Free Rad. Res. Comms., Vol. 3, No. 1–5, pp. 39–46
Photocopying permitted by license only

Printed in Great Britain

VIRUS-INDUCED FORMATION OF REACTIVE OXYGEN INTERMEDIATES IN PHAGOCYTIC CELLS

E. PETERHANS, M. GROB, TH. BÜRGE and R. ZANONI

Department of Virology, Institute of Veterinary Bacteriology, University of Berne, Länggass-Str. 122, Postbox 2735, CH-3001 Berne, Switzerland

(Received July 21st 1986)

Viruses cause disease by a wide variety of mechanisms. These include the impairment of differentiated host cell functions and the killing of infected cells. The latter is referred to as cytopathic effect and is exemplified by Polio virus infection where paralysis results from the loss of neurons killed by the virus. Host immune response as a factor contributing to disease is evident in the skin rashes in measles and rubella. Virus-immune complexes occur in many infections and may be associated with glomerulonephritis and arthropathy.

We describe two mechanisms by which viruses activate the generation of reactive oxygen intermediates (ROI) in polymorphonuclear leukocytes. The first is mediated by antiviral antibody and hence is controlled by the immune system. The second mechanism depends on a direct interaction of viral antigen with the plasma membrane of the phagocyte. It is suggested that the direct activation of ROI generation by paramyxo- and influenza viruses may be related to their well-known toxic effects *in vivo*.

KEY WORDS: Oxygen, toxicity, virus; infection, phagocyte.

INTRODUCTION

Progress in molecular and cellular biology has facilitated the detailed investigation of viral effects on host cells. For this reason, and because of the hope of delineating therapeutic approaches against viral diseases from such studies, the field of viral pathogenesis has attracted considerable interest over the past few years and has led to the conception that the symptoms of viral disease are caused by two main groups of mechanism.

The first group is characterized by detrimental effects of viruses on the cells in which they multiply. Viral infection may lead to the loss of specialized "luxury" functions of host cells or may result in cell death. As an example of the former, C3H mice persistently infected with lymphocytic choriomeningitis virus develop hypoglycemia and grow slower than uninfected control animals. The virus replicates in cells of the anterior pituitary gland and reduces the amount of growth hormone produced without killing the cells.[1,2] The consequences of cell death as a result of viral infection similarly depend on the particular function of the host cell and, as shown in Polio in humans, may even persist after the virus has been cleared from the body. Paresis or

paralysis is the result of loss of neurons and is largely permanent.[3] As a third type of mechanism that is directly due to an effect of the virus on the host cell, transformation, resulting in tumor formation[4] may be cited.

The second group of pathogenic mechanisms is characterized by detrimental effects of the host's immune response to viral infection. Well-known examples include the skin rashes observed in several viral infections, which, wholely or in part, reflect hypersensitivity reactions to viral antigens.[5]

In this paper we have used the measurement of luminol-dependent chemiluminescence (CL) to study virus-induced production of reactive oxygen intermediates (ROI) in polymorphonuclear leukocytes (PMN). CL depends on myeloperoxidase[6,7] and most likely originates from the cooxidation by H_2O_2 and OCl^- of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) to aminophthalate.[8] The experiments show that viruses activate the generation of ROI by two clearly different mechanisms. The possible role of ROI in the pathogenesis of viral disease is discussed.

MATERIALS AND METHODS

Target cells

Target cells to be infected with Sendai or Berne viruses were free from mycoplasmas as assayed by the CL method.[9]

To prepare target cells with Sendai virus surface antigens, HeLa cells were detached from the culture flasks (Falcon, Oxnard, CA, USA) by incubation for 10 min. at 37°C with Ca^{2+} and Mg^{2+}-free PBS containing 0.15% EDTA and 0.025% trypsin. After washing twice, the cells were incubated with egg-grown Sendai virus purified by differential and sucrose density gradient centrifugation[10] (400 hemagglutinating units/10^6 cells) at 0°C for 30 min. and subsequently for 10 min. at 37°C to allow fusion between the viral envelopes and plasma membranes. Cell–cell fusion was minimized by gently pipetting at 1 min. intervals. The cells were then washed twice and kept at 0°C until use in the CL experiments or further incubated for 1 h at 0°C with rabbit IgG or chicken Immunoglobulin directed against Sendai virus. The cells were subsequently washed twice to remove unbound antibody. Target cells presenting antigens of Berne virus were obtained from infected equine dermis cell cultures (multiplicity of infection: 1 $TCID_{50}$) by incubation at 37°C for 10 min. with Ca^{2+} and Mg^{2+}-free PBS containing 0.15% EDTA. Coating with antibody to Berne virus was as described above for Sendai virus target cells.

