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# Tolerance and immunity in mice infected with herpes simplex virus: simultaneous induction of protective immunity and tolerance to delayed-type hypersensitivity

A. A. NASH, P. G. H. GELL & P. WILDY *Division of Virology, Department of Pathology, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge*

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**Summary.** Unresponsiveness to delayed type hypersensitivity was induced in mice following an intravenous injection of herpes simplex virus. The principal tolerogens used were thymidine kinase-deficient virus mutants which grow poorly *in vivo*; u.v.-inactivated and to a lesser extent formalin-inactivated virus were also tolerogenic. The tolerance induced was specific for the virus type.

Despite the tolerance to delayed hypersensitivity, anti-viral immunity is present as determined by the rapid inactivation of infectious virus. The mechanism of tolerance to herpes virus and the importance of these observations for the pathogenesis of viral disease is discussed.

## INTRODUCTION

The persistence of certain virus infections, such as lymphocytic choriomeningitis virus (LCMV) was considered by Burnet & Fenner (1949) to be a consequence of the host becoming immunologically tolerant to the virus antigens. Although tolerance no longer explains the phenomenon of persistent LCMV infections (Oldstone & Dixon, 1967), it is reasonable to suggest that viruses might persist in a host by unwittingly blindfolding part or all of the hosts defences. The possibility that herpes simplex virus (HSV) could induce tolerance to part of the immune system has not really been considered. It has, however, been suggested that the failure of lymphoid cells to produce lymphokines, such as macrophage migration inhibition factor (MIF), may be important in patients undergoing herpes recurrences (Shillitoe, Wilton & Lehner, 1977).

Host responses to herpes simplex involve humoral and cell-mediated immune mechanisms. The clearance of infectious virus and protection against acute HSV infections in mice appears to involve cytotoxic T cells in conjunction with delayed-type hypersensitivity (DTH) cells and/or anti-HSV antibody (Nash, Field & Quartey-Papafio, 1980a; Nash, Phelan & Wildy, 1981b; Howes, Taylor, Mitchison & Simpson, 1979) whereas protection from re-infection is most probably mediated by neutralizing antibody with or without immune cell involvement (Howes *et al.*, 1979).

In this paper we investigate the possibility of tolerizing mice to herpes virus in order to discover whether impairment of certain immune functions could lead to a breakdown (permanent or temporary) in immunity to that virus. For this purpose we have used methods developed for the production in mice of suppressor cells to hapten- or protein-coated lymphoid cells (Bach, Sherman, Benacerraf & Greene, 1978; Miller, Wetzig & Claman, 1979) or to SRBC (Ramshaw, Bretscher & Parish, 1976) following intravenous injection of the antigens.

Correspondence: Dr A. A. Nash, Department of Pathology, University of Cambridge, Laboratories Block, Addenbrooke's Hospital, Hills Road, Cambridge.

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We here demonstrate that tolerance to DTH can be induced following an intravenous injection of infectious herpes simplex virus and it is specific for the virus type used. Furthermore this tolerance does not interfere with protective immunity.

## MATERIALS AND METHODS

*Animals*

Female BALB/c mice aged 5–6 weeks were obtained from Bantin and Kingman Ltd (Aldbrough, Hull) and used when 6–8 weeks old.

*Viruses*

Herpes simplex type 1 strains: SC16 Cl is a recent oral isolate which produces a well characterized infection in mice (Field, Bell, Elion, Nash & Wildy, 1979; Nash, Quartey-Papafio & Wildy, 1980b). Strain Cl (101) and its BUdR-selected thymidine kinase (TK)-deficient mutant Cl (101) $TK^-$ were originally isolated by Dubbs & Kit (1964). Herpes simplex type 2 strains: strain Bry and its BUdR-selected TK-deficient mutant Bry $TK^-$ were isolated by Skinner and Thouless (Thouless, 1972). The pathogenicity for mice of both Cl (101) and Bry and their $TK^-$ mutants has been studied (Field & Wildy, 1978).

