J. Hyg., Camb. (1982), **89**, 335–345
Printed in Great Britain

A non-lethal test of the resistance of inbred mice to herpes simplex virus type 1

By L. H. COLLIER and Q. J. SCOTT

Department of Virology, The London Hospital Medical College, Turner Street, London E1 2AD

(*Received* 28 *May* 1982; *accepted* 2 *July* 1982)

SUMMARY

Inbred strains of mice varying in susceptibility to intraperitoneal (i.p.) inoculation of HSV-1 were tested by inoculating 10^5 p.f.u. of the SC 16 strain into the ear pinna. Increase in ear thickness was less in resistant than in susceptible strains. In the resistant C57BL/6 strain, local replication of virus, spread to cervical ganglia and development of latent infections of the ganglia were all less than in susceptible DBA/2 mice; there was also less cellular infiltration of the inoculation site. Like resistance to i.p. inoculation, resistance to ear inoculation appears to be inherited as a dominant characteristic. The test provides a non-lethal method of distinguishing between resistance and susceptibility of inbred strains of mice to HSV-1 and may also be useful in defining resistance factors.

INTRODUCTION

Herpes viruses are very common pathogens of man and are receiving increasing attention for several reasons; the mechanisms by which they become latent and reactivate are beginning to be understood; the rôle of some herpes viruses in oncogenesis, including herpes simplex type 2, is becoming clearer; and the last few years have seen remarkable advances in the development of highly specific anti-herpes drugs. Nevertheless, despite investigations by many workers, the factors governing susceptibility to infection with herpesviruses are poorly understood and many features of the pathogenesis of such infections are also obscure. In man, primary infection with herpes simplex type 1 (HSV-1) often takes place in early childhood, but some individuals escape infection throughout their lives; we do not know if this is in the main due to chance or to inherent resistance. The studies reported in this and subsequent papers were designed to elucidate some of the factors determining resistance to HSV-1 by comparing the events following infection in mice that differ in susceptibility to this virus.

The intraperitoneal (i.p.) injection of a pathogenic strain of herpes simplex virus into a susceptible mouse is followed within a week or so by illness and death with signs of central nervous system involvement; and the resistance of mice to infection is usually assayed by determining the LD_{50} of a given strain of virus after i.p.

inoculation. In terms of lethal dose, the range between the extremes of resistance and susceptibility may be of the order of a million-fold (Lopez, 1975) so that these terms are largely relative. With this *caveat*, some strains of mice are generally regarded as 'resistant' to HSV-1, a common indicator being ability to survive the i.p. inoculation of 10^6 or more infective units (e.g. Lopez, 1978). Although this somewhat arbitrary dose obviously depends on the strain of virus used, we have adopted it in practice as a reasonably good criterion for defining highly resistant mice such as those with a C57BL background.

Since we are particularly interested in the genetic control of pathogenesis and resistance in HSV-1 infections, a non-lethal test enabling the selection of both resistant and susceptible mice for breeding experiments would be valuable. In the investigation described here, we show that the response to intradermal injection of an appropriate dose of HSV-1 into the ear provides such a test and should also be useful in studying factors that determine resistance and susceptibility.

MATERIALS AND METHODS

Cell cultures. African green monkey kidney (VERO) cells were grown in Eagles' modified minimum essential medium (MEM) containing 2 mM-L-glutamine, non-essential amino-acid solution 1%, sodium bicarbonate 0·075%, penicillin 100 units/ml and streptomycin sulphate 100 μg/ml. Growth and maintenance media were supplemented with 5% and 2% fetal calf serum respectively. VERO cells, MEM, non-essential amino acids and foetal calf serum were from Flow Laboratories, Irvine, Scotland.

Herpes simplex virus type 1 was strain SC16 (Hill, Field & Blyth, 1975) isolated from recurrent labial herpes (W. A. Blyth, personal communication) and was used after four or five passages in VERO cells. Stock suspensions were prepared from VERO monolayers infected 18 h previously at a multiplicity of approximately 1·0. The cells were frozen and thawed 3 times, suspended in maintenance medium (MM), and centrifuged at low speed to remove debris. The supernatant fluid was stored in small aliquots at -70 °C.

