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Lipopolysaccharide-induced suppressor cells for delayed-type hypersensitivity to herpes simplex virus: nature of suppressor cell and effect on pathogenesis of herpes simplex

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Summary. Treatment of mice with LPS at the time of priming with herpes simplex virus type 1 (HSV1) causes the preferential activation of virus-specific T suppressor (Ts) cells. These Ts cells can transfer suppression to the efferent limb of a DTH response. Priming under these conditions is associated with enhanced cell-recruitment to the inoculation site, but had no effect on virus clearance. The induction of suppression was abrogated by pretreatment of mice with cyclophosphamide or indomethacin. LPS had no effect on the antibody response to HSV1 during acute infection, although treated mice showed a raised antibody titre one month after inoculation. Susceptible mice inoculated with HSV1 and given LPS showed protection, both from lethal herpes encephalitis and from demyelination within the CNS as reflected by ear paralysis. These results imply that, during some stages of acute infection, T cell effector mechanisms may themselves mediate tissue damage. At such times, Ts cells may perform a beneficial role leading to a reduction in pathology.

INTRODUCTION

The interactions between herpes simplex virus (HSV) and the host immune response are clearly multifactorial, with various immunological effector mechanisms involved at different stages of pathogenesis, some perhaps themselves contributing to virus-induced pathology. For example, the delayed-type hypersensitivity (DTH) response is implicated in clearance of HSV from the skin (Nash & Ashford, 1982) yet, with respect to tissue damage within the central nervous system (CNS), T cell-suppressed mice infected with HSV are protected compared to normal infected mice in some models (Nahmias *et al.*, 1969; Townsend & Baringer, 1979; Zawatzky, Hilfenhaus & Kirchner, 1979).

The finding that T suppressor (Ts) cell-mediated 'split tolerance' (affecting DTH but not antibody or cytotoxic T lymphocyte responses) can be induced by intravenous virus inoculation (Nash *et al.*, 1981) offers a highly specific tool for probing the contribution made by T cell effector mechanisms to pathogenesis of herpes simplex. For example, it is possible to mimic in animal models and under controlled conditions some of the events in clinical HSV infections, where susceptibility appears to be associated with abnormal OKT4/OKT8 ratios (Sheridan *et al.*, 1982; Schooley *et al.*, 1983).

While conducting such studies, we have developed a complementary model for activation of specific suppressor cells, using the bacterial endotoxin, lipopolysaccharide (LPS). This model affords some advantage in studies of HSV pathogenesis, since virus need only be inoculated via the relatively physiological, intrader-

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mal route, and intravenous inoculation is avoided. There are many previous reports of LPS-induced suppressor cells. For example, specific B suppressor cells acting on the DTH response to sheep red blood cells (SRBC) are activated by simultaneous inoculation of LPS and SRBC (Gill & Liew, 1979). In other models, however, LPS-induced suppression has been attributed to Ts cells, a paradoxical finding in view of the predominant mitogenic effect of LPS on B cells (Ivanyi, 1976).

Here, we report that simultaneous inoculation of HSV1 and LPS activates HSV1-specific Ts cells which have a functional requirement for prostaglandin synthesis and can transfer suppression to the efferent limb of a DTH response. Susceptible mice inoculated under these conditions show protection from lethal infection.

MATERIALS AND METHODS

Mice

NIH/Ola inbred mice of either sex, aged 6–10 weeks and age and sex-matched in each experiment, were originally obtained from Olac 1976 Ltd and maintained as a breeding colony in this department. Swiss outbred white mice (males) were used at 4 weeks of age from a breeding colony maintained in this department.

Virus

HSV1, strain SC16, or HSV2 'Lab strain' were used for intradermal (i.d.) inoculation, which was in the right pinna, as previously described (Harbour, Hill & Blyth, 1981).

Lipopolysaccharide

LPS from *Escherichia coli* serotype no. 026:B6 (Sigma Chemicals) was injected intravenously (i.v.), 100 μg/animal in a volume of 100 μl phosphate-buffered saline (PBS) at the time of HSV1 inoculation or up to 24 hr previously.

