Comparative Mitogenic Effects of Herpes Simplex Virus and Mycoplasma on Murine Lymphocytes

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SUMMARY

Previous work indicated that herpes simplex virus type 1 (HSV-1) is a mitogen for mouse spleen cultures, as monitored by uptake of $^3$H-thymidine. We observed variable responses of mouse spleen cultures to different viral preparations. The variable responses, which did not follow normal dose-response relationships, were not due to HSV-1 strain differences, altered response kinetics or the presence or absence of defective viral particles, but to mycoplasma contamination of viral stocks. Mycoplasma-free (MF) HSV-1 stocks were prepared by transfection of MF NHF cells with HSV-1 DNA. MF HSV-1 infection of spleen cultures resulted in a five- to sixfold stimulation of DNA synthesis and stimulation of a polyclonal antibody response. Heat treatment (56 °C for 1 h) and antibiotics were used to distinguish mycoplasma and HSV-1-induced spleen culture mitogenic responses. The mycoplasma-induced mitogenic activity was found to be heat labile and sensitive to gentamicin and chloramphenicol. In contrast, the HSV-1-induced response was not affected by gentamicin or chloramphenicol and heat treatment resulted in only a 50 % loss of activity.

Previously, we and others (Mochizuki et al., 1977; Kirchner et al., 1978) reported that herpes simplex virus type 1 (HSV-1) infection of murine spleen cultures stimulates DNA synthesis in B lymphocytes. The results obtained by CsCl gradient centrifugation of DNA extracted from HSV-1- and control lipopolysaccharide (LPS)-stimulated cultures indicated that most of the DNA being synthesized at 48 h post-treatment is of host origin. The non-permissive nature of the spleen cell response to HSV-1 infection indicated that this system might be useful for studying oncogenic potential of HSV or the mechanisms of viral latency. However, there is variability in the mitogenic responses in murine lymphocyte cultures using different preparations of HSV-1. This variability could be due to the presence of mycoplasma in the cells or viral inoculum used to propagate virus, since mycoplasmas are able to dramatically alter normal lymphocyte function. Mycoplasma infection can result in either activation or inhibition of normal lymphocyte cultures (Copperman & Morton, 1966; Barile & Leventhal, 1968). Mycoplasma infection of murine B lymphocytes causes blast transformation and results in polyclonal activation (Ginsberg & Nicolet, 1973; Biberfeld & Gronowicz, 1976; Stanbridge & Weiss, 1978), while infection of murine T lymphocytes can result in induction of lymphocyte-mediated cytotoxicity for syngeneic cells (Aldridge et al., 1977).

Mouse spleen cells prepared as described elsewhere (Watson & Riblet, 1974) were resuspended at a density of 2.5 × 10⁶ cells/ml in either Eagle's minimum essential medium (MEM; Irvine Scientific, Irvine, Ca., U.S.A.) or RPMI 1640 medium (Gibco) supplemented with 5 % foetal calf serum (Irvine Scientific), 5 × 10⁻⁵ M-2-mercaptoethanol, 1 mM-glutamine and 50 mg/ml gentamicin (Schering, Kenilworth, N.Y., U.S.A.), unless otherwise indicated. Amounts of 0.2 ml (5 × 10⁵ cells) were plated into each well of a microtitre plate (microtest II, Falcon Plastics, Oxnard, Ca., U.S.A.) and incubated at 37 °C in a gas mixture of 7% oxygen, 10% carbon dioxide and 83% nitrogen. Mitogenesis control cultures were treated...
Fig. 1. Stimulation of DNA synthesis in Balb/c spleen cultures by various HSV-1 viral preparations relative to untreated cultures. Each culture contained 10^6 spleen cells in 0.2 ml medium at the multiplicity of infection indicated. After 48 h, cultures were labelled with 0.25 µCi ³H-thymidine for 6 h and then harvested. Each bar represents the mean of triplicate cultures. Stimulation was tested using (a) two preparations of HSV-1 strain JH designated JH 7/19 and JH 7/8 and (b) HSV-1 Muckbeck 9/4 and Muckbeck 2/19.

with 2.5 to 5 µg/ml Escherichia coli K235 LPS (generously provided by Abbott Laboratories, Chicago, Ill., U.S.A.). After 24, 48, 72 or 96 h, cultures were radioactively labelled for 6 h with 0.25 µCi ³H-thymidine (12 Ci/mmol; New England Nuclear) and harvested using the Mash II (Microbiological Associates, Los Angeles, Ca., U.S.A.) harvester.

