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Role of Macrophage Oxidative Metabolism in Resistance to Vesicular Stomatitis Virus Infection

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The role of oxygen metabolites in mediating virucidal activity was studied in two cloned macrophage-like cell lines. The parental cell line, J774.16, upon appropriate stimulation with either phorbol myristate acetate (PMA) or aggregated immunoglobulin, is induced to oxidize glucose via the hexose monophosphate shunt and produce O_2^- and H_2O_2. A variant derived from it, clone C3C, is defective in oxidative metabolism and cannot be stimulated to produce O_2^- or H_2O_2. Significant differences in yields of vesicular stomatitis virus (VSV) between stimulated clone 16 cells and unstimulated cells could be obtained only when low multiplicities were used for infection. Under the same conditions, PMA stimulation of the variant clone C3C produced no reduction in yields. The effect of PMA on virus yields in clone 16 was short-lived and dose dependent. PMA stimulation of either cell line had no effect on the number of infectious centers, suggesting that the antiviral effect was likely to be an extracellular, rather than an intracellular, one. Using glucose oxidase plus glucose to generate H_2O_2 in solution, we observed that H_2O_2 alone is capable of killing limited amounts of VSV. The inactivation of VSV, both by H_2O_2 in solution and by activated clone 16 cells, could be inhibited by catalase. We conclude that intracellular resistance to VSV is primarily mediated through nonoxidative mechanisms, since activated macrophages can kill only a limited number of infectious virus particles extracellularly by means of secreted H_2O_2.

There is abundant evidence that macrophages play a significant role in both nonspecific and specific immune responses to viral infections (4, 15, 20). Macrophages are often the first cells to take up inoculated virus at the site of entry (14) and are frequently the predominant cells in the inflammatory infiltrates found in target organs affected by virus (13). In the course of cell-mediated immune reactions, activated macrophages are known to restrict viral spread from the site of infection, perhaps best exemplified by the vaccination reaction (4). Indeed, in the case of some viruses, activated macrophages have been known to inhibit cell-to-cell spread of virus in vitro (15). Macrophages can be intrinsically resistant to the replication of many viruses, but when viruses are capable of replication in macrophages, they often produce lethal infections in the same host (4). Viruses which replicate productively within macrophages without causing cytopathic effects often cause persistent viral infections (15). Finally, mice known to be resistant to a variety of viruses can be rendered susceptible by selective depletion of macrophages by colloidal silica (22, 28, 29).

Although there is ample evidence that macrophages play a role in resistance to viral infections, very little is known about the molecular mechanisms by which macrophages mediate antiviral activity. There are a number of properties of mononuclear phagocytes which are well known to contribute to their microbicidal and parasiticidal activity, including low intracellular pH, lysozyme, cationic proteins, lysosomal hydrolases, and interferon (1, 9, 12). Of particular interest in recent years has been the role of oxygen metabolites and the oxidative cytocidal mechanisms (2, 11, 26). It has long been established that phagocytosis in neutrophils and macrophages is associated with stimulation of a respiratory burst, which is associated with increased hexose monophosphate shunt activity and production of the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxy radical (·OH) (11). In the presence of peroxidase and halides, hypohalide is generated (12). Primary mouse macrophages activated by BCG or LPS and known to produce elevated levels of O_2^- and H_2O_2 showed enhanced microbicidal capacity against trypanosomes, toxoplasma, and

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candida (17, 26). Moreover, Belding et al. (3) showed that vaccinia virus and poliovirus were susceptible to cell-free enzyme systems able to generate either H_2O_2 or hypohalide, but the role of these oxygen metabolites in mediating virucidal activity in the macrophage has not yet been fully elucidated.

We have previously developed a murine continuous macrophage-like cell line that, upon appropriate stimulation with either phorbol myristate acetate (PMA) or aggregated immunoglobulin, is induced to oxidize glucose by the hexose monophosphate shunt to produce O_2^- and H_2O_2 in amounts of the same order of magnitude as primary macrophages (5). From this parental line, termed J774.16, we have derived clones defective in oxidative metabolism, which provide a model for the rare genetic deficiency diseases in which oxygen metabolism is absent or defective, such as chronic granulomatous disease (10, 19). In the present study, we have examined the replication of vesicular stomatitis virus (VSV) in the parental clone, J774.16, under conditions in which oxidative metabolism is stimulated by PMA and in the oxidative variant, clone C3C. The results will suggest that oxidative metabolism and H_2O_2 production may play a role in resistance to VSV, not primarily by intracellular killing as would be expected to be the case in bacterial or parasitic infections, but by extracellular killing of VSV.