Delayed-type hypersensitivity

Mice were challenged in the left pinna with heat-inactivated virus, as indicated. Results are expressed as the percentage increase in ear thickness at 24 hr, as measured using a Mitotoyo engineer's micrometer.

Neutralizing antibody

This was determined by a plaque reduction test of HSV1 SC16 in Vero cell monolayers, as previously described (Darville & Blyth, 1982).

Lymphocyte depletion

Spleen cell suspensions were depleted of B cells by passage through nylon wool columns (Julius, Simpson & Herzenberg, 1973) or by affinity chromatography on rabbit anti-mouse $F(ab')_2$-coupled Diakon beads (ICI) (Shand, 1975). The latter separation was performed with Mr G. Watt, Department of Pathology, University of Bristol, who also donated the reagents. Diakon beads were incubated with 75 mg of sodium sulphate-precipitated mouse immunoglobulin for 1 hr at 45°, then overnight at 4°. After washing in PBS, beads were loaded into a column and incubated with 15 ml of 1 mg/ml rabbit anti-mouse $F(ab')_2$ for 1 hr at 37° and then washed with PBS. Spleen cells were added to the column at 4×10^7 cells/ml in Eagle's medium and incubated for 1 hr at 37° before non-adherent cells were washed off with warmed medium. Nylon wool-purified cells were $< 8\%$ Ig^+ by immunofluorescence, affinity-purified cells $< 1\%$ Ig^+. T cells were depleted by *in vitro* incubation with a monoclonal anti-Thy 1·2 monoclonal antibody (Olac 1976 Ltd) followed by low-toxicity normal rabbit serum.

Drugs

Cyclophosphamide (Cy) (Koch Light Laboratories Ltd) was administered intraperitoneally in PBS. Indomethacin (IND) (No. 1-7378; Sigma Chemicals, St. Louis, MO, U.S.A.) was dissolved in a few drops of dimethyl sulphoxide and then in PBS for intraperitoneal injection.

Detection of infectious or latent virus in mouse tissue

Infectious virus in the pinna was detected and measured by cultivation of disrupted mouse cells on Vero cell monolayers. Latency was detected by culture of ganglia for 4 days, followed by transfer of homogenates of the 2nd, 3rd and 4th cervical dorsal root ganglia (C2, C3, C4) on Vero cell monolayers. Both techniques have been described elsewhere (Harbour *et al.*, 1981).

Induction of recurrent disease

Twenty-eight days or more after priming (by which time the infected pinna appeared clinically normal), cellophane tape was repeatedly (six times) applied to the skin, as previously described (Harbour *et al.*, 1981). Mice were then observed daily for the emergence of herpetic vesicles on the skin.

Statistics

Significance of difference between means was assessed by Student's *t*-test.

RESULTS

Effect of LPS on recruitment of cells to the site of HSV1 inoculation

NIH mice (6 per group) were primed with 1×10^4 PFU HSV1 SC16 having received various doses of LPS a few hours before (Fig. 1). On each of the days shown, the primary response of mice receiving any of the three LPS doses was more vigorous than that of the control group receiving virus only. All groups showed peak swelling 6–7 days after infection, the skin returning largely to normal by day 12.

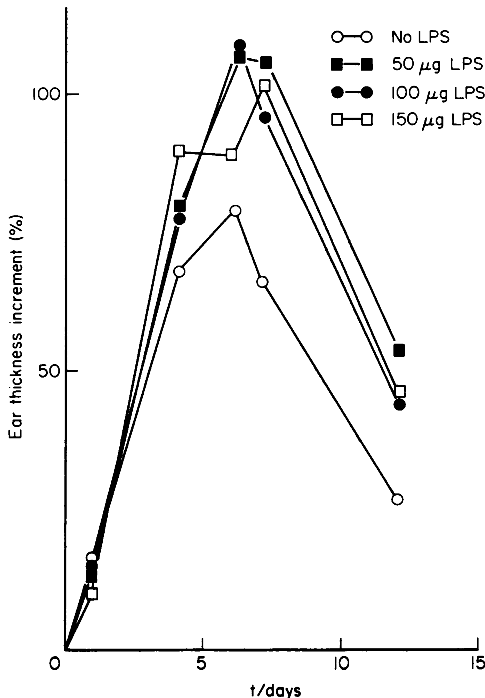

Figure 1. Effect of LPS on recruitment of cells to the site of inoculation.