All viruses were propagated in BHK-21 cells. Cells were infected at low multiplicity incubated at 32° for 48 hr, disrupted by ultrasound and stored in aliquots at −70°.

*Inactivation of virus*

SC16 Cl was subjected to the following procedures in order to inactivate live virus.

*Heat inactivation.* One millilitre containing $2 \times 10^9$ p.f.u. was held at 56° for 45 mins.

*Ultraviolet inactivation.* $1 \times 10^9$ p.f.u. in 2 ml PBS was u.v. inactivated using a 30 W lamp, 43 cm from the source for 20 mins. Dose rate was approximately 1000 $mm^2$/min and surviving infectivity was less than $10^{-5}$.

*Formalin inactivation.* $2 \times 10^9$ p.f.u. was mixed with 10% paraformaldehyde solution in PBS to give a 0·2% solution. Before use the preparation was dialysed in order to remove excess formalin.

*Inoculation of virus and infectivity assays*

The inoculation of virus into the ear pinna has been described in detail elsewhere (Nash *et al.*, 1980a). In all cases the left ear pinna was injected for measuring primary ear swelling responses. Inactivated virus preparations or $TK^-$ mutants were inoculated intravenously in 0·1 ml PBS by the tail vein route. Individual ears were assayed for infectious virus on BHK cells as described elsewhere (Nash *et al.*, 1980a).

*Measurement of primary ear swelling and DTH responses*

This procedure has been described in detail elsewhere (Nash *et al.*, 1980a). Briefly ear thickness was measured using an engineer's micrometer and the results expressed in units $\times 10^{-2}$ mm as the difference between infected (or skin tested) and uninfected ears. In all experiments reported herein, the DTH skin test was performed in the right ear pinna using $10^4$ p.f.u. of infectious virus. Heat-killed virus at the appropriate concentration gives comparable results (Nash *et al.*, 1980a).

## RESULTS

### Effect of the route of infection on the induction of DTH responses

Groups of BALB/c mice were inoculated with $10^5$ p.f.u. of the avirulent strain Cl (101) $TK^-$ either intravenously or subcutaneously into the ear pinna. After 7 days the mice were challenged with $10^4$ p.f.u. SC16Cl subcutaneously and the resulting ear thickness measured on successive days (Fig. 1). Mice sensitized

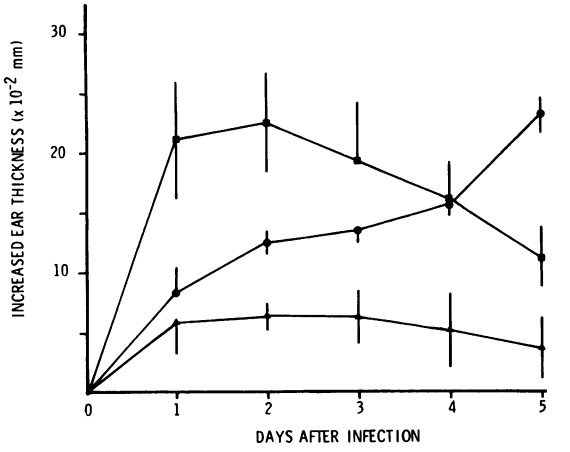


**Figure 1.** Effect of the route of infection on the induction of DTH responses to herpes simplex virus type 1. Mice were inoculated subcutaneously (ear pinna, ■———■) or intravenously (▲———▲) with $10^5$ p.f.u. Cl (101) $TK^-$. After 7 days the two groups were challenged with $10^4$ p.f.u. SC16Cl and the ear thickness response measured on successive days. Control mice received challenge virus only (●———●). Each point represents the mean ± SD of ear thickness of five mice/group.