Virus titrations were done by plaque assay in confluent VERO cell monolayers prepared in Linbro tissue culture plates containing twelve wells, each 4·5 cm^2 (Flow Laboratories). After removing growth medium, 0·1 ml volumes of suitably diluted suspension were added (four wells per dilution) and allowed to adsorb for 1 h at 37 °C; 1·5 ml of MM containing 0·75% carboxymethyl cellulose was then added to each well and incubation was continued for 3–4 days. Plaques were counted after fixation with 4% formalin and staining with 1% naphthalene black. The titre in terms of $\log_{10}$ plaque-forming units (p.f.u.)/ml was calculated from the mean count of four wells inoculated with a dilution producing 10–50 plaques. In seven replicate titrations of a suspension with a mean titre of $10^{7\cdot3}$ p.f.u./ml, the range of standard deviations of the counts in the four replicate wells was $\pm$5–29% with a mean of $\pm$16%, (or $\log_{10}$ 0·07 for this suspension).

Mice. C57BL/KS, B10.M, B10.HTT, BALB/c, CBA/H and A/J mice were bred in The London Hospital Medical College animal house by single line, brother–sister

mating. C57BL/6, DBA/2 and 129 mice were obtained from Olac Laboratories (Blackthorn, Bicester). All mice were 6–8 weeks old when used.

Intraperitoneal (i.p.) inoculation. Mice were inoculated by the i.p. route with 0·1 ml volumes of virus appropriately diluted in MM. For susceptibility tests, 10-fold dilutions were inoculated into groups of six mice and 50% lethal doses (LD_{50}) were calculated by the moving averages method (Thompson 1947).

Ear inoculations. With a 250 μl syringe and 16 × 0·5 mm needle, 10 μl of virus suspension containing 10^5 p.f.u. was inoculated into the pinna of the right ear through the external surface and 10 μl of MM as a control into the left ear; for this procedure mice were anaesthetised with sodium pentobarbitone, 0·05 mg/g body weight given i.p.

Ear thickness was measured with a dial gauge micrometer (Quicktest AOLT, Carbronze Ltd., London); this was calibrated in 0·1 mm intervals and could be accurately read by eye to 0·025 mm. Each measurement on an individual mouse is the mean of four readings. Results are expressed as increase in thickness of the test ear (T) as a percentage of the control (C), i.e. $((T-C)/C) \times 100$. (The thickness of an uninoculated ear in a 6 week old mouse is about 0·2 mm).

Assay of infective virus in ears and ganglia. After killing the mouse by cervical dislocation, the epidermis was scraped from the entire top surface of the pinna, homogenized in 0·5 ml of MM in a ten Broeck tissue grinder, frozen and thawed once and then titrated by plaque assay. The second, third and fourth right cervical dorsal root ganglia were removed under a dissecting microscope, pooled and assayed for virus in the same way as epidermis. Results are expressed as p.f.u. per ear or per three ganglia.

Assay of virus in latently infected ganglia. One month after ear inoculation, some mice were killed and tested for latent infection of the ipsilateral cervical ganglia by an explant method based on that of Stevens & Cook (1971). The ganglia were removed, incubated whole in growth medium for four days and then assayed for infective virus as described above. Ganglia from some of these infected mice were assayed immediately after removal as controls for the persistance of infective virus; the results were invariably negative.

Immunofluorescence staining. Frozen sections of ear 8 μ thick were stained for HSV-1 antigen by the indirect method. The anti-herpes serum was prepared in rabbits; the antigen was a homogenate of rabbit kidney (RK 13) cells grown in medium supplemented with normal rabbit instead of fetal calf serum and heavily infected with HSV-1. The first dose (day 0) was virus inactivated with 0·02% formaldehyde, emulsified in Freund's incomplete adjuvant (FCA) and injected intramuscularly. A similar dose of live virus was given on day 14 and a final dose of live virus without FCA was given i.p. on day 30. The rabbit was bled on day 40 to provide the antiserum.