LPS induces suppression of the DTH response which can be abrogated by Cy or IND

NIH mice given LPS at the time of priming with HSV1 SC16 subsequently show greatly suppressed DTH compared to controls without LPS ($P < 0{\cdot}001$) (Table 1). Since LPS is known to induce the release of prostaglandin E_2 (PGE_2) from macrophages, and PGE_2 has well known immunoregulatory effects, it was of interest to see if responsiveness could be restored by pretreatment with the prostaglandin synthetase inhibitor, IND (Goldyne, 1983). Pretreatment of mice with IND does indeed abrogate the induction of suppression. In adoptive transfer experiments, pretreatment of donors with IND before killing was able to abrogate the expression of DTH-suppression in recipients (data not shown). Since many suppressor cell pathways have a precursor cell in common which is sensitive to Cy, mice were pretreated with the drug to see if this too would abrogate suppression. No significant suppression was seen in Cy-pretreated mice which had been inoculated with LPS and HSV1 SC16.

Suppression is adoptively transferable and specific for HSV1

The experiment shown in Table 2 demonstrates that suppression is attributable to splenic suppressor cells,

Table 1. Effect of drugs on induction of suppression

Pretreatment of mice	Priming	DTH (% increase)
None	HSV1 SC16	74·1 ± 12·4
None	HSV1 SC16+LPS	39·7 ± 11·5
Indomethacin (5 mg/kg i.p.)*	HSV1 SC16	84·9 ± 9·8
Indomethacin (5 mg/kg i.p.)*	HSV1 SC16+LPS	77·4 ± 8·9
Cyclophosphamide† (100 mg/kg i.p.)	HSV1 SC16	87·4 ± 12·8
Cyclophosphamide† (100 mg/kg i.p.)	HSV1 SC16+LPS	74·1 ± 12·4

Mice were primed in the right pinna with 1×10^4 PFU HSV1 SC16 (with or without 100 μg LPS i.v.) 6 days before challenge.

*24 hours before priming.

†96 hours before priming.

NIH females aged 10 weeks were used, six mice per group.

Table 2. Specificity of suppressor cells

Donor priming	Recipient priming	DTH (% increase) (*n*)
None	HSV1 SC16	74·1 ± 4·9 (4)
HSV1 SC16	HSV1 SC16	62·6 ± 4·6 (4)
HSV1 SC16+LPS	HSV1 SC16	53·6 ± 3·3 (5)
None	HSV2/Lab	52·1 ± 6·7 (4)
HSV2/Lab	HSV2/Lab	52·5 ± 17·0 (4)
HSV1 SC16+LPS	HSV2/Lab	67·6 ± 16·0 (4)

Recipients were primed with 2×10^2 HSV2 Lab. strain or 1×10^4 PFU HSV1 SC16 7 days before transfer. Donors received 1×10^4 PFU HSV1 or 2×10^2 PFU HSV2 Lab. strain i.d. with or without 100 μg LPS i.v. 4 days before transfer. Challenge of HSV2-primed groups was with 2×10^2 PFU of heat-inactivated HSV2 Lab strain, or of HSV1-primed groups with 5×10^5 PFU of heat-inactivated HSV1 SC16. Spleen cells, 1×10^8, were transferred immediately before challenge.

NIH females aged 11 weeks were used.

Table 3. Transfer of DTH suppression by spleen cells

Cell Donor	Treatment of cells	DTH (% increase)
Experiment 1		
Unprimed	None	63·5 ± 14·0
HSV-primed	None	66·4 ± 3·9
LPS-treated	None	68·3 ± 3·6
HSV1-primed+LPS	None	36·6 ± 12·0
HSV1-primed+LPS	Passage through nylon wool	59·5 ± 15·0
Experiment 2		
Unprimed	None	57·3 ± 14·0
HSV1-primed+LPS	None	35·6 ± 9·8
HSV1-primed+LPS	Passage through anti F(ab′)2 column	18·5 ± 8·0
Experiment 3		
Unprimed	None	59·4 ± 5·7
HSV1-primed+LPS	None	44·7 ± 9·6
HSV1-primed+LPS	Anti-Thy 1·2+ complement	62·3 ± 5·2

1×10^8 whole spleen cells (or 5×10^7 or B-enriched cells) were adoptively transferred from donors inoculated in the right pinna with 1×10^4 PFU HSV1 SC16 (with or without i.v. LPS) 5 days previously. Recipients were primed with 1×10^4 PFU 7 days before transfer.

