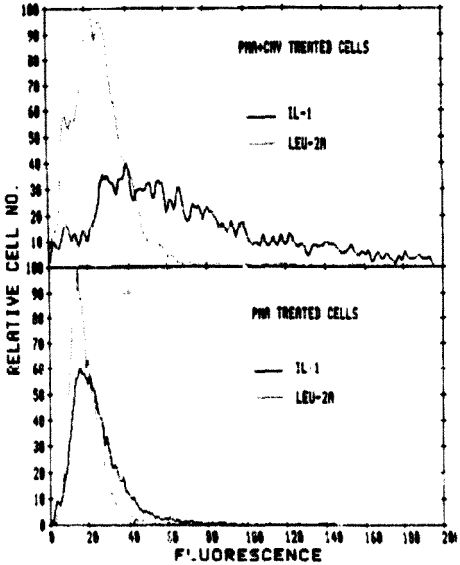

Figure 7. Flow cytometric analysis of IL-1β expression in ML-3 cells treated with PMA with or without HCMV. Both the IL-1β mAb and the Leu-2a control are IgG1 subclasses.

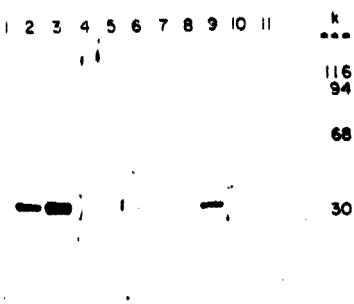

Figure 8. Western transfer analysis of IL-1β translation products in monocytes (*lanes 1–5*) or ML-3 cells (*lanes 6–11*): *lane 1*, uncultured monocytes, and *lane 6*, untreated ML-3 cells; PMA-treated ML-3 cells pulsed 0.4 h alone (*lane 7*), monocytes or ML-3 treated cells for 2 h with heat-inactivated (*lanes 2* and *8*), infectious virus (*lanes 3* and *9*), 72 h with heat-inactivated (*lanes 4* and *10*), or infectious HCMV (*lanes 5* and *11*).

induced with PMA only produced detectable protein 24 h after exposure to live HCMV.

IE gene of HCMV transactivates the IL-1β promoter in PMA-differentiated ML-3 cells. CMV infection of monocytes seldom leads to a complete replication cycle. Rather if any virus genes are detected, they are restricted to those designated IE (1, 7). We were therefore interested in determining if the enhanced expression of IL-1β mRNA seen in ML-3 cells could result from transactivation of the IL-1β gene by IE gene products. To examine this possibility we used chimeric plasmids containing pro-IL-1β genomic DNA sequences fused to the CAT gene. The insertion of the pro-IL-1β promoter in front of the promoterless CAT gene allows for promoter testing by the expression of CAT in transfected cells. IL-1β-CAT constructs were cotransfected into ML-3 cells with various HCMV-IE gene region constructs. Results in Figure 9A show the effect of HCMV-IE region expression plasmids on IL-1β promoter function in ML-3 cells that were induced to differentiate with PMA before transfection. When 3M-AT DNA was transfected singly into PMA-differentiated ML-3 cells there was no CAT activity. When plasmid 3M-AT containing 524 bp of pro-IL-1β genomic DNA was cotransfected with the HCMV-IE region 1 and 2 expression plasmid, pHD101SV1, it stimulated the activity of the IL-1β promoter. To ascertain whether regions 1 and 2 of the HCMV-IE region were both required for transactivation of 3M-AT, HCMV plasmids encoding either region 1 or region 2 were cotransfected with 3M-AT. The region 1 products alone do not support IL-1β promoter activity. In contrast, region 2 products resulted in activity equivalent to that obtained

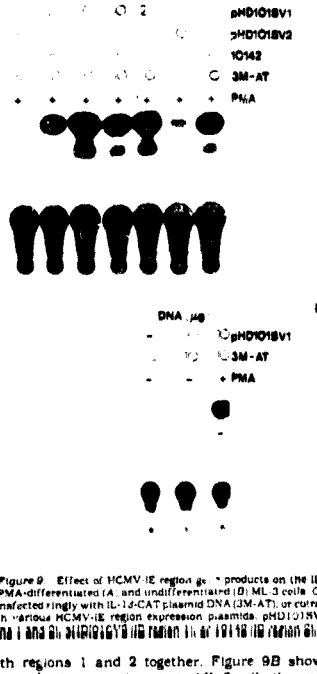

Figure 9. Effect of HCMV-IE region gene products on the IL-1β gene in PMA-differentiated (A) and undifferentiated (B) ML-3 cells. Cells were transfected singly with IL-1β-CAT plasmid DNA (3M-AT), or cotransfected with various HCMV-IE region expression plasmids. pHD1018V1 (IE regions 1 and 2) and pHD1018V2 (IE region 1) or 1C142 (IE region 2).