The data in Fig. 1 demonstrate the variability observed in response to different virus preparations, as measured by their ability to induce DNA synthesis. Over a range of multiplicities of infection, between 0.3 and 10, the Muckbeck 9/4 and JH 7/8 HSV preparations did not stimulate DNA synthesis more than twofold above untreated cultures (stimulation index ≤ 2). The Muckbeck 2/19 and JH 7/19 HSV preparations were highly mitogenic at comparable multiplicities of infection. The source of observed variability was not due to HSV-1 strain differences, because different preparations of the same HSV-1 strain, Muckbeck or JH, had dissimilar effects on murine lymphocytes (Fig. 1). Another source of the observed variability could be due to altered response kinetics between preparations. This was not the case with two weakly mitogenic viral preparations assayed on days 2, 3 and 4. The maximum stimulation of DNA synthesis was observed at the same time in both preparations.

It is possible that the mitogenic agent was derived from the cells used to propagate the virus. Undiluted and concentrated culture supernatants from NHF cells were tested and found to have no influence on cellular DNA synthesis.

One virus preparation which was mitogenic and displayed expected dose-response relationships was tested for mycoplasma contamination. Mycoplasma was grown from aliquots of virus preparations by plating in PPLO broth (Difco) supplemented with 20% horse serum (Irvine Scientific), 10% yeast extract, 0-2% phenol red and 100 units/ml penicillin.
Mycoplasma titres were determined on PPLO agar, supplemented as for the broth. The virus preparation was found to be contaminated with $1.52 \times 10^3$ colony-forming units (c.f.u.) per ml of mycoplasma. Other highly mitogenic virus preparations were also found to be contaminated with mycoplasma. It thus became necessary to determine which agent, mycoplasma, HSV or the two agents in concert, were critical for induction of DNA synthesis in splenocytes.

The culture supernatants recovered at different times (24 and 48 h) after infection with a contaminated HSV-1 (Savage 8/2) preparation were assayed for virus and mycoplasma. The HSV-1 titre decreased from $1.33 \times 10^8$ p.f.u./ml in the initial inoculum to $4.75 \times 10^3$ p.f.u./ml after 48 h. The mycoplasma titre increased from $1.52 \times 10^3$ c.f.u./ml in the initial inoculum to $2.85 \times 10^7$ c.f.u./ml after 48 h. The mitogenic activity also increased in the 24 and 48 h supernatants. The rate of DNA synthesis in cultures infected with the initial Savage 8/2 preparation was 11-fold greater than untreated cultures. Cultures infected with the 24 and 48 h preparations incorporated radioactively labelled thymidine at a rate 21 and 34 times greater than untreated cultures respectively. In LPS-treated cultures the stimulation index was 9.8. Thus, the mycoplasmal contaminant in the Savage 8/2 preparation was the probable mitogenic agent. The isolation of the mycoplasma contaminant in supplemented PPLO broth, and the demonstration of its mitogenic potential confirmed that the mycoplasma contaminant was mitogenic and did not require the presence of HSV for activity. Mixing experiments indicated that the two agents did not act synergistically, but additively in stimulating murine cultures. The response to mycoplasma was comparable to that seen with LPS. In all instances the mycoplasma isolated from other mitogenic viral preparations were mitogenic for spleen cell cultures.