Polymorphonuclear leukocytes

Horse and human PMN were prepared from blood obtained by venipuncture as described previously.[9]

Measurement of CL

PMN were suspended at 100 000/ml in Hanks' balanced salt solution (HBSS) containing 5.6 mM glucose and buffered with 20 mM HEPES instead of bicarbonate. To enhance CL, 7.5 μl/ml of 4% bovine serum albumin saturated with luminol was added (final concentration of luminol 5–10 μM). Aliquots of 750 μl of the PMN suspension were filled in siliconized Pico vials (Packard, Downers Grove, USA) and dark-adap-

ted for 15–30 min. The CL experiments were performed as described previously using a modified Kontron Betamatic I liquid scintillation spectrometer operated in the out of coincidence mode and connected on line with a Hewlett-Packard 9816S microcomputer for data acquisition and analysis.[9]

RESULTS

Equine dermis cells infected with Berne virus were detached from culture dishes at various times post-infection and added to horse PMN after incubation with heat-inactivated horse immune serum to Berne virus. Cells harvested at 12 hours post-infection stimulated a slight (approx. 15,000 CPM) increase in CL above light emission observed in the presence of uninfected control cells that had been incubated with horse immune serum to Berne virus (Figure 1). Infected cells not incubated with immune serum, or incubated with horse serum lacking antibody to Berne virus, consistently failed to stimulate CL (results not shown). Cells harvested at 15 and 18 hours post-infection induced significant CL emission in the PMN. Titration of the cell

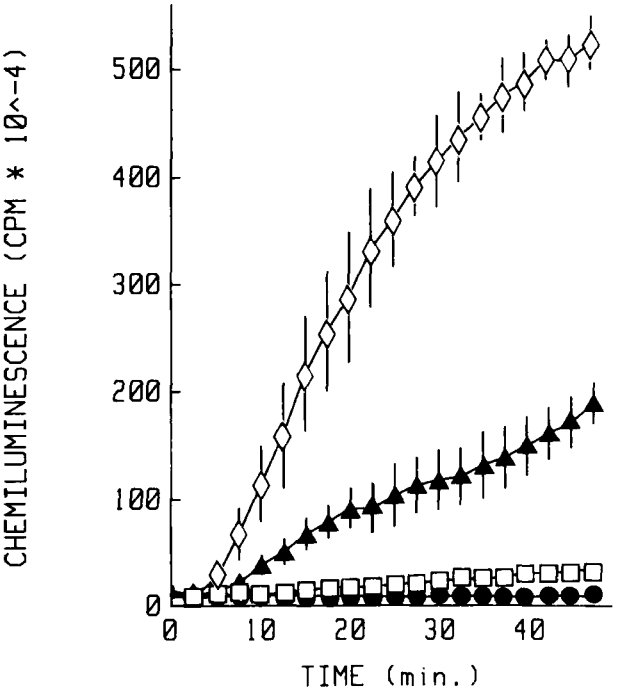

FIGURE 1 The induction of CL by antibody-coated cells infected with Berne virus. Equine dermis cells infected with Berne virus (BEV) were harvested from the culture dishes, incubated with horse immune serum to BEV and added to horse PMN at an effector to target cell ratio of 1:5. CL emission was measured in a liquid scintillation counter. Zero time refers to addition of target to effector cells. Points represent the means of 3 replicate samples and bars indicate standard deviations. ●—● PMN + uninfected cells incubated with horse immune serum to BEV; □—□ PMN + cells harvested at 12 hours post-infection, antibody-coated; ▲—▲ PMN + cells harvested at 15 hours post-infection, antibody-coated; ◇—◇ PMN + cells harvested at 18 hours post-infection, antibody-coated.

culture supernatants indicated that the extent of CL correlated with the replication cycle of Berne virus. Thus, infectious virus was first detected at 12 hours post-infection and reached a maximum at 18 hours post-infection (results of titration not shown). Taken together, these observations indicated that CL induction depended on the presence of antigens of Berne virus on the surface of the target cells and was mediated by antigen-bound antibody.