**Table 1.** Dose of virus required to induce DTH unresponsiveness and concomitant immunity

| Dose of Cl(101)TK$^-$ injected intravenously ($\log_{10}$ p.f.u.) | Primary ear thickness ($\times 10^{-2}$ mm) at day 2 post challenge† | Virus titre at day 2 post challenge ($\log_{10}$ p.f.u./ear) | *DTH response at day 1 ear thickness ($\times 10^{-2}$ mm) |
|---|---|---|---|
| Control (no virus) | 20±3·6 | 4·12 (3·9–4·2) | 27·5±1·7 |
| 1·0 | 17·3±2·2 | 3·70 (3·6–3·9) | 26·0±1·8 |
| 2·0 | 16·8±2·8 | 3·60 (3·3–3·8) | 26·7±1·7 |
| 3·0 | 13·0±2·7 | 2·60 (2·2–3·0) | 17·6±1·7 |
| 4·0 | 10·4±1·4 | 2·35 (2·0–2·7) | 16·6±1·7 |
| 5·0 | 7·5±2·2 | 1·58 (1·2–2·3) | 11·5±2·0 |

* Skin test used $10^4$ p.f.u. SC16Cl into right pinna 6 days after primary challenge.
† Mice were challenged with $10^4$ p.f.u. SC16Cl injected into the left pinna 7 days after intravenous sensitization.
All ear thickness results expressed as mean ± SD of five mice/group.

subcutaneously with the Cl (101) TK$^-$ produced a DTH response, whereas those sensitized intraveneously produced ear swelling well below the control level. The data imply that following an intravenous injection of virus a state of unresponsiveness to DTH induction is achieved. This effect is long lasting with unresponsiveness to DTH still present after more than 100 days (data not shown).

**Dose of virus required to produce DTH unresponsiveness and protective immunity**

Various dilutions of Cl (101) TK$^-$ were injected intravenously into groups of mice. After 7 days all mice were challenged subcutaneously with $10^4$ p.f.u. SC16Cl into the ear pinna. As shown in Table 1 there is a progressive decrease in the primary ear swelling response with increasing dose of virus injected intravenously. Similarly the infectivity titres on day 2 post challenge were reduced by 2–3 $\log_{10}$ p.f.u. in the groups of mice receiving 3–5 $\log_{10}$ p.f.u. intravenously. In this range of virus inoculum there is also marked inhibition of the induction of DTH responses when measured 6 days later. Consequently at doses as low as $10^3$ p.f.u. injected intravenously unresponsiveness to DTH is produced in addition to anti-herpes immunity.

**Effect of injecting virus simultaneously in the pinna and intravenously on the induction of DTH unresponsiveness**

It is clear from the data presented in Table 1 that intravenous injection of Cl(101)TK$^-$ leads to induction of DTH unresponsiveness and concomitant immunity. The possibility that the immune status of

**Table 2.** Effect on DTH responses of simultaneously injecting virus intravenously and subcutaneously

| Route of infection | †Virus strain | Primary ear thickness at day 4 ($\times 10^{-2}$ mm) | Infectivity titres at day 4 ($\log_{10}$ p.f.u./ear) | *DTH response ear thickness at 24 hr ($\times 10^{-2}$ mm) |
|---|---|---|---|---|
| s.c. | SC16Cl | 24·2±1 | 4·20 (4·04–4·41) | 20±1 |
| i.v. | Cl (101) TK$^-$ | — | — | 9·6±2 |
| s.c.+ i.v. | SC16Cl+ Cl (101) TK$^-$ | 20±3 | 3·83 (3·39–4·59) | 8·2±3 |

Ear thickness 24 hr after injection of $10^4$ p.f.u. SC16Cl into normal mice was 10·4±1
* $10^4$ p.f.u. SC16Cl injected into right ear pinna 6 days after sensitization.
† Dose of virus used: SC16Cl $10^4$ p.f.u., Cl (101) TK$^-$ $10^5$ p.f.u.
Results expressed as mean ± SD or mean (range) of 6 mice/group.

intravenously injected mice could lead to a lowering of the threshold for DTH induction by rapidly inactivating an infectious virus challenge was investigated in mice simultaneously injected intravenously and subcutaneously. As shown in Table 2 this procedure results in primary ear thickening and infectivity titres comparable with those of mice injected in the pinna only. However the DTH response measured 6 days after the simultaneous injection was markedly suppressed and comparable to the response produced when mice received only intravenous injections. This implies that tolerance to DTH is the result of the intravenous injection of virus and is independent of the amount of virus growth in the pinna.