with regions 1 and 2 together. Figure 9B shows that cotransfection experiments in ML-3 cells that were not induced to differentiate before transfection resulted in no stimulation of IL-1β promoter activity.

Transactivation of the IL-1β gene by HCMV-IE gene products is therefore dependent on induction of differentiation by PMA before transfection, suggesting the need for a host-derived competence factor(s).

Cotransfection experiments using plasmid 3M-PT, which has an additional 500 bp of upstream pro-IL-1β sequence, gave no increase in CAT activity, indicating that the additional upstream DNA sequence did not increase efficiency of CAT expression (data not shown).

DISCUSSION

There is increasing evidence for monocytes playing an important part not only in host defense against virus infection, but also of being a "silent" host harboring virus and awaiting the appropriate challenge before breaking out of the latent state (43). The highly variable state of differentiation/activation that cells of the mononuclear phagocyte series may exhibit and the influence that this may have on the outcome of virus exposure and latency are issues that yet remain to be addressed.

HCMV has been shown to express only part of its genome in human PBMC. Among these cells, the expression of IE and early classes of viral genes is highest in monocytes, in the range of 0.1 to 5% (1–3, 5, 7). A latent infection has been established in monocytes after in vitro HCMV infection with gene expression restricted to IE and early proteins only (1, 7). The functional effects of this infection, albeit nonproductive, have been shown by several investigators who suggest that monocytes may mediate the immunosuppressive properties caused by HCMV. Monocytes infected in vitro or in vivo with HCMV have been shown to suppress the proliferation of T lymphocytes in response to Con A (6). Kapasi et al. (5) were able to show significant depression of respiratory burst activity(≥90%) in HCMV-infected monocytes, although no more than 5% of the cells could be shown to express the IE gene product.

Because monocytes can rapidly respond to most infections by secretion of inflammatory mediators, several investigators have looked at the effect of HCMV infection on production of functional IL-1 activity (2–4), i.e. the ability of IL-1 function as a co-mitogen together with T cell mitogens such as PHA or Con A in the murine thymocyte proliferation assay. HCMV infection of monocytes suppresses the bioactivity of IL-1. Rodgers et al. (2) have associated this with the release of an IL-1 inhibitor. Kapasi et al. (4) have suggested that the inability of monocytes and lymphocytes to produce and respond to IL-1 and IL-2 is the result of metabolic derangement in these cells caused by HCMV. Thus, at the functional level, the effect of HCMV infection on production of IL-1 has been well documented.

We have been interested in investigating the monocyte response to HCMV infection at the transcriptional level, particularly in view of the fact that the IE proteins possess transactivating properties and have been shown to transactivate transcription from viral and cellular promoters (19–22). Inasmuch as the state of activation and differentiation of the host cell are likely to be of utmost importance when monocytes/macrophages are involved, we have examined the effect of various differentiation states of a promyelocytic cell line on monocyte mediator production in response to HCMV infection. In spite of the altered regulatory processes that are likely to be present in transformed cell lines such as HL-60, ML-3, and U937, cell lines can be induced to differentiate along different cellular lineages, and have provided much valuable information with regard to virus monocyte interactions. The promyelocytic cell lines, ML-3 and HL-60, possess some monocytic-like characteristics, but require exposure to the differentiating agent PMA to exhibit most monocytic traits (37, 44, 45). PMA has a multitude of influences, not only as a ligand for protein kinase C (46), but also in perturbing the cell membrane and altering receptor localization (47). Weinshenker et al. (8) showed that TPA-induced differentiation of the monocytic cell line, THP-1, resulted in a full cycle of HCMV replication. TPA-induced differentiation of the promyelocytic cell line, HL-60, did not result in productive infection, thereby demonstrating the importance of cell lineage. In this study, PMA-induced differentiation of the ML-3 cell line resulted in expression of HCMV- IE Ag only. The results