MATERIALS AND METHODS

Reagents. PMA (Consolidated Midland Corp., Brewster, N.Y.) was stored in 0.2-ml portions at −90°C at a concentration of 10 mg of PMA per ml of dimethyl sulfoxide and diluted with medium before use. Catalase (from bovine liver) and glucose oxidase (type II, from *Aspergillus niger*) were purchased from Sigma Chemical Co. (St. Louis, Mo.) and prepared just before use.

Cell lines. The cell lines used for these experiments were continuous macrophage-like cell lines, clones J774.16 (clone 16) and C3C, derived from the murine reticulum cell sarcoma J774 (24). These cells were propagated as previously described (5). The cells were cultured in 90-mm petri dishes (no. 1029; Falcon Plastics, Oxnard, Calif.) in a humidified incubator in 5% CO_2-balanced air at 37°C. Medium was changed twice a week. For best results, cells were cultured for at least 2 weeks in the same petri dish; 3- to 4-day-old cultures were used in all experiments. L929 cells, used for virus plaque assay, were grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, glutamine, and antibiotics.

Virus and virus titrations. The Indiana (HRC) serotype of VSV was used. Stock virus was grown in primary chicken embryo fibroblasts and partially purified, and samples were stored at −70°C. The titer of the stock virus was 2.5×10^8 PFU per ml. Gradient-purified B particles of VSV, free of defective interfering particles, were kindly provided by P. I. Marcus. Virus plaque assays were performed in L-cell monolayers as described by Rager-Zisman and Merigan (23).

VSV infection of clones 16 and C3C. Cells (5×10^5/ml) of either clone 16 or clone C3C were dispersed into wells of 16-mm cluster dishes (76-033-05; Linbro Scientific Inc., Hamden, Conn.). After an incubation of 2 to 3 h at 37°C in 5% CO_2, monolayers were formed, and 0.1 ml of VSV was added to the wells in different dilutions.

Stimulation of cells with PMA. Unless stated otherwise, stimulation of 5×10^5 cells with 1 μg of PMA per 0.01 ml of 1% dimethyl sulfoxide was performed 10 min before infection with VSV. Dimethyl sulfoxide alone at comparable volumes had no detectable effect on cell lines studied.

Anti-VSV serum. Rabbit anti-VSV Indiana serum was a gift from J. Holland. The antiserum had a neutralization titer of 1:1000.

Infectious centers assay. Cell suspensions were infected with virus at a multiplicity of infection (MOI) of 5 to 0.05 per cell. After adsorption for 60 min at 37°C, the cells were treated with anti-VSV serum for 30 min at 4°C, washed, resuspended in 1 ml of medium, and reincubated at 37°C for an additional 1 to 3 h. Different numbers of infected cells were then assayed on L-cell monolayers (21).

CCP. Cytochrome *c* peroxidase (CCP) was prepared as described by Damiani et al. (5). The enzyme was frozen and stored in liquid nitrogen at a concentration of ≈1 mM CCP.

Measurement of H_2O_2 production by CCP. Measurement of H_2O_2 production by CCP was performed as described by Damiani et al. (5). Clones 16 and C3C were infected with VSV at a MOI of 3 and incubated at 37°C in 5% CO_2. After 1 or 3 h, VSV-infected or control uninfected macrophages were washed with Krebs-Ringer phosphate buffer glucose, resuspended to 10^6 cells per ml in Krebs-Ringer phosphate buffer glucose, and kept on ice until assayed.

Assay for [1-^{14}C]glucose oxidation. The assay was performed as described by Damiani et al. (5). Radioactive glucose was obtained from New England Nuclear Corp. (NEC043; Boston, Mass.). [1-^{14}C]glucose had a specific activity of 0.05 mCi/1.3 mg. VSV-infected cells (MOI of 3; 1 or 3 h after infection) or uninfected cells were labeled with ~1.25 nmol of glucose in Krebs-Ringer phosphate buffer without glucose that contained 0.5% bovine serum albumin and resuspended to a cell density of 10^6 cells per ml. Cells, unstimulated or stimulated with different amounts of PMA, were incubated for 30 min. CO_2 was released by the addition of 0.5 ml of 1 M H_2SO_4. The CO_2 was trapped with 0.2 ml of DL-phenylamine on filter paper and later counted in a scintillation counter. Counts were converted to nanomoles of $^{14}CO_2$ released. Less than 1 nmol of $^{14}CO_2$ was produced by cells not stimulated with PMA.