### Specificity of DTH unresponsiveness and immunity to type 1 and type 2 herpes virus

Groups of mice were infected intravenously with either Bry TK$^-$ (type 2) or Cl(101)TK$^-$ (type 1) at a dose of $10^5$ p.f.u. The infected mice were left for 7 days and then inoculated in the pinna with the parental strains of each virus type. On successive days the ear thickness was measured and on the 4th day after infection some mice had their ears removed to assay for infectious virus (Table 3).

Mice sensitized intravenously with type 2 virus and challenged subcutaneously with either virus type give markedly reduced primary ear swelling response at day 4 (data not shown) and also no infectious virus of either type. Similarly mice sensitized intravenously with type 1 virus and challenged subcutaneously with either type show reduced primary ear swelling and no type 1 infectious virus on day 4 while variable though reduced amounts of infectious type 2 virus were present. Of particular interest in this table is the DTH response of mice sensitized intravenously with either type of virus and challenged with the heterologous herpes type which was also used in the DTH skin test. These mice showed an intermediate DTH response between complete suppression as seen in the homologous system and the normal DTH response obtained after subcutaneous sensitization.

The possibility that tolerance to one herpes type could be reversed by challenging with the other type thus leading to a positive skin test with the first was investigated and the results shown in Table 4. Although mice sensitized subcutaneously with Bry (type 2) were able to respond equally to both virus types in a DTH skin test, mice initially tolerized to type 1 virus remained unresponsive to type 1-induced DTH, despite a primary challenge with type 2.

Together with the data presented in Table 3 this strongly suggests that type 1 virus, for example, injected intravenously renders DTH cells unresponsive to type 1 specific antigens and presumably type 1 and type 2 common antigens. However a subcutaneous challenge with type 2 virus may still induce a DTH cell response to type 2 specific antigens.

**Table 3.** Specificity of DTH unresponsiveness and immunity to type 1 and type 2 herpes simplex virus

| *Virus type used for i.v. injection (day 0) | †Virus type used for s.c. challenge (day 7) | Virus titre on day 4 post challenge, $\log_{10}$ p.f.u./ear (day 11) | ‡Virus type used in DTH skin test (day 13) | DTH response ear thickening at 24 hrs§ ($\times 10^{-2}$ mm) |
|---|---|---|---|---|
| none | type 1 | 4·03 (3·6–4·3) | type 1 | 21 ± 1·5 |
| none | type 2 | 2·5 (2·0–2·7) | type 2 | 26 ± 2 |
| type 2 TK$^-$ | type 2 | 0 | type 2 | 9·5 ± 2 |
| type 2 TK$^-$ | type 1 | 0 | type 1 | 16·5 ± 4 |
| type 1 TK$^-$ | type 2 | 1·1 (0–2·3) | type 2 | 15 ± 1 |
| type 1 TK$^-$ | type 1 | 0 | type 1 | 7·5 ± 1·5 |

* $10^5$ p.f.u. Cl (101)TK$^-$ (type 1) or Bry TK$^-$ (type 2) injected intravenously.

† $10^4$ p.f.u. Cl (101) (type 1) or Bry (type 2) injected in left ear pinna 7 days after intravenous injection.

‡ $10^4$ p.f.u. Cl (101) (type 1) or Bry (type 2) injected in right ear pinna 6 days after primary challenge. Ear thickness expressed as mean ± SD of 5 mice/group.

§ Normal Balb/c mice inoculated with $10^4$ p.f.u. Cl (101) (type 1) or Bry (type 2) in the ear pinna produced 8 ± 1 units or 7·5 ± 1·5 units increase in thickness, respectively, at day 1.