indicate that these levels of HCMV infection are sufficient to modify mRNA expression of select monocyte mediator genes in ML-3 cells induced to differentiate before infection. Induction of the granulocytic pathway of differentiation with DMSO did not support a CMV- dependent upregulation of IL-1β expression. The apparent requirement for PMA differentiation of the host cell suggests the need for a host-derived competence factor, one that is apparently present in monocytes but not in DMSO-induced ML-3 cells. Genes that are constitutively expressed, such as lysozyme and actin, were unaffected by virus, indicating a direct relationship between genes that are induced by PMA differentiation and enhancement by the IE gene products of HCMV. Folks et al. (48) have reported that cytokine-induced expression of HIV-1 in U937 cells, in which persistent but apparently latent infection had been induced, resulted in selective upregulation of IL-1β only.

Results of investigating regulation in more detail, of IL-1β in blood monocytes, showed that levels of steady state IL-1β mRNA were similarly enhanced and sustained for up to 96 h postinfection. The fact that the observed induction of IL-1β mRNA with LPS and HCMV was not as great as that compared to CMV alone, indicates possible diffusible biochemical pathways of induction. At the single cell level we observed a small percentage of monocytes (1 to 8%) chronically expressing IL-1β mRNA (>100 grains/cell for one donor, and 200 grains/cell for two other donors tested). This percentage is in the same range as that reported for viral Ag expression in monocytes after in vitro HCMV infection. Future experiments using double labeling in situ hybridization for detection of HCMV-IE mRNA and IL-1β mRNA will attempt to answer whether the cells expressing IE genes are the same as those chronically expressing IL-1β.

In contrast to our studies which focused on steady-state levels of pre IL-1β mRNA, the transfection experiments involving putative pro-IL-1β regulatory region-CAT fusion DNA demonstrate that the regulation of the HCMV phenomenon is probably at the level of gene transcription. Furthermore, the observed transactivation appears to primarily require region 2 of the HCMV genome. The pHDICISV2 DNA supported little activation as judged by CAT activity. The observation that IC142, which contains some region 1 sequences in addition to region 2, has some effect on activity suggests a possible role for region 1 DNA, perhaps in supporting region 2 gene expression. The HCMV DNA used in this study have also been shown to be effective at transactivating HIV-LTR transcription in a TAT/TAR-independent fashion (20). We have now demonstrated that the pro-IL-1β upstream sequences, like the upstream HIV-LTR DNA, can also be transactivated by HCMV region 2. We have been unable to find any extensive sequence homology between the upstream pro-IL-1β and HIV-LTR responsive sequences. However, some homology does exist, and it is possible that a short factor binding sequence may be shared between these two DNA. Of course, it is also possible that the HCMV region 2 gene product is indirectly involved in the activation pathway.

Enhanced mRNA expression did not necessarily lead to sustained translational activity as shown by flow cytometric and Western blot analysis. Enhanced levels of intracellular IL-1β protein were observed at 24 h postinfection and not at later time points. This supports the view that the IL-1β protein is either degraded or rapidly exported after expression in contrast to previous observations with PMA-stimulated THP-1 cells (49). The data derived from flow cytometric analysis indicates that the majority of monocytes have enhanced levels of IL-1β protein suggesting, in agreement with others (5), that the effect of virus is manifested in the majority of the population. Although IL-1β expression is rapidly induced following adherence of monocytes, little, if any, protein is secreted in the absence of a second signal as is usually produced following exposure to LPS (50). This present data indicates that HCMV can probably also serve that role.

Taken together these data suggest that HCMV-IE gene products might be directly enhancing mRNA expression of mediator genes such as IL-1β. We have shown that there is transactivation of the IL-1β gene in ML-3 cells induced to differentiate before transfection, it appears that limited virus-encoded responses are sufficient to induce significant alterations in monocyte-associated mediator expression. Furthermore, the overall inflammatory mediator response to HCMV infection is manifested in selective upregulation of monocyte-associated mediator genes and may thereby contribute to the pathology of this viral infection.