Susceptibility of VSV to H_2O_2 produced by glucose oxidase. Direct virucidal activity of H_2O_2 against VSV was assesed by exposing the virus to a glucose-glucose oxidase system in solution and measuring VSV titers. A 0.1-ml amount of various dilutions of VSV was resuspended in Krebs-Ringer phosphate buffer (pH 7.2) containing 4.5×10^{-4} M glucose and increasing amounts of glucose oxidase in 1 ml. The suspension was incubated for 60 min at 37°C and assayed for infectious virus. The rate of H_2O_2 production by

glucose-glucose oxidase was independently determined by the CCP assay.

RESULTS

Two macrophage-like cell lines were used for these studies. The parental clone 16, which was derived from the murine reticulum cell sarcoma J774, shows a marked increase in Nitro Blue Tetrazolium reduction, [1-^{14}C]glucose oxidation via the hexose monophosphate shunt, and O_2^- and H_2O_2 production after stimulation by PMA or aggregated immunoglobulin. The second cell line was an oxidative variant selected from clone 16 and termed clone C3C and is incapable, under appropriate stimulation, of showing a respiratory burst or producing O_2^- or H_2O_2 (5).

Susceptibility of parental clone 16 and the oxidative variant, clone C3C, to VSV. Monolayers of clone 16 or C3C were infected at a range of multiplicities of 1 to 10^{-5}. Both lines were found to be extremely susceptible to VSV. Between 24 and 48 h after infection, both cell lines were completely lysed by the virus. The rate and extent of lysis could be correlated with MOI. Whereas complete destruction occurred within 24 h at multiplicities of 1 to 10^{-3}, it required 48 h for lysis to be observed in cultures infected with a MOI of 10^{-4} or less. The susceptibility of both clones was very similar; the 50% tissue culture infective dose of VSV was estimated to be $10^{-6.5}$ in clone 16 and $10^{-6.0}$ in clone C3C. Infection of clone 16 by VSV produced unusual cytopathic effects, including the formation of multinucleate giant cells and marked vesiculation before final lysis, which were not seen in the variant (Fig. 1).

When VSV yields were measured at 24 h after infection (Table 1), there were few differences in yields between the two macrophage clones, and little effect of MOI was seen. For example, the titer of the infectious VSV yields dropped only about 10-fold after a 1,000-fold diminution in MOI. If anything, the variant C3C clone was slightly less permissive than the parental clone when infected with 500 or 50 PFU of virus.

Effect of PMA activation on VSV yields from clone 16 or clone C3C. Our previous studies (5) indicated that clone 16 produced O_2^- and H_2O_2 only after appropriate stimulation, e.g., induced by PMA or aggregated immunoglobulin. Since viruses may not be sufficient in themselves to trigger the respiratory burst in macrophages, experiments were undertaken to examine viral replication in the macrophage clones subsequent to treatment with PMA. In the experiments summarized in Fig. 2, parental clone 16 or variant C3C was stimulated for 10 min with 1 μg of PMA per ml before virus infection and then infected with VSV at several multiplicities. Whereas at a high multiplicity of 5×10^3 PFU, no significant difference could be seen in VSV yields between PMA-stimulated cells of either clone, at the lower multiplicities of 500 or 50 PFU, there was a highly significant reduction ($P > 0.001$) in a Student's t test in VSV yields in the PMA-treated clone 16 cells, on the order of 2 to