Phosphate-buffered saline solution A (PBSA), pH 7·2 (Dulbecco & Vogt, 1954) was used as diluent and for washing. Sections were air-dried, fixed in methanol at −25 °C for 10 min and treated with anti-herpes serum diluted 1 in 40 for 30 min at 37 °C. After three 10 min washings the section was covered with swine anti-rabbit serum conjugated with fluorescein isothiocyanate (Nordic Laboratories)

Table 1. *Resistance of eight inbred strains of mice to intraperitoneal inoculation of HSV-1*

Mouse strain	LD_{50} (log_{10} p.f.u.)
C57BL/6	>6·3
C57BL/KS	>6·3
B10.M	>6·3
B10.HTT	>6·3
129	5·5
CBA/H	4·5
DBA/2	4·1
A/J	3·3

diluted 1 in 40; the incubation and washings were repeated and after a final rinse in distilled water sections were mounted in 10% glycerol in PBSA for ultraviolet microscopy. Sections of uninoculated ears and appropriate controls for non-specific fluorescence with normal rabbit serum and anti-rabbit conjugate were included.

Sections were examined under code; the degree of staining was allotted an arbitrary score of 1 (a single small focus) to 4 (extensive brilliant fluorescence).

Histological examinations. In parallel with the tests for immunofluorescence, ear sections stained with haematoxylin and eosin were examined for the extent of cellular infiltration and vesicle formation. Sections were examined under code and both types of lesion were allotted arbitrary scores of 1–4 according to severity.

RESULTS

Comparison of the resistance of inbred strains of mice to intraperitoneal (i.p.) inoculation of HSV-1

Groups of six mice of various strains were inoculated i.p. with tenfold serial dilutions of HSV-1 in 0·1 ml amounts and then observed twice daily. In susceptible mice, illness and death followed the well-known pattern of hunched back and staring fur, first seen 6–10 days after inoculation according to the inoculum and followed a day or two later by limb paralysis and death. Occasionally, a mouse with premonitory signs recovered completely and was considered as resistant. Table 1 shows the resistance of a number of strains in terms of the 50% lethal dose (LD_{50}). On the basis of these results we selected C57BL/6 and DBA/2 as respectively representing comparatively resistant and comparatively sensitive strains for routine use. Strain A/J mice were more susceptible than DBA/2 but were not available in the numbers needed for most experiments.

Responses to intradermal inoculation of the ear. Preliminary experiments, not reported here, suggested that the degree of swelling and erythema following inoculation of 10^5 p.f.u. of HSV-1 into the pinna was greater in DBA/2 and BALB/c mice than in C57BL/6. This observation was confirmed in further more detailed experiments.

We first compared the course of infection in resistant and susceptible mice after inoculation of 10^5 p.f.u. of virus into the ears of equal numbers of DBA/2 and