NIH males aged 8–10 weeks were used, four to six mice per group.

rather than to some form of effector cell sequestration away from the challenge site in LPS-treated mice. Spleen cells (1×10^8) from donors given LPS with HSV1 SC16 confer significant suppression on the efferent limb of a DTH response in recipients ($P = 0{\cdot}02$) whereas cells from unprimed donors or donors primed only with virus confer no suppression. It was not possible to transfer suppression to the afferent limb of a DTH response using either spleen cells or draining lymph node cells from donors given HSV1 SC16 with LPS (data not shown). In addition, cells from mice primed with HSV1 SC16 and LPS could not transfer suppression to mice primed with HSV/Lab. Thus, LPS-induced suppression of DTH is mediated by splenic suppressor cells which are HSV-serotype-specific.

Suppression is mediated by an adherent Ts cell

Experiments were then conducted to characterize further the nature of the suppressor cell (Table 3). Again, spleen cells from donors given LPS and HSV1 SC16 can transfer suppression (Experiment 1: $0{\cdot}01 < P < 0{\cdot}02$, comparing with unprimed donors). Suppression was lost following passage of cells through nylon wool, suggesting that the effect may be B cell mediated. However, affinity chromatography on anti-F(ab′)2 coated beads depleted the suspensions of virtually all B cells, but they still caused substantial enhancement of the suppression transferable (Experiment 2: $P < 0{\cdot}001$ comparing affinity-purified T cells and unprimed cells). To confirm the probable T cell identity of the suppressor cell, suspensions were treated *in vitro* with monoclonal anti-Thy 1·2 and complement (Experiment 3). This treatment abrogated the transfer of suppression by spleen cells ($0{\cdot}001 < P < 0{\cdot}01$ comparing suppression transferred by naive cells with suppression transferred by cells from donors primed with LPS and HSV1 SC16); slight enhancement is observed (not significant) when T-depleted cells are compared to controls. Thus, suppression is probably mediated by a nylon wool-adherent Ts cell.

Antibody production in LPS-treated mice

NIH mice were inoculated with HSV1 SC16, with or without an i.v. inoculum, and then tested for serum HSV-antibody at various times after infection (Table 4). LPS caused no suppression of the antibody response and, indeed, a slight enhancement was seen on days 14 and 28 (not significant on day 14; $P = 0{\cdot}02$ on day 28). During the first week of infection (during which time Ts cells acting on DTH are transferable) there is no difference between groups in antibody production.

Table 4. Effect of LPS on antibody production

Priming	Neutralizing antibody titre (log_{10} reciprocal geometric mean)		
	Day 7	Day 14	Day 28
HSV1 SC16 only	1·08±0·34	1·38±0·46	1·57±0·21
HSV1 SC16+LPS	1·07±0·50	1·89±0·43	1·92±0·23

Animals were primed i.d. in the right pinna with 3×10^5 PFU HSV1 SC16, one group having received 100 μg LPS i.v. a few hours before.

NIH males aged 7 weeks were used, six mice per group.

Effect of LPS on clearance of acute HSV1 infection

Although LPS caused enhancement recruitment of cells to the site of inoculation with concomitant generation of specific Ts cells, it had no obvious effect on clearance of infectious virus from the skin and dorsal root ganglia (Table 5). While it is hard to draw firm conclusions considering the fairly small groups examined, there is a suggestion that LPS-treated NIH mice are able to clear infectious HSV1 from the dorsal root ganglia faster than normal infected mice (comparing each group on day 8 after infection).