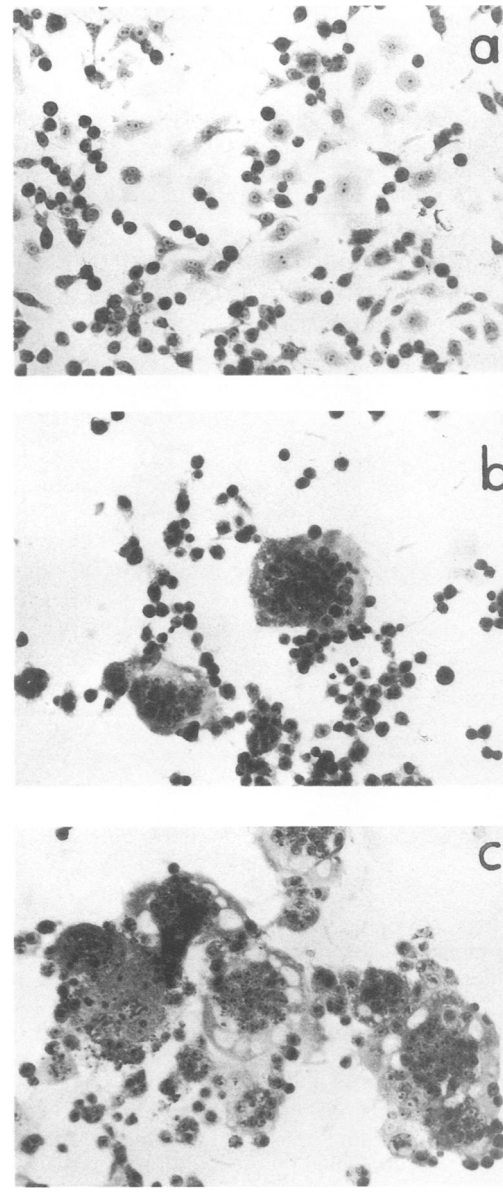

FIG. 1. Cytopathic effects of VSV infection in clone 16. Clone 16 cells were stimulated with 1 μg of PMA per ml and infected with 500 PFU of VSV. Cytopathic effects were seen 24 h after infection. Note the vesiculation and formation of giant multinucleate cells in PMA-stimulated cultures. (a) Clone 16, uninfected; (b) clone 16 after infection with VSV; (c) clone 16 stimulated with PMA and infected with VSV (×320).

TABLE 1. VSV yields from clones 16 and C3C (24 h after infection)[a]

Input virus (PFU)	MOI	Virus yields (PFU/5 × 10^5 cells)	
		Parental clone 16	Variant clone C3C
5 × 10^4	0.1	6.0 × 10^7 ± 1.8 × 10^7	5.0 × 10^7 ± 1.4 × 10^7
5 × 10^3	0.01	3.0 × 10^7 ± 0.8 × 10^7	3.0 × 10^7 ± 2.0 × 10^7
5 × 10^2	0.001	1.0 × 10^7 ± 3.0 × 10^7	2.0 × 10^6 ± 2.2 × 10^6
5 × 10^1	0.0001	4.5 × 10^6 ± 1.4 × 10^6	1.0 × 10^6 ± 0.3 × 10^6

[a] A 1-ml volume containing 5 × 10^5 cells was dispersed into the wells of 16-mm cluster dishes and incubated at 37°C in 5% CO_2. After monolayers were formed (2 to 3 h later), the cells were infected with different amounts of VSV (Indiana, HRC serotype). Results are an average of six experiments.

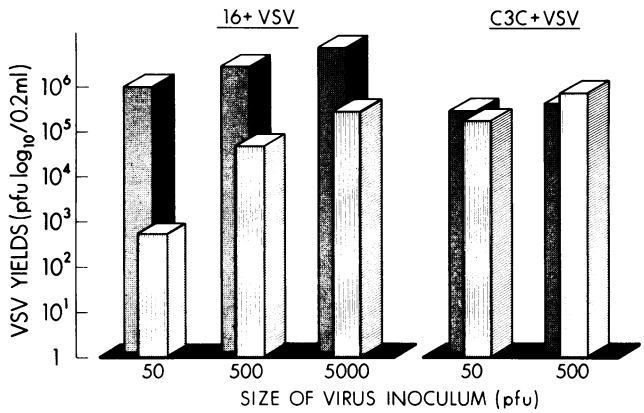

FIG. 2. Effect of PMA stimulation on VSV yields. Monolayers of clone 16 or C3C were stimulated with 1 μg of PMA per ml 10 min before infection with VSV. Cell culture supernatants were collected 24 h later, and VSV yields were measured. Dots, No PMA; lines, with PMA. Results are an average of six experiments.

3 logs. No reduction was found in yields of VSV in clone C3C similarly treated with PMA, and indeed, there was occasionally an increase in viral yield in C3C cells triggered with PMA, possibly due to increased internalization of membranes and an increased uptake of virus or stimulation of cell division. PMA stimulation and virus infection of clone 16 cells led to the formation of giant cells (Fig. 1). This cytopathic effect was abolished by the addition of anti-VSV serum and was not seen in cells treated with PMA alone.