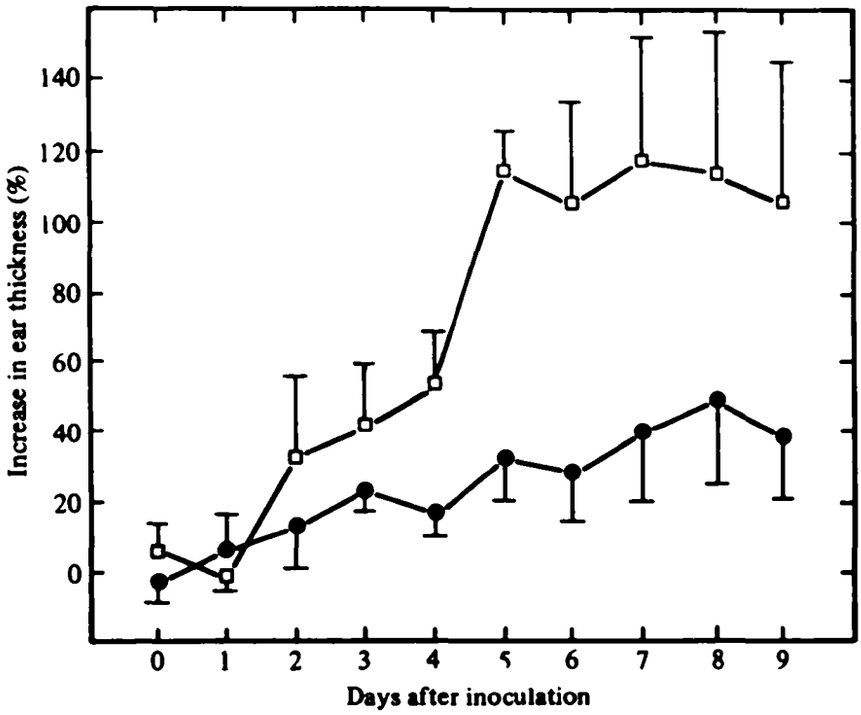

Fig. 1. Ear thickness measurements in DBA/2 (□) and C57BL/6 (●) mice after inoculation of 10^5 p.f.u. HSV-1. Points are means for six mice, expressed as percentage increases over control ear measurements. Vertical bars, 1 standard deviation.

C57BL/6 mice. Ear thickness measurements were made daily on the same group of six mice of each strain, the first being made immediately before inoculation. A further four mice of both strains were killed and their ears and cervical ganglia were assayed for virus; the first such determinations were made immediately after inoculation.

Measurements of ear thickness (*ET*). Fig. 1 shows the mean increases in ET in six DBA/2 and six C57BL/6 mice inoculated into the ear with 10^5 p.f.u. of virus. On day 5 after inoculation and subsequently, values were about three times higher in the more susceptible DBA/2 mice, the differences being greatest on days 5–7 after inoculation.

Recovery of virus from ears and cervical ganglia. Fig. 2(*a*) shows that up to and including day 6, the ears of all DBA/2 tested yielded virus in comparatively large amounts; thereafter, there was a pronounced diminution in yield and none was detectable on day 9. The yields from C57BL/6 mice were smaller throughout; virus was detectable in only 2 mice by day 6 and none was recovered on days 7–9.

These findings were reflected by infection of the ipsilateral ganglia. From both strains of mice, some virus was recovered on day 4; on days 5 and 6 about 10^3 p.f.u. were recovered from all the DBA/2 mice, after which both the proportion infected and yield diminished sharply (Fig. 2(*b*)). By contrast, fewer C57BL/6 mice were infected in the ganglia and the amount of virus recoverable was 10–1000 times less than in DBA/2.

Early stages of virus replication. The amount of virus recoverable from C57BL/6 on the day following inoculation was much less than from DBA/2 ears; to obtain a more detailed picture of the early stages of infection we compared virus