In Figure 2 is shown the effect on CL in human PMN of HeLa cells expressing Sendai virus antigens on their surface. In contrast to Berne virus-infected cells, HeLa-Sendai virus targets evoked CL generation in the absence of antiviral antibody. Target cells that had been incubated with rabbit IgG to Sendai virus induced a CL burst characterized by a slower onset, decreased peak and prolonged duration. Interestingly, the CL burst induced by antibody-free target cells could be abrogated by preincubation with chicken immunoglobulin used at the same antiviral activity as the rabbit IgG (as measured in a hemagglutination inhibition assay).

DISCUSSION

Our experiments show that viruses induce the generation of ROI in PMN by two clearly different mechanisms (see Figure 3). The first, exemplified by berne virus,

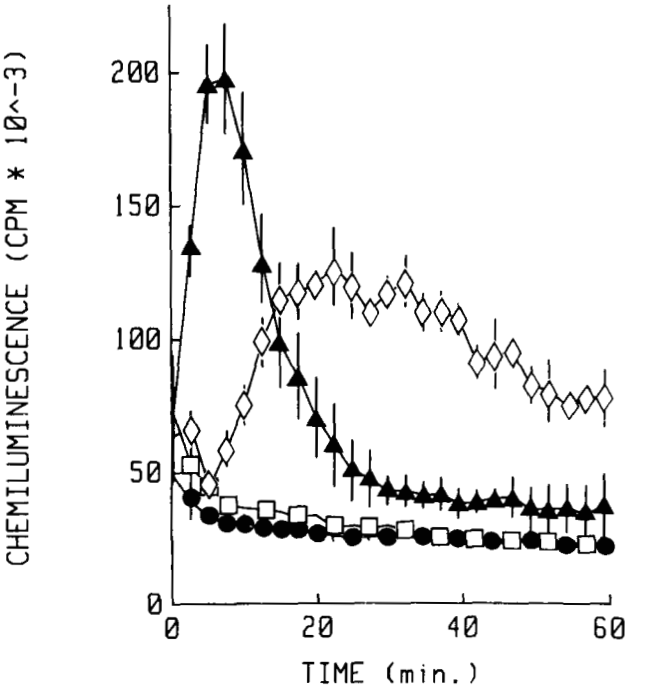

FIGURE 2 The induction of CL by HeLa cells infected with Sendai virus. HeLa cells carrying Sendai virus surface antigens were prepared as described in Materials and Methods. At time = 0 target cells were added to human PMN at an effector to target cell ratio of 1:20 and CL was measured as described in Materials and Methods. ●—● PMN alone; ▲—▲ PMN + HeLa-Sendai virus targets; ◇—◇ PMN + HeLa Sendai virus targets coated with rabbit antibody to Sendai virus; □—□ PMN + HeLa Sendai virus targets coated with chicken antibody to Sendai virus.