The effect of PMA on VSV yields was expressed only within a critical time period. Stimulation with 1 μg of PMA per ml was effective only when PMA was added 10 min before, simultaneously with, or 10 min after virus infection. Treatment at earlier or later intervals had no effect (Fig. 3a). The optimum dose of PMA that was effective and nontoxic appeared to be 1 μg/ml (Fig. 3b).

Effects of VSV on [1-^{14}C]glucose oxidation and H_2O_2 production. Since VSV infection in permissive cells has been demonstrated to result in inhibition of host cell metabolism (7), it was of interest to ascertain whether the respiratory burst and oxidative cytocidal mechanisms could be impaired as a consequence of VSV infection. Accordingly, clone 16 or clone C3C cells, uninfected or infected by VSV at a MOI of 3 for 1 or 3 h, were stimulated by PMA as described above. [1-^{14}C]glucose oxidation (Fig. 4a) and H_2O_2 production detected by the CCP assay (Fig. 4b) were measured. Virus infection appeared to have no effect on [1-^{14}C]glucose oxidation or H_2O_2 production either with suboptimal (0.1 μg) or optimal (1 μg) concentrations of PMA, which is not surprising in light of the low percentage of productively infected cells. As expected, uninfected or virus-infected clone C3C cells exhibited minimal levels of [1-^{14}C]glucose oxidation or H_2O_2 production after stimulation with PMA.

Role of H_2O_2 in killing VSV. Since it was found that 10^6 clone 16 cells stimulated with (1 to 3 μg) PMA released approximately 0.13 nmol of H_2O_2 per min (Y. Tanaka, C. Kiyotaki, H. Tanowitz, and B. R. Bloom, Proc. Natl. Acad. Sci. U.S.A., in press), it was possible to indirectly test the role of H_2O_2 in reducing yields of VSV by carrying out the experiment of PMA stimulation and virus infection in the presence of 1 mg

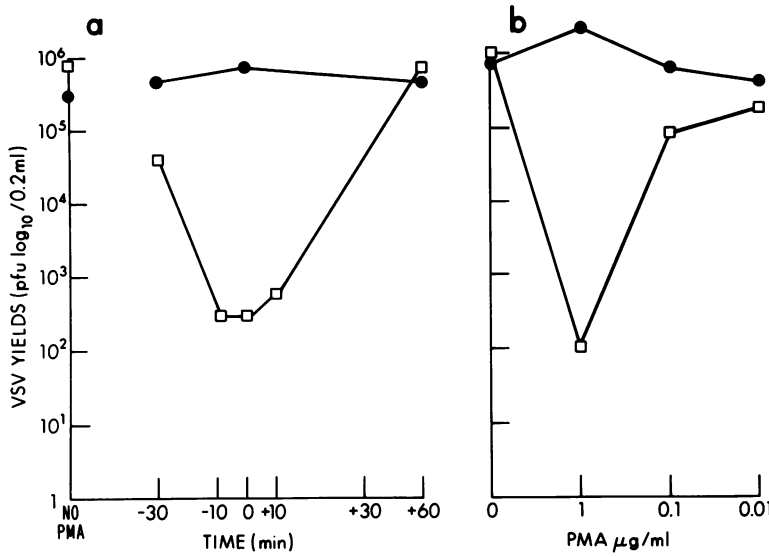

FIG. 3. Effect of PMA dosage and time of stimulation on VSV yields. (a) Monolayers of clone 16 or C3C were stimulated with 1 μg of PMA per ml at different time intervals before or after infection with 50 PFU of VSV. (b) Monolayers of clone 16 or C3C were stimulated with different doses of PMA 10 min before infection with 50 PFU of VSV. Symbols: □, clone 16; ●, clone C3C. VSV yields in cell culture supernatants were measured 24 h after infection. Results are an average of three experiments.