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Interaction of Strain AD169 and a Clinical Isolate of Cytomegalovirus with Peripheral Monocytes: The Effect of Lipopolysaccharide Stimulation

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The effect of human cytomegalovirus (CMV) infection on lipopolysaccharide (LPS)-stimulated and unstimulated monocytes from seronegative donors was studied by using the laboratory-adapted strain AD169 and a recent clinical isolate. LPS-stimulated and unstimulated monocytes infected with the isolate showed expression of immediate-early CMV antigens and were significantly more suppressive for lymphocyte proliferation than were strain AD169–infected monocytes, which rarely expressed detectable viral protein. Human CMV infection of LPS-stimulated and unstimulated monocytes resulted in abrogation of interleukin-1 activity, with the effect being marked in LPS-stimulated monocytes infected with the clinical isolate of CMV. Addition of interleukin-1 to infected, stimulated monocytes completely restored lymphoproliferative responses to phytohemagglutinin, whereas addition of this leukokine to infected, unstimulated cells could not restore this response.

Human cytomegalovirus (HCMV) infection is associated with immunosuppression [1–3]. Among peripheral blood mononuclear cells, the monocyte appears to be the cell most frequently infected with clinical isolates of cytomegalovirus (CMV) [4, 5]. Studies on monocytes from patients with CMV mononucleosis and normal monocytes infected with HCMV in vitro have shown that monocytes can suppress the lectin-induced proliferation of autologous lymphocytes [1, 6]. A recent report has shown that challenge of unstimulated monocytes with HCMV strain AD169 abrogates their constitutive production of interleukin-1 (IL-1) activity [7]. This abrogation was associated with the release from virus-challenged monocytes of an inhibitor of IL-1 activity, and it was proposed that this inhibitor partially accounts for the previously observed immunosuppressive properties of HCMV. However, in organ transplant recipients, CMV infection is frequently accompanied by the presence of other pathogens [8–11]. Peripheral blood monocytes are stimulated by bacteria and bacterial products such as lipopolysaccharide (LPS), the endotoxin released by *Escherichia coli* and other gram-negative bacteria [12, 13]. IL-1 production by such cells is greater than for resting monocytes.

We have therefore compared the effects of HCMV challenge on unstimulated and LPS-stimulated monocytes with two strains of CMV: the laboratory-adapted strain AD169 and a clinical isolate strain. Recent work has suggested differences in the degree of infection of leukocytes obtained with clinical isolates and laboratory strains [4, 5]. Production of IL-1 activity by the monocytes, as assessed by lymphoproliferative assays, was investigated, as was expression of immediate-early antigen.

Materials and Methods

Strains of cells and virus. CMV strain AD169 with a titer of 10^8 pfu/ml in fibroblasts was passaged in primary human embryonic lung fibroblasts. The clinical isolate of CMV was isolated from the urine of a renal transplant recipient and cultured in human embryonic lung fibroblasts maintained in Eagle's MEM (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum (M. A. Bioproducts, Walkersville, Md), with this virus being used between passage 3 and 5, when the titer was 10^8 pfu/ml.

Preparation of mononuclear cells from human peripheral blood. All donors, 21–50 years of age, were seronegative for CMV as determined by ELISA. Human peripheral blood treated with heparin (15 U/ml) was separated into mononuclear and polymorphonuclear cell fractions by using density gradients

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of Ficoll-Isopaque® (Pharmacia, Uppsala, Sweden) [14]. The mononuclear cells were washed twice in PBS and once with RPMI 1640 medium (GIBCO) containing penicillin (100 U/ml), streptomycin (200 μl/ml), and L-glutamine (2 m*M*). Mononuclear cells were resuspended in this supplemented RPMI 1640 medium containing 10% pooled type AB serum that had been heat inactivated at 56 C for 30 min and was free of antibodies to CMV. Monocytes were removed by adsorption onto the flat surfaces of 60-mm plastic tissue culture dishes (Costar, Cambridge, Mass) at 37 C for 90 min. They were recovered by incubation of the plates with Versene® solution (dilution, 1:5,000; GIBCO) for 20 min at 4C, followed by gentle pipetting of supplemented RPMI 1640 medium onto the surface of dishes so that monocytes that were still adherent after Versene treatment were removed. After removal, monocytes were washed twice in supplemented RPMI 1640 medium. The resulting cell population was >95% monocytes as determined by nonspecific esterase staining [15], and the viability was >95% as determined by trypan blue dye exclusion.

T lymphocytes were separated from B lymphocytes and other nonadherent cells by differential centrifugation of lymphocytes rosetted with sheep red blood cells treated with 2-aminoethylisothiouronium bromide [16]. Less than 5% were non-T cells as determined by immunofluorescent staining with the pan-T cell monoclonal antibody OKT3 (Ortho Pharmaceuticals, Raritan, NJ).