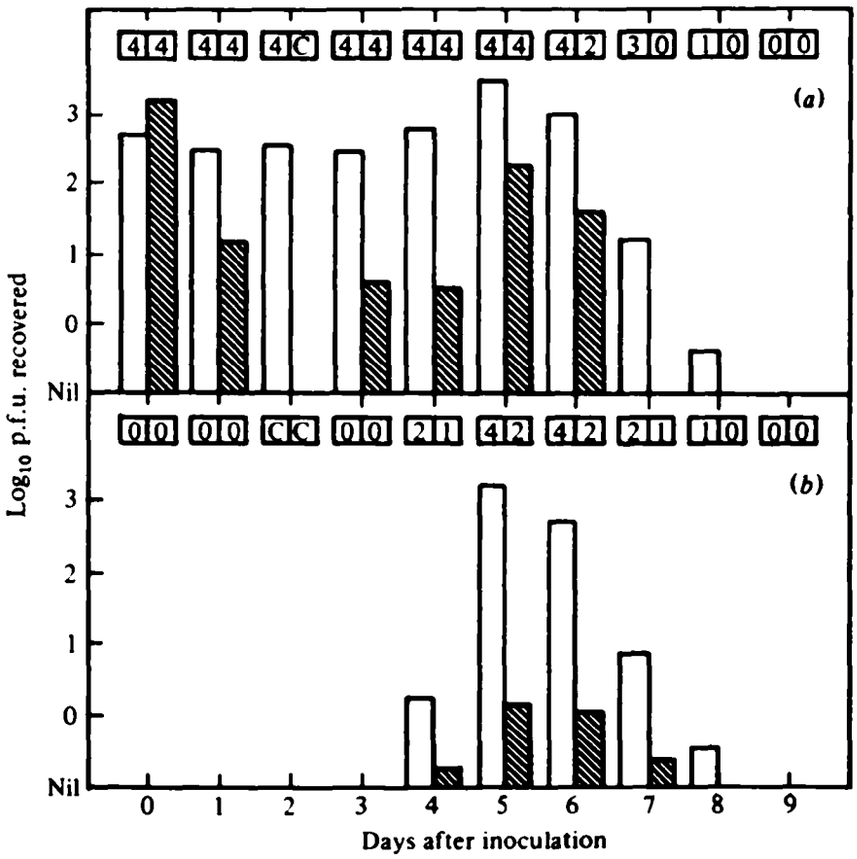

Fig. 2. Geometric mean titres of virus recovered from ears (*a*) and cervical ganglia (*b*) of DBA/2 and C57BL/6 mice after inoculation of 10^5 p.f.u. HSV-1 into the pinna. Open bars, DBA/2; shaded bars, C57BL/6. Boxes over bars indicate the number of mice infected of the four tested. C, cultures contaminated.

replication in the two strains of mice at intervals during the first 24 h after inoculation of 10^5 p.f.u. into the ear (Fig. 3). The rate of loss of infectivity was similar for both strains, the lowest values being obtained at 6 h. Therefore, the rate of replication was somewhat higher in DBA/2 and by 12 h the amount of infective virus recoverable from the ear was noticeably greater than from C57BL/6. By 24 h there was nearly 20 times more infective virus in the DBA/2 ears.

Immunofluorescence staining. On days 3–7 after inoculation, frozen sections from the ears of two mice of each strain were examined for immunofluorescing virus antigen. It was present from day 4 onwards in the DBA/2 mice; the total score for immunofluorescence for days 4–7 was 19 out of a possible 32. In the C57BL/6 mice the degree of staining was considerably less, with a total score of 7 for this period.

Histological appearances. Although the extent of vesiculation was rather more difficult to evaluate than inflammatory infiltration, the scores for both lesions were clearly greater in the DBA/2 mice. In this strain, maximal scores for infiltration were recorded on days 4–7 after inoculation and the total for days 3–7 was 36 out of a possible 40. The total score for vesicle formation, which was most pronounced on days 3 and 4, was 13/40. By contrast, the scores for infiltration and vesicles in C57BL/6 mice were respectively 19/40 and 9/40. It may however be significant

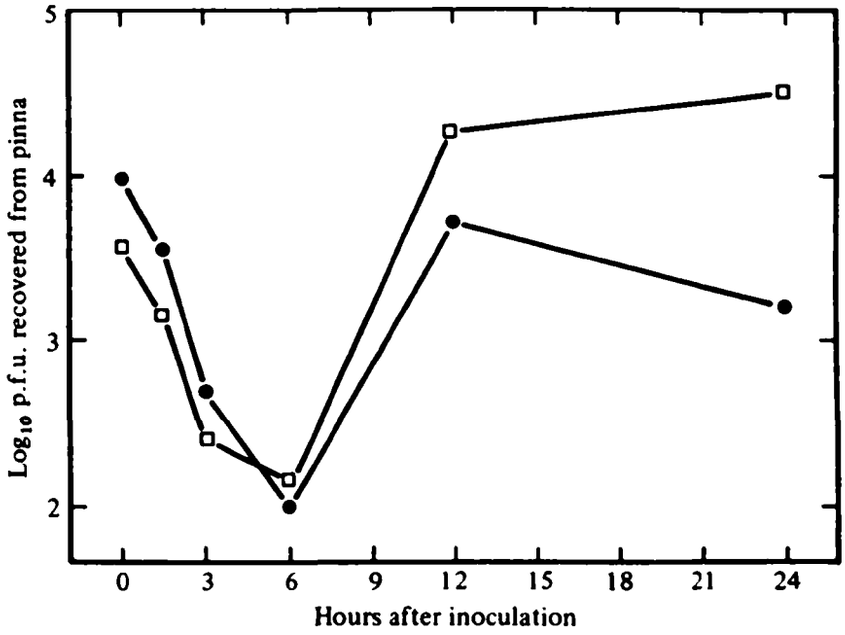

Fig. 3. Replication of HSV-1 in ears of DBA/2 (□) and C57BL/6 (●) mice after ear inoculation with 10^5 p.f.u. Each point represents the geometric mean titre of virus recovered from four mice.

that in C57BL/6 mice, the scores for infiltration did not decrease over the period of observation as did the amount of virus present.