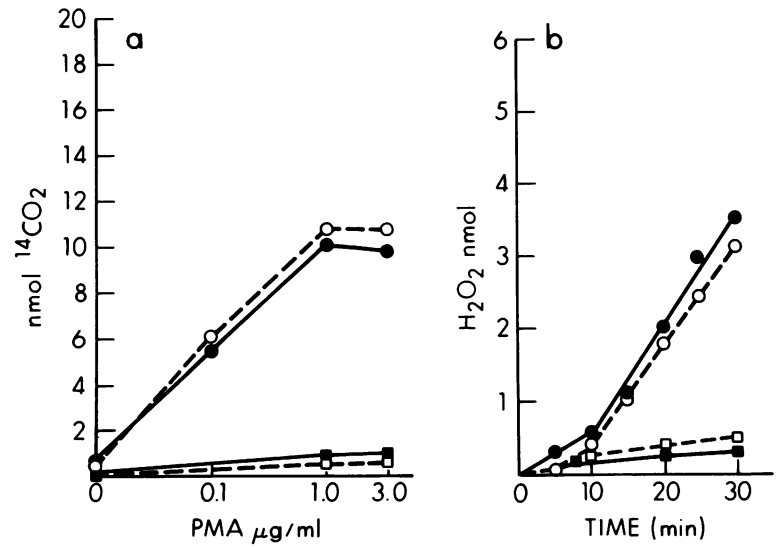

FIG. 4. Effect of VSV infection on [1-^{14}C]glucose oxidation and H_2O_2 production. (a) [1-^{14}C]glucose oxidation and (b) H_2O_2 production by VSV-infected clone 16 or C3C cells (10^6 cells). H_2O_2 production was detected by the CCP assay. Symbols: ○, clone 16; ●, clone 16 plus VSV; □, clone C3C; ■, clone C3C plus VSV. Results are an average of three experiments.

of catalase. Catalase catalyzes the breakdown of H_2O_2 to O_2 and H_2O. As can be seen in Table 2, stimulation of clone 16 cells with PMA resulted in this experiment in a 4-log diminution in VSV yields. Thus, catalase partially inhibited the inactivation of VSV, suggesting that H_2O_2 was involved in the reduction in virus yields. In contrast, boiled catalase had no effect.

Direct evidence of the virucidal activity of H_2O_2 was obtained by exposing VSV to an H_2O_2-generating system consisting of glucose and glucose oxidase in Krebs-Ringer phosphate

TABLE 2. Catalase inhibition of VSV inactivation

Treatment[a]	VSV yields (PFU/5 × 10^5 cells)	
	Parental clone 16	Variant clone C3C
None	2 × 10^6	1.2 × 10^6
1 μg of PMA	2 × 10^2	3.7 × 10^6
1 μg of PMA plus 1 mg of catalase[b]	5 × 10^3	3.7 × 10^6

[a] A 1-ml volume containing 5 × 10^5 cells was dispersed into the wells of 16-mm cluster dishes and incubated at 37°C in 5% CO_2. After monolayers were formed, the cells were infected with 50 PFU of VSV. PMA was added 10 min before VSV infection. Cell culture supernatants were collected 24 h later, and VSV yields were measured.

[b] PMA stimulation and virus infection were carried out in the presence of 1 mg of catalase. Results are an average of three experiments.

buffer. The virus was exposed to varying amounts of H_2O_2 for 1 h at 37°C, and the results of viral titrations are depicted in Fig. 5. Reduction in viral infectivity was detected only with low initial numbers of infectious virus (PFU); with higher amounts of virus, little reduction in infectivity could be detected. When 2.4 × 10^4 PFU of VSV were incubated with H_2O_2 produced by glucose oxidase in solution for 1 h, 97% of VSV was killed at an H_2O_2 level of 100 nmol/min per ml, and the 50% lethal dose (1.2 × 10^4 PFU) was at the level of 18.2 nmol/min per ml. (With the limitation, we are able to measure only rates rather than absolute levels of H_2O_2.) A similar rate of VSV inactivation in the glucose-glucose oxidase system was obtained with gradient-purified VSV B particles, confirming the observation on crude virus preparations that H_2O_2 is directly virucidal for a limited number of infectious particles. A second exposure of the same virus population to H_2O_2 killed VSV to the same extent. It therefore appears that a given amount of H_2O_2 has the ability to kill only a limited number of PFU. The simplest assumption is that since reductions in yields were seen only at low MOIs, H_2O_2 produced by the macrophages probably acts on the small extracellular input virus, rather than on the intracellular viral progeny.

This hypothesis was tested by the infectious centers assays. If viruses were killed intracellularly by an oxidative cytocidal mechanism after PMA stimulation, one would expect to find a diminution in infectious centers in parental clone 16 but not in the oxidative variant C3C. If only extracellular input virus was killed, although one might anticipate a diminution in yields in PMA-stimulated clone 16 cells at low MOIs, one would not expect a diminution in infectious centers. The results summarized in Table 3 show that only 0.9 to 7.5% of clone 16 cells produced infectious centers. Addition of PMA to VSV-infected cells had no effect on the number of infectious centers.