Monocyte culture and production of IL-1-containing supernatants. Monocytes (10^6 cells/ml) were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated, pooled type AB serum for 20 hr at 37 C in the presence or absence of LPS from *E. coli* (20 μg/ml; Difco, Detroit) [17]. After a wash in supplemented RPMI 1640 medium these cells were challenged with virus at the appropriate moi or were mock-infected for 2 hr at 37 C. They were subsequently washed three times; resuspended in supplemented RPMI 1640 medium containing 10% heat-inactivated RPMI 1640 medium before seeding in microtiter wells (Falcon Plastics, Oxnard, Calif), with or without glass coverslips, at the appropriate concentration, and cultured for three days in a humidified atmosphere containing 5% CO_2 at 37 C for the required time.

Control and IL-1-containing supernatants were collected by centrifugation at 1,000 *g* for 10 min and frozen at −20 C for three to five days until used. No deterioration in IL-1 activity was observed.

T lymphocyte stimulation. Autologous T lymphocytes were prepared as described above and diluted to a concentration of 10^6 cells/ml in supplemented RPMI 1640 medium and 10% heat-inactivated, pooled type AB serum, with or without a previously determined optimal dose of 2 μg of phytohemagglutinin-P (PHA-P, purified grade; Wellcome Reagents, Beckenham, England)/ml. T lymphocytes (2×10^4 per well) were incubated alone, with monocytes previously cultured for three days, with IL-1-containing supernatants, or with ultrapure IL-1 (a gift from L. Traub, South African Institute for Medical Research, Johannesburg). After 72 hr, lymphocyte stimulation was measured by addition of 0.5 μCi of [^{3}H] thymidine (specific activity, 19.3 Ci/mmol; New England Nuclear Corp., Boston) to each well for 16 hr. Cells were harvested with a multiple automatic harvester (MASH® II; M. A. Bioproducts) on glass fiber filters (Whatman, Clifton, NJ), air-dried, and immersed in toluene scintillation fluid. [^{3}H]Thymidine incorporation was assessed by scintillation counting (Packard Instrument Co., Downers Grove, Ill). Results were determined by subtracting the mean dpm of cells without added PHA *from the mean dpm of cells with added PHA for* triplicate samples.

Assay for IL-1 activity. Single-cell thymocyte suspensions from BALB/c mice (six to eight weeks old), at a concentration of 1.5×10^6 cells per 200-μl well, were cultured for three days in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 2.5×10^{-4} *M* 2-mercaptoethanol, and 2 μg of PHA-P/ml in the presence of serially diluted culture supernatants collected from unstimulated and LPS-stimulated monocytes infected or mock-infected with CMV three days earlier. Cultures were pulsed with 0.5 μCi of [^{3}H]thymidine per well for the final 16 hr of incubation, and incorporation was assessed by liquid scintillation counting.

Immunofluorescence. Monocytes were rinsed once in PBS, rapidly air-dried, and fixed in cold acetone for 10 min before staining for immediate-early viral antigens by indirect immunofluorescence. The cells were blocked with human type AB serum and incubated at 37 C for 1 hr with the HCMV immediate-early antigen-specific monoclonal antibody E3 at a dilution of 1:1,000 (a gift from L. Goldstein, Fred Hutchinson Cancer Research Center, Seattle).

Table 1. Percentage of monocytes expressing the major 72-kilodalton immediate-early CMV polypeptide after infection with CMV strain AD169 or a clinical isolate.

CMV strain	moi (pfu/cell)*	E-3 positive (%)†
AD169	0.5	0
	1.0	0–0.01
	5.0	0.01–0.1
V3474-84‡	0.001	1–2
	0.005	3–5
	0.01	4–6
	0.1	7–9

* The moi values were estimated by plaque-forming assays on fibroblasts.

† Monoclonal antibody E-5 detects the major 72-kilodalton immediate-early CMV polypeptide.

‡ CMV clinical isolate V3474-84 was from a renal transplant recipient.

After a rinse in PBS they were exposed to FITC-labeled goat antibody to mouse immunoglobulin (Cappel Laboratories, Cochranville, Pa) for 1 hr and again rinsed in PBS. Stained cells were observed with a microscope (Dialux® 20; Leitz, Darmstadt, Federal Republic of Germany) equipped with incident light UV illumination.