Effect of LPS on pathogenesis during acute, latent and recurrent herpes simplex

Swiss outbred mice, which show a higher susceptibility to lethal infection by HSV1 than the NIH inbred strain, were then used to study the effect of LPS on pathogenesis following infection by the virus (Table 6). Paradoxically perhaps, treatment with LPS causes greatly enhanced survival when mice are infected with 3×10^5 or 4×10^5 PFU HSV1 SC16. Mice dying as a result of infection tended to do so in the first 6–10 days following the development of various signs of neurological disease (e.g. circling, paralysis).

Another manifestation of central nervous system (CNS) involvement in this model is the occurrence of paralysis of the infected ear; this has been shown to correlate with demyelination of the 7th cranial nerve root on the CNS side (Hill *et al.*, 1983). LPS-treated mice were less likely than controls to develop ear paralysis during acute infection (e.g. 41% compared to 75% after giving 3×10^5 PFU HSV1 SC16).

No clear trend could be established for the effect of LPS on viral latency detectable in dorsal root ganglia of mice infected more than 100 days previously; when infecting with 3×10^5 PFU, LPS appears to predispose to a lower incidence of latency, while when infecting with 4×10^5 PFU, LPS-treated mice have a higher incidence of latency than controls.

Having recovered from acute infection, surviving mice were observed for recurrent disease following application of a reactivation stimulus to the skin. To do this, the right ear was cellophane tape stripped (CTS). Mice that had been infected at the same time as receiving LPS showed a slightly higher incidence of recurrent disease than controls.

DISCUSSION

Clinical evidence suggests that, in considering the success of HSV at spreading through human popula-

Table 5. Acute infection in mice inoculated with SC16 and LPS

Inoculation	Day p.i.	Virus titre (log_{10} PFU) Pinna	Ganglia (C_2, C_3, C_4)
	2	4·4, 4·1, 3·8	<0, <0, <0, 0·6
	4	3·2, 4·3, 4·3, 4·5, 4·9	<0, 0·6, 0·6, 1·3, 2·1
1×10^5 PFU HSV1 SC16 i.d.	6	3·6, 3·7, 3·9, 5·2	<0, <0, 1·5, 1·9, 2·5
	8	<0, <0, 2·9, 5·1, 5·3, 5·5	<0, 0·3, 0·9, 2·4
	2	3·4, 4·4, 4·8	<0, <0, <0
	4	3·8, 3·9, 4·6, 4·9, 5·1	0·6, 1·4, 3·2
1×10^5 PFU HSV1 SC16 i.d.	6	4·9, 5·0, 5·9, 5·9	<0, <0, 1·4, 2·0
+100 μg LPS i.v.	8	<0, <0, <0, 3·5, 4·5	<0, <0, <0, <0, <0, 0·6

Numbers are log_{10} PFU for tissue from individual mice: C_2, C_3, and C_4 were pooled for each animal.

Table 6. Effect of LPS on pathogenesis of herpes simplex during acute, latent and recurrent disease

HSV1 inoculum (PFU)	LPS inoculum*	Survival at day 14 (%)	Incidence of ear paralysis at day 10 (%)	Incidence of latency at day 100+ (%)	Incidence of recurrent disease after CTS † (%)
3×10^5	None	33/67 (49)	18/24 (75)	9/15 (60)	18/85 (21)‡
3×10^5	100 μg (i.v.)	28/37 (76)	14/34 (41)	7/20 (35)	8/26 (31)
4×10^5	None	18/40 (45)	21/40 (52)	3/11 (27)	NT
4×10^5	100 μg (i.v.)	16/20 (75)	7/16 (44)	7/16 (44)	NT

Swiss outbred male mice were used at 4 weeks of age.
*LPS was given immediately before anaesthetizing mice for virus inoculation.
†CTS, cellophane tape stripping of infected pinna.
‡Pooled result from more than one experiment.
NT, not tested.

tions and causing recurrent disease, one should look to its ability to profit from aberrant immunoregulation in the host (Sheridan *et al.*, 1982; Schooley *et al.*, 1983). We have recently been attempting to manipulate the immune response in the mouse ear model of herpes simplex in an attempt to mimic this aberrant regulation. In this paper, we report that the B cell mitogen, LPS, can preferentially stimulate the generation of HSV1-specific Ts cells acting on the DTH response when LPS is given at the same time as priming with the virus. These Ts cells were adoptively transferable in the short term but not more than 2 weeks after priming (data not shown), were sensitive to Cy and IND, and did not suppress the antibody response.