**Table 4.** Specificity of DTH responsiveness and unresponsiveness to type 1 and type 2 herpes simplex virus

| Virus used for i.v. sensitization* (day 0) | Virus used in s.c. challenge (day 7) | Virus used for DTH skin test† (day 13) | DTH response increased ear thickness ($\times 10^{-2}$ mm) 24 hr | 48 hr |
|---|---|---|---|---|
| Cl (101 TK$^-$) type 1 | Bry (type 2) | Bry (type 2) | 14·5±3 | 13±2 |
| Cl (101) TK$^-$ | Bry (type 2) | Cl (101) (type 1) | 8±2·5 | 7±3 |
| — | Bry (type 2) | Bry (type 2) | 24±3 | 18±2·5 |
| — | Bry (type 2) | Cl (101) (type 1) | 24±3 | 21±6 |

* $10^5$ p.f.u. Cl (101) TK$^-$ (type 1) injected intravenously.
† $10^4$ p.f.u. Cl (101) (type 1) or Bry (type 2) injected into pinna and also in DTH skin test.
Results expressed as mean ± SD of five mice/group.

**Induction of tolerance and immunity with various inactivated preparations of type 1 virus**

Various inactivated preparations of SC16 Cl were injected intravenously at a dose of $10^6$ erstwhile p.f.u. into groups of mice. After 7 days the mice were infected subcutaneously with $10^4$ p.f.u. SC16 Cl and the primary ear swelling response, infectious virus titres and DTH response measured (Table 5). Both u.v. and formalin inactivated virus produced suppression of the primary ear swelling response and reduced infectious virus titres on day 2 by 3·5 logs. Heat-inactivated virus appeared not to influence primary ear swelling and produced a reduction of only 0·9 $\log_{10}$ p.f.u. in virus titres. The DTH response varied depending on which inactivated virus preparation was used, for example u.v. treated virus produced complete suppression, whereas with the formalin preparation the response observed was intermediate between normal DTH and no DTH. This implies that the formalin treatment preserves antigens which are important in anti-viral immunity, yet new antigens are seen by the DTH system following infection with live virus. The situation with heat-killed virus, which also produces an intermediate DTH response but little immunity is less clear.

## DISCUSSION

The main conclusion from the experiments reported herein is that the intravenous inoculation of herpes simplex virus produces a state of unresponsiveness with respect to DTH. The principal tolerogens used

**Table 5.** Induction of tolerance and immunity with various inactivated preparations of type 1 herpes simplex virus

| *Sensitizing dose of inactivated virus inoculated intravenously | †Primary ear thickness response 2 days after challenge ($\times 10^{-2}$ mm) | Infectivity titres ($\log_{10}$ p.f.u./ear) | DTH response at 24 hr post skin test ($\times 10^{-2}$ mm) |
|---|---|---|---|
| No virus | 22±4·7 | 4·69 (4·30–4·82) | 22·5±3·4 |
| U.V. inactivated | 10±2·2 | 1·23 (0·95–1·39) | 11·0±1·5 |
| Formalin-inactivated | 7±2·2 | 1·20 (0·77–1·50) | 16·0±1 |
| Heat-inactivated | 19·5±4·6 | 3·77 (3·04–4·00) | 18·0±1·5 |

* SC16Cl was inoculated intravenously after inactivation by various procedures. The dose was $10^6$ p.f.u. measured before inactivation.
† $10^4$ p.f.u. SC16Cl used in subcutaneous ear challenge 7 days after intravenous sensitization, and also in DTH skin testing 6 days after primary challenge.
Results expressed as mean ± SD or mean (range) of 5 mice/group.

were mutants of herpes lacking thymidine kinase activity; such strains are competent in standard cell cultures but are not pathogenic in mice compared with the parental strains (Field & Wildy, 1978). It is, however, also clear that infectious virus is not essential for the induction of tolerance; u.v. and to a lesser extent formalin inactivated viruses are also tolerogenic. This point is particularly relevant because it implies that tolerance is not the result of a deletion of DTH cell precursors produced following infection with the virus. A similar observation on DTH tolerance has been made with u.v. inactivated reovirus, although in this instance live reovirus injected intravenously sensitized mice for DTH (Greene & Weiner, 1980).