All data were analyzed with Student's *t* test at a 1% significance level.

Results

Comparison of monocyte sensitivity to infection by a clinical isolate of CMV and laboratory-adapted strain AD169. Unstimulated or LPS-stimulated monocytes challenged with CMV strain AD169, which has been cultured for many passages in human fibroblasts, showed very low (0.01%–0.1%) expression of CMV immediate-early antigens, even when an moi of 5 pfu per cell was used. The number of immediate-early antigen–positive monocytes was not different for stimulated or unstimulated cultures, with the staining being observed three days after infection and remaining evident for the life of the cultures (10–12 days). However, CMV replication in both unstimulated and LPS-stimulated monocytes was demonstrable after infection with the clinical isolate of CMV, with the percentage of monocytes showing nuclear or perinuclear fluorescence rising slightly with increasing moi (table 1). No staining was observed in uninfected monocytes or in appropriate controls.

Table 2. Suppression of T lymphocyte proliferation by LPS-stimulated or unstimulated, CMV-infected monocytes.

Cell type	CMV strain (moi, in pfu/cell)	[³H]Thymidine incorporation	
		4×10^4 monocytes (%)	8×10^4 monocytes (%)
T lymphocytes only ($2 > 10^5$)	. . .	109.82 ± 7.91 (45.5)	109.82 ± 7.91 (45.5)
T lymphocytes and unstimulated monocytes	. . .	241.12 ± 15.15 (100)*	275.17 ± 13.56 (114)
	AD169 (0.5)	174.62 ± 12.46 (72.4)	223.82 ± 9.64 (92.8)
	AD169 (1.0)	110.46 ± 10.01 (45.8)	133.43 ± 8.75 (55.3)
	AD169 (5.0)	55.38 ± 8.25 (22.9)	60.10 ± 6.25 (24.9)
	V3474-84 (0.001)	150.41 ± 9.52 (62.3)	230.40 ± 9.46 (95.5)
	V3474-84 (0.005)	70.56 ± 6.48 (29.3)	120.55 ± 8.93 (49.9)
	V3474-84 (0.01)	28.81 ± 2.82 (11.9)	40.33 ± 5.21 (16.7)
T lymphocytes and LPS-stimulated monocytes	. . .	360.52 ± 24.9 (150)	380.27 ± 26.1 (158)
	AD169 (0.05)	210.21 ± 15.12 (87.1)	201.40 ± 14.32 (83.4)
	AD169 (1.0)	31.49 ± 4.97 (13.0)	21.20 ± 2.01 (8.8)
	AD169 (5.0)	15.25 ± 2.44 (6.3)	10.01 ± 1.04 (4.2)
	V3474-84 (0.001)	300.61 ± 21.62 (124.7)	310.65 ± 21.08 (128.8)
	V3474-84 (0.005)	34.46 ± 4.09 (14.3)	45.63 ± 5.12 (18.9)
	V3474-84 (0.01)	22.39 ± 2.21 (9.3)	25.42 ± 2.71 (10.5)

NOTE. [³H]Thymidine incorporation is expressed as mean ± 1 SD change in dpm values $\times 10^{-3}$ for triplicate samples (percentage stimulation). CMV clinical isolate V3474-84 was from a renal transplant recipient.

* Percent stimulation of T lymphocytes in the presence of 5×10^4 unstimulated, uninfected monocytes and PHA (2 μg/ml) is represented as 100%. All other percentages are calculated relative to this value.

Suppression of T lymphocyte proliferation by autologous, LPS-stimulated or unstimulated, CMV-infected monocytes. The number of unstimulated, uninfected monocytes needed to augment the proliferative response of purified autologous T cells in a coculture assay system was established (table 2). For all donors tested, the ratio of monocytes to T lymphocytes that significantly increased T lymphocyte stimulation was 1:5 (0.4×10^5 monocytes:2×10^5 T lymphocytes), and this ratio was used for all subsequent experiments. Addition of LPS-stimulated monocytes to autologous T lymphocytes significantly augmented the T cell proliferative response to PHA when compared with that achieved with unstimulated monocytes ($P < .01$ by Student's t test).