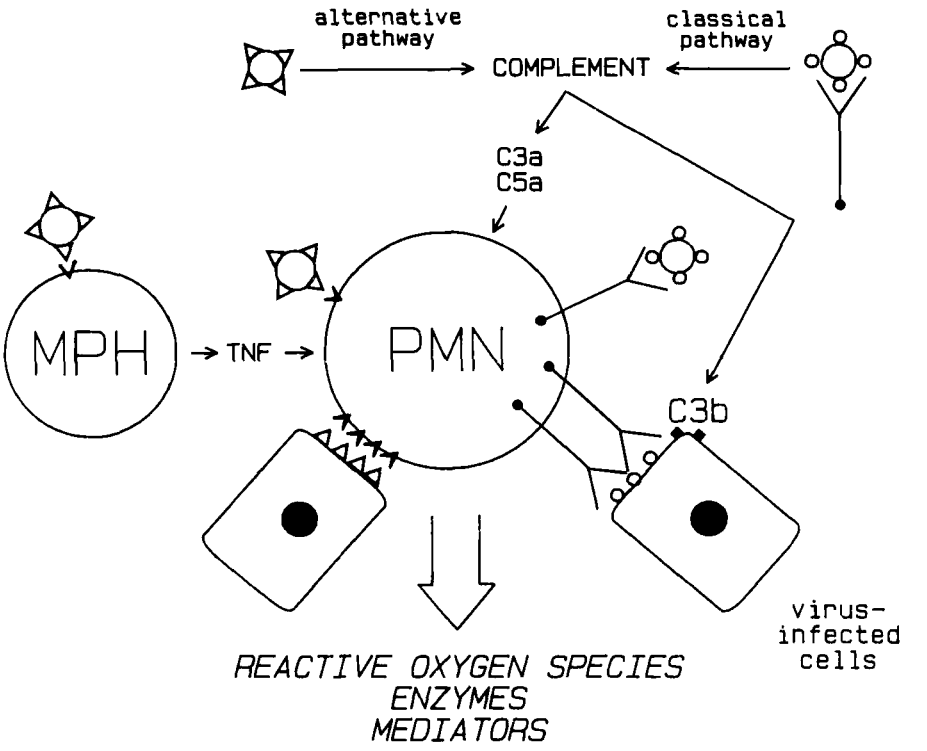

FIGURE 3 The activation of phagocytic cells by viral particles and by virus-infected target cells. On the left hand side is shown the direct activation of PMN effector functions by Sendai virus particles and by infected cells presenting viral surface proteins. Sendai virus may also induce the release of tumor necrosis factor (TNF) from macrophages (MPH)[36] and may activate the alternative complement pathway.[43] On the right is shown the activation of PMN effector functions by virus-immune complexes; virus-infected antibody-coated cells and by C3a, C5a and C3b arising from complement activation through the classical pathway.

depends on the simultaneous presence of viral antigen and antigen-bound antibody. The increase in CL seen during the viral replication cycle can be explained by the production of mature virus particles some of which remain attached to the plasma membrane of the host cell. Thus, cells harvested late, i.e. at 15 and 18 hours post-infection, present more viral antigen and hence bind more antibody. As shown earlier with bovine Herpes virus 1 (BHV-1), viral antigen may also be presented by free viral particles.[11] Others have reported that also Herpes simplex[12] and respiratory syncytial viruses[13] require antiviral antibody for the induction of ROI generation in phagocytic cells. In contrast to these viruses, Sendai virus stimulates ROI production independently of antiviral antibody (Figure 2) through a mechanism mediated by the two viral surface glycoproteins, Hemagglutinin-Neuraminidase and Fusion protein.[10,14] The experiment shown in Figure 2 indicates that also virus-infected target cells are capable of directly activating the production of ROI. In the additional presence of antiviral antibodies of chicken, but not rabbit, the virus-induced CL burst was abrogated. In line with the earlier demonstration that antibody-induced CL depends on the Fc portion,[11] and in view of the fact that rabbit and chicken antibodies were used at

identical and saturating concentrations, the abrogation of CL indicates a species-specific failure of chicken antibody to activate the Fc receptor of human PMN.

What is the significance of direct and antibody-mediated activation of ROI generation?

The induction in PMN of ROI production by virus-infected, antibody-coated cells bears a striking similarity to antibody-dependent cell-mediated cytotoxicity (ADCC). In fact, ROI have been demonstrated to contribute to ADCC[15,16] and PMN are able to serve as effector cells in killing BHV-1-infected target cells.[17] Moreover, virus-antibody complexes occur transiently in many, if not all, viral infections and are thought to contribute significantly to the pathological alterations of chronic infections, e.g. in B-type hepatitis,[18] feline infectious peritonitis,[19] equine infectious anemia[20] and hog cholera.[21] It is conceivable that immune complexes may also indirectly stimulate the generation of ROI in PMN by activation of complement which results in the generation of an array of biologically active products, among them the anaphylatoxin C5a and membrane-bound C3b which stimulate the respiratory burst.[22,23]

The direct activation of ROI formation by viral glycoproteins implies a kind of recognition of "non-self" that is not controlled by the immune system, i.e. a structure of the microorganism rather than antibody bound to this structure activates a powerful host defense mechanism. It is noteworthy that target cells infected with Sendai[24] and Parainfluenza-3 viruses[25] (which is closely related to Sendai virus and also directly activates ROI generation) were shown to be killed in the absence of antiviral antibodies by mouse spleen and bovine effector cells, respectively. In contrast, the destruction of BHV-1 virus-infected target cells requires antibody[17] which we also found to be essential for CL activation by this virus.[11] Moreover, human lymphocytes treated with Sendai virus, but not untreated lymphocytes were reported to kill uninfected "bystander" cells.[26] This report suggests that the virus may also activate cytotoxic effector mechanisms in non-phagocytic cells.