While the demonstrable effect of LPS *in vitro* is predominantly on B cells, and previous reports of LPS-induced suppression have indeed attributed the suppression observed to these cells (Persson, 1977; Gill & Liew, 1979), other evidence points to effects on T cells and antigen-presenting cells (APC). For example, the antibody enhancement induced when challenging primed mice with a hapten-carrier, together with LPS, is carrier specific (Amerding & Katz, 1974). Furthermore, mixed lymphocyte reactions generated against H-2K, D differences are enhanced by LPS, suggesting an effect on CTL generation (Narayanan & Sundharadas, 1978). While much work has centred on the adjuvant properties of LPS, many studies suggest that it can also be suppressive. Portnoi, Motta & Truffa-Bachi (1981) reported suppressed responses to T-dependent antigens in Mishell-Dutton cultures after pretreating mice with LPS, the defect apparently arising at the T helper cell/APC interaction. In some circumstances, LPS may have an analogous effect to concanavalin A, inducing Ts cells capable of suppressing lymphocyte proliferative responses (Miller, Gartner & Kaplan, 1980).

From the experiments reported here, we can only speculate on the mechanism of Ts cell-induction. We favour the idea that LPS may act initially on APC, stimulating the release of prostaglandins which leads to the loss of both Ia antigen expression and the ability to present antigen (Goldyne, 1983; Snyder, Beller & Unanue, 1982). This may then lead to activation of Ts cells by presentation in an alternative (suppressive) manner, or without presentation by nature of the fact that Ts cells can bind antigen (D. M. Altmann, unpublished observations). Alternatively, prostaglandin released from LPS-stimulated APC may act on Ts cell precursors, leading to the generation of suppression (Goldyne, 1983).

Various workers have previously reported the generation of Ts cells acting on the DTH response to HSV1 by intravenous inoculation of virus (Nash *et al.*, 1981; Schrier, Pizer & Moorhead, 1983). In our hands, the events triggered by LPS differ in many respects from the suppression activated by intravenous inoculation. Ts cells activated by the latter means are not sensitive to Cy, can act only on the afferent or efferent limb of a DTH response (whereas LPS-induced cells act only on the efferent limb), do not adhere to nylon wool and are associated with an enhanced antibody response in the presence of greatly suppressed DTH (D. M. Altmann & W. A. Blyth, manuscript in preparation). While it is hard to draw firm conclusions without further membrane phenotyping, the evidence to date suggests that different pathways are involved in the two modes of Ts cell activation.

When susceptible mice were inoculated with HSV1 together with LPS, there was slightly enhanced recruit-

ment of cells to the inoculation site, but largely normal spread of virus in the skin and to the peripheral nervous system. Mice receiving LPS were less likely to suffer ear paralysis or lethal encephalitis than controls, and marginally more likely to get recurrent lesions after the application of trauma to the skin. Clearly, one cannot define whether or not the various aspects of protection observed here are the direct consequence of Ts cell activation, or of some form of LPS-induced enhancement (such as enhanced interferon production) which has not been measured. However, this work demonstrates that protection from CNS pathology is, at least, not incompatible with the preferential activation of Ts cells acting on the DTH response. Furthermore, parallel studies involving the activation of Ts cells by intravenous virus inoculation which, as mentioned above, differs in many other respects from the events following administration of LPS and virus, also showed protection in mice carrying 'split tolerance' (D. M. Altmann & W. A. Blyth, manuscript in preparation). This protection was transferable by T cells and was not dependent on anti-viral responses restricting the spread of HSV1 to the CNS. It is inferred from the findings in both of these models of Ts cell activation that, during some stages of virus spread through the CNS, DTH-mediated pathology may, itself, be more harmful than the direct, cytopathic effect of virus and, in this case, Ts cell activation has a beneficial effect.

Perhaps it is precisely this need to generate a T cell response to the virus which can be highly regulated with respect to the restriction of neural spread, and yet extremely swift at limiting the spread of reactivated virus in the skin, which creates the circumstances for the virus to profit from very slight changes in immunoregulation, causing frequent recrudescence in otherwise normal individuals.

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