The response of spleen cultures to HSV-1 preparations with very low mycoplasma contamination indicated that HSV-1, if added at sufficiently high multiplicities of infection, might be mitogenic. It was not possible to test the effects of HSV-1 free from contaminating mycoplasma organisms since both are surrounded by membranes and tend to aggregate when mixed, thus hindering purification. Instead, mycoplasma-free (MF) HSV-1 stocks, designated JH-T, were prepared in MF NHF cells, using as inocula HSV-1 stocks produced by transfection by the procedure of Stow & Wilkie (1976), with sheared human placental DNA as carrier.

This preparation of HSV was capable of stimulating DNA synthesis in spleen cultures (Fig. 2b, d). The rate of DNA synthesis was maximal at 48 h, as was the case with mycoplasma. All JH-T viral preparations showed a linear dose-response relationship. U.v. irradiation (1 s to 3 min at 53 ergs/s/mm$^2$) of the JH-T stocks resulted in a loss of infectious activity which corresponded to loss of mitogenic activity (not shown). Although the maximum response to HSV was below the control LPS response (incorporation of 14431 ct/min compared to 24453 ct/min of radioactively labelled thymidine), it was well above background incorporation of thymidine (2138 ct/min).

JH-T-infected spleen cultures were assayed for their ability to mount a polyclonal antibody response to trinitrophenyl (TNP) determinants. Polyclonal antibody responses were determined using a microscope slide assay (Mishell & Dutton, 1967), in which sheep erythrocytes (SRBC) (Colorado Serum Company, Denver, Co., U.S.A.), or TNP-SRBC were used as indicator cells (Watson & Riblet, 1975). An increase in plaque-forming cells specific for TNP was observed on days 2 and 3. The polyclonal response was 25 to 40% of the response stimulated by LPS, and 10- to 17-fold above background.

The possibility remained that MF viral stocks contained low levels of mycoplasma contamination, which were amplified in spleen cultures. Although we screened infected cell cultures using the sensitive DAPI staining (Russell et al., 1975) and UdR/U ratio methods (Schneider et al., 1976), low levels of mycoplasma contamination might not have been
detected. This led us to determine assay conditions which might distinguish between effects of virus and mycoplasma using the antibiotics gentamicin and chloramphenicol, and with heat treatment.

Most mycoplasma strains are susceptible to the mycoplasmacidal effects of gentamicin (Braun et al., 1970; Rahman et al., 1967), whereas the drug does not interfere with the replication of HSV (Fischer, 1975; Schafer et al., 1972). Chloramphenicol has also been shown to be an effective anti-mycoplasmal agent (Stanbridge, 1971); there have been no reports of chloramphenicol resistance in mammalian mycoplasmas.

The data presented in Fig. 2 demonstrate the effects of chloramphenicol and gentamicin on the mycoplasma- or HSV-1-induced spleen culture response. In control medium containing the antibiotics penicillin and streptomycin (100 units/100 µg), maximum stimulatory responses to both mycoplasma and HSV-1 were observed. The response to mycoplasma infection was abolished when the culture medium contained either 10 or 20 µg/ml
chloramphenicol or 50 μg/ml gentamicin. Conversely, the response to HSV-1 in media containing either of these antibiotics was not affected.

Heat treatment at 56 °C for 1 h also had differential effects on virus- and mycoplasma-induced responses. The mitogenic potential of the mycoplasma species utilized was heat labile, while the stimulatory activity of heat-inactivated HSV was reduced to 50%.

We have now demonstrated comparable mitogenic properties of both HSV-1 and mycoplasma on murine spleen cells. The ability of both MF HSV-1 and mycoplasma to stimulate a polyclonal antibody response in infected murine spleen cultures indicated that a target cell for both agents was a B lymphocyte. However, it was possible to distinguish mycoplasma- and HSV-induced effects through the use of antibiotics and heat treatment. Given the ability of microorganisms to affect lymphocyte function, it is critical that all possible contaminants be eliminated before an effect is ascribed to a particular agent or factor.

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REFERENCES


Short communications


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