In contrast, the proliferative response of autologous T cells to PHA was significantly suppressed in the presence of monocytes infected with either strain of virus (table 2), with the suppression being greater with increasing moi. LPS-stimulated monocytes infected with either strain of CMV at an moi of 0.005 and 0.01 pfu per cell were more suppressive for autologous T lymphocyte proliferation than were infected, unstimulated monocytes at the same moi. Infection of LPS-stimulated monocytes with the clinical isolate of CMV at an moi of as low as 0.005 pfu per cell completely abrogated their ability to augment the mitogenic response of T lymphocytes.

Effect of CMV infection on production of IL-1 activity by unstimulated and LPS-stimulated monocytes. Infection of both unstimulated and stimulated monocytes abrogated their production of IL-1 activity as assessed in the conventional bioassay, where 50% of the medium was supernatants collected from the relevant monocyte cultures three days after infection.

Uninfected, unstimulated monocytes constitutively produced low levels of IL-1 activity, with this production being reduced by three to five times when monocytes were infected with either strain of CMV. The IL-1 activity of LPS-stimulated monocytes was higher (figure 1), but the abrogation of this activity after infection with either strain of CMV was more marked than for unstimulated cells, with a reduction of 17–35 times being observed. The supernatants of stimulated, infected monocytes did not augment [^{3}H]thymidine incorporation by mouse thymocytes at all.

Effect of addition of IL-1–containing supernatants or pure IL-1 on T lymphocyte proliferation in the presence of CMV-infected monocytes. Addition of various dilutions of pure IL-1 or IL-1–containing supernatant (collected from LPS-stimulated monocytes after overnight culture) to T lymphocytes cultured in the presence of autologous, stimulated CMV-infected monocytes completely restored their proliferative response to levels in the presence of stimulated uninfected monocytes (figure 2, top). This effect was observed for all concentrations of pure IL-1 used, ranging from 2 to 0.25 U/ml. Addition of IL-1–containing supernatant restored the normal PHA response even at a 1:16 dilution.

Addition of this leukokine to T lymphocytes cultured with autologous unstimulated, CMV-infected monocytes did not restore their proliferative response (figure 2, bottom).

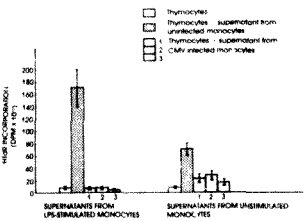

Figure 1. Effect of CMV infection on production of IL-1 activity by unstimulated and LPS-stimulated monocytes. Supernatants from LPS-stimulated and unstimulated, uninfected or CMV-infected monocytes were assayed in the conventional bioassay, which measures the ability of IL-1 to augment the PHA-induced proliferation of mouse thymocytes. IL-1 activity is expressed as the mean ± 1 SD (bars) incorporation of [^{3}H]thymidine (^{3}HTdR) in dpm for triplicate samples. *1* = clinical isolate at an moi of 0.005 pfu per cell; *2* = strain AD169 at an moi of 1 pfu per cell; and *3* = strain AD169 at an moi of 5 pfu per cell.

Discussion

Previous reports on the interaction of HCMV with monocytes have only described effects of CMV infection on unstimulated monocytes [4–7]. This in vitro study investigated the effects of CMV challenge on both unstimulated and LPS-stimulated monocytes in an attempt to examine more closely the mechanisms of immunosuppression that may accompany CMV infection in vivo.

CMV infections have been shown to increase susceptibility to superinfection by other organisms. This

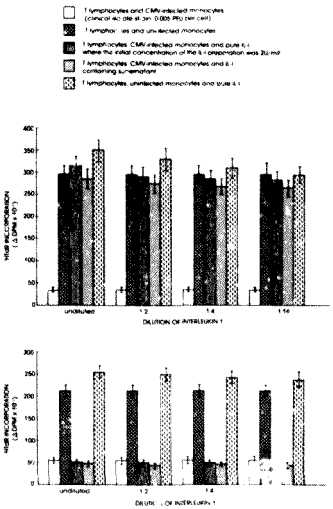

Figure 2. Effect of addition of IL-1–containing supernatant or pure IL-1 on PHA-induced proliferation of T lymphocytes in the presence of (*top*) LPS-stimulated and (*bottom*) unstimulated, CMV-infected and uninfected monocytes. Culture supernatants from CMV-infected or uninfected monocytes were removed after four days and replaced with fresh, autologous T lymphocytes in the presence of various dilutions of IL-1–containing supernatant or ultrapure IL-1. T lymphocyte proliferation was assessed by addition of [^{3}H]thymidine (^{3}HTdR) three days later, and incorporation after 16 hr was expressed as the mean ± 1 SD dpm of triplicate samples.