There is evidence suggesting that a direct activation of phagocytic effector functions may play a role in viral infection *in vivo*. Influenza- and Newcastle disease viruses, both of which activate ROI generation in the absence of antiviral antibody,[14,27] (unpublished observation), exert toxic effects *in vivo*. The mechanism of toxicity depends on a interaction of viral particles with phagocytic cells and is independent of viral replication. Injection of virus in the blood stream results in fever and hence endogenous pyrogen, now classified as Interleukin-1,[28] was thought to be an important mediator of viral toxicity.[29–31] However, also pulmonary edema, multiple hemorrhages and in extreme cases death were recorded as manifestations of viral toxicity.[32–35] These findings, some of which were published 40 years ago, have very recently been complemented by a report on the release of tumor necrosis factor (TNF) from macrophages induced by Sendai virus.[36] TNF is now known to be a key factor in the pathogenesis of endotoxin shock[37] which in its pathology resembles hemorrhagic viral fevers,[38] suggesting that common mechanisms operate in certain bacterial and viral diseases and also in the pathogenesis of infestations with parasites such as malaria.[39]

Although the activation of effector functions in phagocytic cells seems to be a common denominator in these conditions, it should be kept in mind that this is not *per se* a pathologic event. To illustrate this point, Berne virus which we showed to activate ROI generation in PMN is of unknown pathogenicity in horses.[40] The activation of phagocyte effector functions may be hazardous only when the activating agent is present over an extended time period and/or at high concentration.

In summary, we have shown two mechanisms by which viruses can trigger the

production of ROI, also referred to as "toxic oxygen", in PMN. The precise role of ROI in viral disease remains to be established. Indirect evidence, however, suggests that ROI may be involved in viral pathogenesis. The activity of the antioxydant enzyme superoxide dismutase was shown to increase in human lymphocytes during the early stages of influenza.[41] Moreover, butylated Hydroxytoluene (BHT), an antioxydant widely used in foodstuffs, protected chicken from the lethal effect of infection with Newcastle Disease virus.[42] The latter report also demonstrated that animals protected by BHT failed to mount an antibody response, highlighting a possible beneficial role of ROI in the infection with this virus.

Acknowledgments

This work was supported by the Swiss National Science Fund, grant Nr. 3.429.0.83. We thank Drs F. Hesford and M. Vandevelde for critically reading the manuscript, Dr. M. Weiss for providing Berne virus and Dr. I.A. Clark for stimulating discussions.