Latent infection of the cervical ganglia. During the acute phase of infection, the cervical ganglia of C57BL/6 mice were less heavily infected than those of the DBA/2 strain; it was therefore of interest to determine whether latent infection was less readily established in the more resistant strain. One month after inoculation of 10^5 p.f.u. HSV-1 into the right pinna, the ipsilateral cervical ganglia of twelve DBA/2 and ten C57BL/6 mice were tested for the presence of latent virus. Fig. 4 shows that all the DBA/2 mice developed latent infection of the ganglia, whereas only four of the C57BL/6 did; and in them the amount of virus that could be reactivated was much less than in the DBA/2 mice.

Inheritance of resistance to ear inoculation. Further ear thickness tests with relatively susceptible (CBA/H, A/J) and resistant (C57BL/S, B10.M) strains gave differences similar to those between DBA/2 and C57BL/6 mice. Since these results correlated well with the differences in susceptibility to HSV-1 elicited by i.p. inoculation, it was important to determine whether resistance to ear inoculation is also inherited as a dominant characteristic.

In the first instance, groups of 6 mice of strains A/J (susceptible), B10.M (resistant) and A/J × B10.M (F1 generation) were tested in parallel. All mice were 6–8 weeks old when inoculated. The results (Fig. 5) show that the offspring behaved like the resistant parent strain. In a second similar experiment with DBA/2 and B.10M mice (Fig. 6) the F1 cross again behaved more like the resistant B10.M parental strain. On this occasion the B10.M mice showed an uncharacteristic degree of ET, although there was still a substantial difference between their response and that of the susceptible DBA/2.

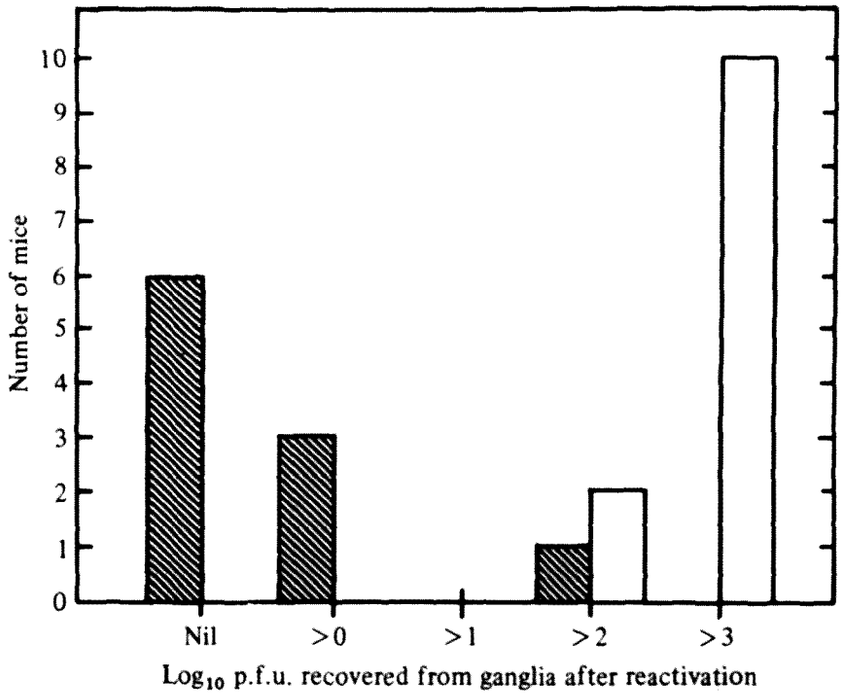

Fig. 4. Latent HSV-1 in cervical ganglia of DBA/2 and C57BL/6 mice one month after ear inoculation with 10^5 p.f.u. Open bars, DBA/2; shaded bars, C57BL/6.