The induction of tolerance to DTH is specific with respect to the virus type initially used for intravenous sensitization. For example type 1 virus tolerizes DTH to type 1 strains but only partially inhibits the DTH response to type 2 strains. Since we know that the two types of herpes virus possess type specific and type common determinants (Honess & Watson, 1977) it follows that tolerance is produced against the homologuous type-specific antigens and to type-common antigens but not at all to heterologous type-specific antigens. This indicates a very fine discrimination of herpes antigens during the induction of tolerance and contrasts with the subcutaneous sensitization of DTH by either virus type in which the magnitude of the DTH reaction is roughly equivalent whichever virus type is used for skin testing. One could explain this difference by arguing that type common antigens are abundant and frequent, while type specific are scarce and rare. Hence DTH (a positive reaction) tends to show up type common effects while tolerance ( a negative reaction) tends to distinguish negative effects. It is worth noting that once an animal has been tolerized by an intravenous injection with one type of virus, a subcutaneous challenge with the heterologous type will not sensitize for the original type.

Despite the specific tolerization of the DTH system, concomitant immunity to herpes is also present as shown by the rapid clearance of infectious virus from the ear. This immunity may well involve antibody, since neutralizing antibodies are detected in these mice (A. A. Nash, unpublished data). Because of the rapid clearance of infectious virus it could be argued that the antigenic threshold required for the induction of DTH is not attained thus accounting for the apparent unresponsiveness. However the simultaneous intravenous and subcutaneous injection of virus results in the normal growth of virus in the pinna, but the induction of DTH to herpes is nevertheless suppressed. This indicates that the induction of tolerance is a real phenomenon which is dependent upon the intravenous injection of virus.

Suppression of DTH with concomitant immunity has been observed using non-viral antigens such as SRBC (Ramshaw *et al.*, 1976). The inhibition of DTH is this system was mediated by suppressor T cells. Suppressor T cells were also observed in the spleens of mice 6 days after a tolerizing injection of u.v. inactivated reovirus (Greene & Weiner, 1980). Similarly in mice infected by aerosol with influenza virus suppressor T cells develop which interfere with the induction of DTH (Liew & Russell, 1980). In contrast to these suppressor systems mice latently infected with herpes simplex virus type 1 contain a population of Ig positive, thy-1·2 negative lymphocytes which can suppress the established DTH response to the virus (Nash & Gell, 1980). In the present model of herpes-induced tolerance, the preliminary evidence suggests that there are two mechanisms causing DTH unresponsiveness, one appears early (within 1 day) after virus injection, is resistant to cyclophosphamide and adult thymectomy and is long lasting ($>100$ days), and a second mechanism is transferable by spleen cells from 14 days onwards and suppresses the induction of DTH (Nash, Phelan, Gell & Wildy, 1981a).

Immune surveillance in hosts latently infected with herpes is considered an important requirement for controlling the emergence of infectious virus. Breakdown in the host's defence system often results in recurrent herpes infections such as seen in immunosuppressed leukaemia and transplant patients (Merigan & Stevens, 1971). Experiments are currently under way to study the effects of tolerizing doses of herpes simplex virus on the establishment and maintenance of latent infections in mice, as well as investigating their effects on other aspects of CMI, e.g. cytotoxic T cells. It is too early yet to know whether tolerized hosts are more likely to be predisposed to herpes recurrences. However if this proves to be the case it could offer an explanation for the incidence of recurrences observed in some humans with herpes simplex infections, in which impairment of CMI function has been proposed as a possible cause of the clinical recurrent disease (Shillitoe *et al.*, 1977). In addition by using this technique of paralysing certain areas of immunity to the virus, one could assess the importance of such mechanisms in protection against viral disease.

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