situation has been most clearly shown in transplant recipients [8–11], but it has also been suggested in adults with CMV mononucleosis [18]. Peripheral blood monocytes can be stimulated by bacterial and other antigens and by bacterial products such as LPS, the endotoxin released by *E. coli* and other gram-negative bacteria. The IL-1 production by stimulated cells is greater than for resting, unstimulated monocytes [12, 13]. Therefore, depending on whether CMV infection is accompanied by superinfection with other organisms, one might expect interactions of CMV with both stimulated and unstimulated monocytes in the peripheral blood and in the tissues, where monocytes mature both functionally and morphologically to become typical tissue macrophages [19].

We compared the interaction of HCMV with unstimulated and stimulated monocytes. Two strains of the virus, a clinical isolate and the laboratory-adapted strain AD169, were used because reports suggesting biologic differences between laboratory and clinical strains with regard to degree of infection of leukocytes have been published [4, 5]. The results in this study confirm that HCMV strain AD169 fails to initiate expression of detectable immediate-early antigens in most challenged monocytes [4, 5, 7], even when these cells are stimulated with LPS. However, monocytes challenged with a clinical isolate of CMV did express immediate-early antigens, with the percentage of apparently infected cells rising with increasing moi (table 1).

The ability of low apparent numbers of virions of the clinical isolate (0.1 pfu per cell) to cause expression of immediate-early antigens in 7%–9% of the monocytes raises the question of the validity of such pfu assays as an accurate determination of viable viral particles. It appears that virions unable to replicate in fibroblasts may well be present in the clinical isolate. Similar anomalous results were reported previously [4, 5].

Virus-challenged monocytes were suppressive for T cell blastogenesis. LPS-stimulated monocytes challenged with either strain of CMV were more suppressive for autologous T lymphocyte responses than were virus-challenged unstimulated monocytes, when tested under identical assay conditions (table 2), even when optimal PHA concentrations were used. Stimulated monocytes produce significantly higher levels of IL-1 activity than do unstimulated monocytes, but challenge with virus abrogated this function, as assessed by the conventional mouse thymocyte bioassay [20] with monocyte-conditioned medium (figure 1). Monocytes infected with the clinical isolate of CMV were more suppressive than monocytes challenged with strain AD169, with an moi of 0.005 and 5 pfu per cell, respectively, resulting in similar abrogation of released IL-1 activity (figure 1).

A report by Rodgers et al. [7] suggested that HCMV strain AD169 abrogates the IL-1 activity of unstimulated monocytes by inducing an inhibitor. However, IL-1 was not added back to their ex-

perimental system to assess whether the biologic activity of IL-1 could be restored.

The effect of addition of exogenous IL-1 on the PHA-induced proliferation of autologous T lymphocytes was therefore investigated in the current study. Addition of IL-1, in the form of IL-1–containing supernatants obtained from LPS stimulation of monocytes or of ultrapure IL-1, fully restored the proliferative response of T cells cultured in the presence of stimulated CMV-infected monocytes to that observed in the presence of control uninfected, stimulated monocytes (figure 2, top). Addition of IL-1 to T cells cultured with unstimulated, CMV-infected monocytes did not restore the normal response (figure 2, bottom), a result suggesting that unstimulated, CMV-infected monocytes release an inhibitor of supernatant IL-1 activity [7].

However, the release of an inhibitor cannot explain the abrogation of IL-1 activity of virus-challenged, LPS stimulated monocytes. Addition of IL-1 to T cells cultured with such monocytes totally restored their response in the lymphoproliferation assay, an observation suggesting that the virus interfered with the release of this factor by LPS-stimulated monocytes.

In conclusion, it is suggested that immunosuppressive effects of HCMV infection are mediated in part by an effect of HCMV on the monocyte and that this immunosuppression may occur through different mechanisms for unstimulated and stimulated monocytes. Clinical isolates have a greater immunosuppressive effect than the laboratory strain AD169. These observations indicate the need for examination of CMV interactions with both stimulated and unstimulated monocytes for accurate establishment of the immunosuppressive mechanisms accompanying HCMV infection in vivo.

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