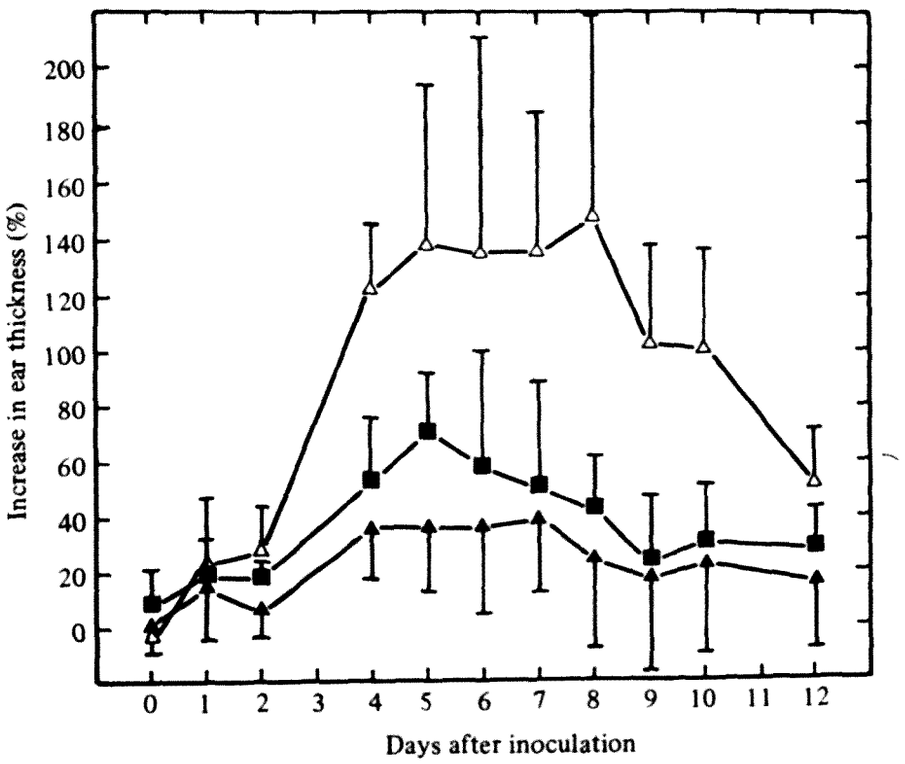

Fig. 5. Ear thickness measurements in A/J (△), B10.M (▲) and B10.M × A/J (■) mice after inoculation with 10^5 p.f.u. HSV-1. Points are means for six mice, expressed as percentage increases over control ear measurements. Vertical bars, 1 standard deviation.

https://doi.org/10.1017/S002217240007087X Published online by Cambridge University Press

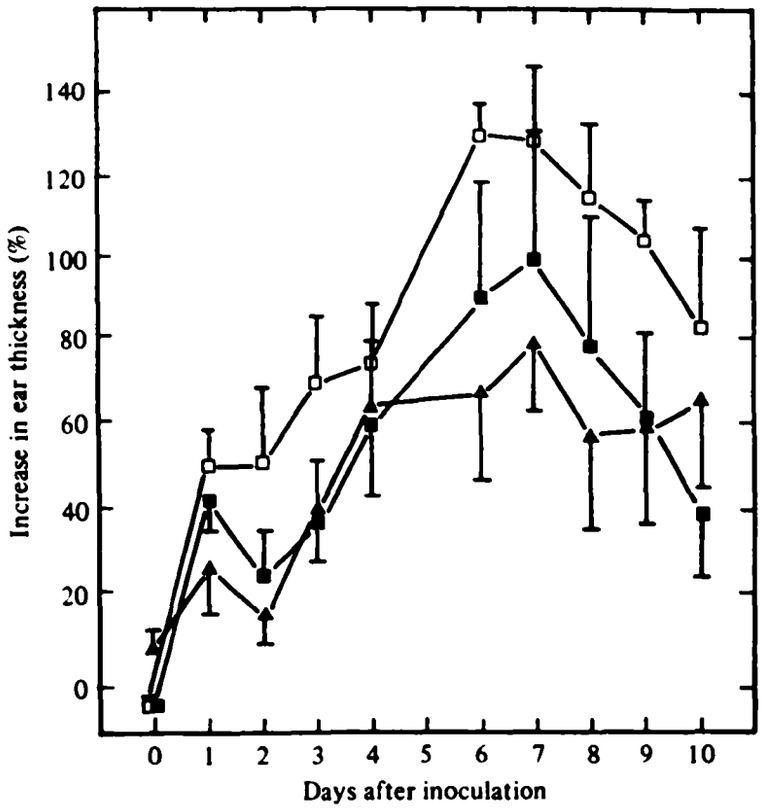

Fig. 6. Ear thickness measurements in DBA/2 (□), B10.M (▲) and B10.M × DBA/2 (■) mice after inoculation with 10^5 p.f.u. HSV-1. Points are means for six mice, expressed as percentage increases over control ear measurements. Vertical bars, 1 standard deviation.