References

1. Oldstone, M.B.A., Sinha, Y.N., Blount, P., Tishon, A., Rodriguez, M., von Wedel, R.J. and Lampert, P.W. *Science*, **218,** 1125 (1982).
2. Rodriguez, R., von Wedel, R.J., Garrett, R.S., Lampert, P.W. and Oldstone, M.B.A. *Lab. Invest.*, **49,** 48 (1983).
3. Melnick, J.L. in *Virology*, ed. B.N. Fields (Raven Press: New York, 1985) p. 739.
4. White, D.O. and Fenner, F. *Medical Virology* (Academic Press: New York, 1986) p. 217.
5. Mims, C.A. and White, D.O. *Viral Pathogenesis and Immunology* (Blackwell Scientific Publications: Oxford, 1984) p. 139.
6. DeChatelet, L.R., Long, G.D., Shirley, P.S., Bass, D.A., Thomas, M.J., Henderson, F.W. and Cohen, M.S. *J. Immunol.*, **129,** 1589 (1982).
7. Dahlgren, C. and Stendahl, O. *Infect. Immun.*, **39,** 736 (1983).
8. Brestel, E.P. *Biochem. Biophys. Res. Commun.*, **126,** 482 (1985).
9. Bertoni, G., Keist, R., Groscurth, P., Wyler, R., Nicolet, J. and Peterhans, E. *J. Immunol. Methods*, **78,** 123 (1985).
10. Peterhans, E., Bächi, T. and Yewdell, J. *Virology*, **128,** 366 (1983).
11. Weber, L. and Peterhans, E. *Immunobiol.*, **164,** 333 (1983).
12. Bingham, E.L., Fenger, T.W., Sugar, A. and Smith, J.W. *Invest. Ophthalmol. Visual. Sci.*, **26,** 1236 (1985).
13. Kaul, T.N., Faden, H. and Ogra, P.L. *Infect. Immun.*, **32,** 649 (1981).
14. Peterhans, E. *Virology*, **105,** 445 (1980).
15. Hafeman, D.G. and Lucas, Z. *J. Immunol.*, **123,** 55 (1979).
16. Nathan, C.F. *Fed. Proc.*, **41,** 2206 (1982).
17. Rouse, B.T., Wardley, R.C. and Babiuk, L. *Infect. Immun.*, **13,** 1433 (1976).
18. Gower, R.G., Sansker, W.F., Kohler, P.F., Thorne, G.E. and McIntosh, R.M. *J. Allergy Clin. Immunol.*, **62,** 222 (1978).
19. Jacobse-Geels, H.E.L., Daha, M.R. and Horzinek, M.C. *Amer. J. Vet. Res.*, **43,** 666 (1981).
20. Banks, K. and Henson, J. *Fed. Proc.*, **28,** 752 (1969).
21. Cheville, N.F. and Mengeling, W.L. *Lab. Invest.*, **20,** 261 (1969).
22. Schreiber, R.D., Dangburn, M.K., Bjornson, A.B., Brothers, M.A. and Muller-Eberhard, H.J. *Clin. Immunol. Immunopathol.*, **23,** 335 (1982).
23. Goldstein, I.M. and Weissmann, G. *Semin. Hematol.*, **16,** 175 (1979).
24. Anderson, M.J. *Infect. Immun.*, **20,** 608 (1978).
25. Campos, M., Rossi, C.R. and Lawman, M.J.P. *Infect. Immun.*, **36,** 1054 (1982).
26. Alsheikhly, A., Oervell, C., Härfast, B., Andersson, T., Perlmann, P. and Norrby, E. *Scand. J. Immunol.*, **17,** 129 (1983).
27. Mills, E.L., Debets-Ossenkopp, Y., Verbrugh, H.A. and Verhoef, J. *Infect. Immun.*, **32,** 1200 (1981).
28. Oppenheim, J.J., Kovacs, E.J., Matsushima, K. and Durum, S.K. *Immunology Today*, **7,** 45 (1986).
29. Bennett, I.L., Wagner, R.R. and Lequire, V.S. *Proc. Soc. Exptl. Biol. (N.Y.)*, **71,** 132 (1949).

30. Kanoh, S. and Kawasaki, H. *Biken J.*, **9,** 177 (1966).
31. Siegert, R., Pollmann, W. and Shu, H.L. *Z. Naturforsch.*, **22b,** 320 (1967).
32. Davenport, F.M. *Fed. Proc.*, **10,** 405 (1951).
33. Ginsberg, H.S. *J. exptl. Med.*, **94,** 191 (1951).
34. Henle, G. and Henle, W. *J. exptl. Med.*, **84,**623 (1946).
35. Henle, W. and Henle, G. *J. exptl. Med.*, **84,** 639 (1946).
36. Aderka, D., Holtmann, H., Toker, L., Hahn, T. and Wallach, D. *J. Immunol.*, **136,** 2938 (1986).
37. Beutler, B., Milsark, I.W. and Cerami, A.C. *Science*, **229,** 869 (1985).
38. Fisher-Hoch, S.P., Platt, G.S., Neild, G.H., Southee, T., Baskerville, A., Raymond, R.T., Lloyd, G. and Simpson, D.I.H. *J. Infect. Dis.*, **152,** 887 (1985).
39. Clark, I.A., Hunt, N.H. and Cowden, W.B. *Adv. Parasitol.*, in press.
40. Horzinek, M.C. and Weiss, M. *Zbl. Veterinärmedizin B*, **31,** 649 (1984).
41. McCue, J.P. *Exp. Hematol.*, **7,** 361 (1979).
42. Brugh, M. *Science*, **197,** 1291 (1977).
43. Okada, H. and Okada, N. *Immunology*, **43,** 337 (1981).

Accepted by Prof. H. Sies