DISCUSSION

In recent years, greater understanding of the intracellular cytocidal mechanisms of activated macrophages has emerged. While a number of potentially effective molecular mechanisms have been identified, particular interest has been fo-

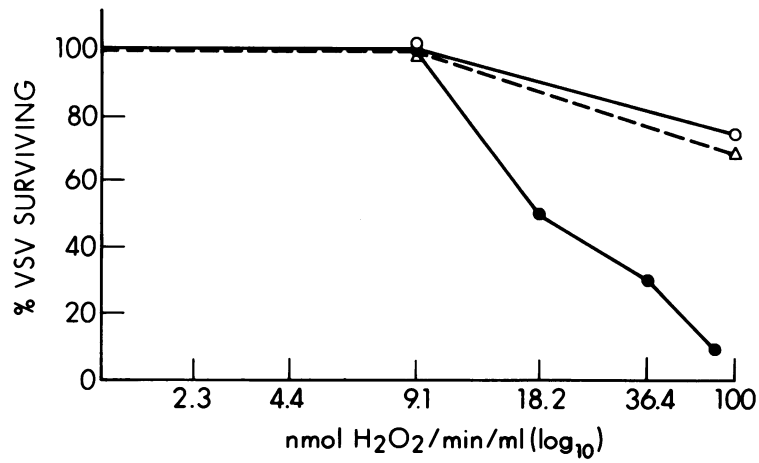

FIG. 5. Virucidal activity of H_2O_2 in a cell-free system. The rate of H_2O_2 production in this system was independently assayed with CCP. Symbols: ○, 3 × 10^5 PFU of VSV; ●, 2.4 × 10^4 PFU of VSV; △, 2 × 10^4 PFU of VSV plus 1 mg of catalase. Results are an average of eight experiments.

TABLE 3. Incidence of VSV infectious centers with or without PMA

Clone	MOI	Total no. of cells plated	No. (%) infectious centers[a]
16	5	10^4	40 (2)
16	5	10^3	9 (4.5)
16 plus PMA[b]	5	10^4	50 (2.5)
16 plus PMA	5	10^3	12 (6)
16	0.5	10^4	45 (2.2)
16	0.5	10^3	11 (5.5)
16 plus PMA	0.5	10^4	60 (3)
16 plus PMA	0.5	10^3	15 (7.5)
16	0.05	10^4	18 (0.9)
16	0.05	10^3	NT[c] NT
16 plus PMA	0.05	10^4	25 (1.2)
16 plus PMA	0.05	10^3	NT NT
C3C	5	10^4	16 (0.8)
C3C	5	10^3	2 (1)

[a] Infectious centers assays were carried out as described in the text. Results of a representative experiment (of four) are presented.

[b] PMA (1 μg/ml) was added to the cells after virus adsorption.

[c] NT, Not tested.

cused on the role of oxidative cytocidal mechanisms (2, 11, 12, 25).

Because of the difficulty in obtaining homogeneous primary macrophage cell populations free of other contaminating cell types, the variability in results from animal to animal, and the limitations in numbers of cells that can be obtained from animals, we have studied, over the past several years, the continuous macrophage-like cloned cell lines 16 and C3C derived from a reticulum cell sarcoma, J774 (5, 24). We have shown that clone 16 cells are capable of killing the protozoal parasite, *Trypanosoma cruzi*, whereas variant C3C cells allow the parasite to grow, indicating that these cell lines can be used for studying the oxidative mechanisms of resistance to intracellular parasites (Tanaka et al., in press).

The present work was designed to examine three questions: (i) the role of oxidative metabolism and oxygen metabolites in resistance to virus infection, with VSV as a model; (ii) whether antiviral activity occurred intracellularly or extracellularly; and (iii) whether viruses are capable of triggering the oxidative metabolic process or are inhibitory in any way.

Initially we observed that, unlike primary mouse peritoneal macrophages (unpublished data), both clones of macrophage cell lines supported the replication of VSV, and the 50% tissue culture infective dose reached levels of $10^{-6.5}$ in clone 16 and $10^{-6.0}$ in clone C3C. Yet, in the infectious centers assay, it emerged that only 0.9 to 7.5% of the cells were capable of replicating virus. From these data it could be estimated that in a single-step growth cycle, each productively infected cell was capable of producing progeny of at least 10^3 PFU. It remains unclear why, within a cloned cell population, such a high percentage of the cells are resistant to replication by VSV. It is possible that as in the case of vaccinia virus (6, 27), VSV is endocytosed very rapidly by the majority of the cells into the vacuole system and is later degraded in lysosomes.

When the effects of PMA stimulation of parental clone 16 and variant clone C3C on virus yields were examined, it became apparent that significant differences between stimulated clone 16 cells and unstimulated cells could be obtained only when low doses of virus were used for the infection. Under the same conditions, PMA stimulation of variant clone C3C had no effect. It thus appeared that cells capable of oxygen metabolism via the hexose monophosphate shunt were able to exert antiviral activity on a limited number of infectious particles. The effect of PMA on virus yields was short-lived and dose dependent. Since PMA stimulation of either cell line had no effect on the number of infectious centers, this suggested that oxidative metabolites may be involved in protection against infection at low multiplicities and that the antiviral effect was extracellular rather than intracellular. Alternatively, it could be proposed that C3C cells, in addition to their defect in oxidative metabolism, have another independent defect which prevents them from killing VSV, although the low figures of obtained oxidative metabolism mutants (5) should suggest that these cells are unlikely to have multiple mutants.

Recent studies on *T. cruzi* indicated that the resistance of BCG-activated macrophages, stimulated with PMA, to this intracellular protozoal parasite, correlated with production of H_2O_2 (17). In previous studies with clones 16 and C3C, we have also found that H_2O_2 was essential for the killing of *T. cruzi* in clone 16 (Tanaka et al., in press). We have therefore examined the role of H_2O_2 in inactivating VSV. With a cell-free system, glucose oxidase plus glucose, to generate H_2O_2, it was found that H_2O_2 alone can kill very limited amounts of VSV. From the inactivation curve with H_2O_2, it could be estimated that 18.2 nmol of H_2O_2 per min per ml was able to inactivate 1.2×10^4 PFU of VSV. For clone 16, 5×10^5 cells produce 0.065 nmol/min per ml after PMA stimulation, which can be estimated to be effective in inactivating approximately 43 PFU of VSV. This value correlates well with the number of input infectious VSV particles used to infect PMA-stimulated clone 16 cells under conditions in which a highly significant reduction in VSV yields was observed. Inactivation of VSV, both by activated clone 16 cells and by glucose

oxidase plus glucose in solution, could be inhibited by catalase, which accelerates the degradation of H_2O_2. This finding is consistent with the conclusion that the principal oxygen metabolite involved in anti-VSV resistance in this system is H_2O_2.

Of interest was the finding that VSV infection did not appear to be a significant stimulator either of the respiratory burst or the production of H_2O_2, nor did virus infection significantly impair the ability of clone 16 cells after stimulation to produce a respiratory burst or H_2O_2.

We conclude that oxygen metabolites confer resistance to VSV in macrophages by extracellular killing, acting on limited numbers of input infecting virus, rather than by intracellular killing, for the following reasons. (i) Reduction in viral yields was detected only if virus was added to cell cultures together with, 10 min before, or 10 min after stimulation with PMA. (ii) Resistance was apparent only at very low input multiplicities, and there was a correlation between the infecting dose of VSV and the number of infectious particles that could be inactivated by free H_2O_2 in solution. (iii) Each infected macrophage can produce a large VSV progeny. (iv) No reduction of infectious centers occurred after stimulation with PMA. Thus, PMA-stimulated cells capable of producing H_2O_2 do not show resistance to intracellular VSV. An observation of extrinsic antiviral activity mediated by activated macrophages has been recently described; this activity was interferon independent and was related to inhibition of herpes simplex virus replication in the target cells (16).

It is possible that the H_2O_2-mediated virucidal effect seen in vitro here could provide some protection locally in areas of primary virus infection and inflammatory sites in which local H_2O_2 production by activated macrophages may contribute to diminishing the initial infectious dose. It is also possible, since H_2O_2 has been reported to be an important mechanism of activated macrophage killing of tumor cells (18), that activated macrophages may exert a cytotoxic effect on virus-infected cells through the production of oxygen metabolites. Finally, because over 90% of these cloned macrophage-like cells were resistant to VSV infection independent of their ability to produce oxygen metabolites, these results suggest that there must be highly effective nonoxidative mechanisms, such as intracellular pH and lysosomal enzymes, which protect macrophages against virus infection; these require further study. In this regard, it is of interest that patients lacking the oxidative cytocidal mechanism, namely patients with chronic granulomatous disease and related metabolic deficiency diseases in oxygen metabolism, while highly susceptible to bacterial infections, appear to be not at comparably greater risk to acute virus infections (8).

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