DISCUSSION

We have demonstrated good correlation between the resistance of inbred strains of mice to the lethal effects of i.p. inoculation of HSV-1 and the degree of ear thickness (ET) following intradermal inoculation of a sublethal dose of virus into the pinna. The validity of this test for discriminating between resistant and relatively susceptible strains is supported by virological studies: the degree of replication in the ear, spread to the cervical dorsal root ganglia and development of latent infections were all considerably less in strains of mice that we classified as resistant to i.p. inoculation.

It is interesting to compare our findings with those of others who used mouse ear inoculation for different purposes. The technique was first used by Hill, Field & Blyth (1975) to compare the pathogenicity of strains of HSV-1 and HSV-2 and in particular, reactivation of latent infections. Type 1 viruses were less virulent than type 2, but it is noteworthy that intradermal inoculation of strain SC16, used in all our experiments, killed a proportion of mice of various strains, including strain A/J, at doses equal to or somewhat less than our standard inoculum of 10^5 p.f.u. (Hill *et al.* 1975; Harbour, Hill & Blyth, 1981). This is difficult to explain since our virus had undergone only one or two further passages in VERO cells after receipt from Dr Hill's laboratory; nevertheless, the observation emphasizes the need for choosing a dose of virus that will infect the ear without killing the mouse;

we were fortunate in that in our hands the dose first selected was satisfactory in this respect and also discriminated well between resistant and susceptible strains.

Hill and co-workers used erythema and ear paralysis as macroscopic criteria of infection. We found that thickness measurements provided a satisfactory index of infection and were more suitable for quantitative analysis; this method was used by Nash, Field & Quartey-Papafio (1980) to study delayed type hypersensitivity to HSV-1. They noted that in the primary response to 10^5 p.f.u. inoculated into the pinna, virus titres in the ear were highest 5–6 days after inoculation; the subsequent fall was preceded by a large infiltration of mononuclear cells. In our experiments, the titre of virus in the ears of the resistant C57BL/6 mice diminished earlier and more abruptly than in DBA/2 mice but, as far as our limited observations went, the degree of cellular infiltration was greater in the susceptible DBA/2 strains in which infective virus persisted at higher titre and for a longer period. Together with oedema and hyperaemia, cellular infiltration obviously contributes to the degree of ET, but more work is needed to elucidate the relative importance of these factors and the qualitative changes in the cell population that must occur during the course of a primary infection. Until this has been done, it will be difficult to interpret the significance of the different patterns of ET observed in resistant and susceptible strains of mice.

As well as merely discriminating between resistant and susceptible strains of mice, examination of the responses to intradermal inoculation of the ear should prove as useful in studies of the mechanisms of strain-dependent resistance as they have in researches on reactivation and delayed hypersensitivity. Our finding that resistance to ear inoculation appears to be inherited as a dominant characteristic is interesting: it suggests that the mechanism – or at least some component of it – is similar to that involved in resistance to i.p. inoculation and supports the use of primary ear inoculation as a means of studying strain-related resistance to HSV-1. Since HSV-2 also induces inflammatory lesions in the mouse ear (Hill *et al.* 1975), the intradermal test might also be useful for comparing the resistance of strains of mice to this virus.

We thank Miss Bonnett for her excellent technical assistance; Mr A. Weir and his staff in the Medical College Animal House; Dr Stella Barnass for preparing the rabbit anti-HSV-1 serum; Dr W. A. Blyth for the SC16 strain of HSV-1; and the Lister Institute of Preventive Medicine for the grant that supported this work.

REFERENCES

DULBECCO, R. & VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *Journal of Experimental Medicine* **99**, 167–182.

HARBOUR, D. A., HILL, T. J. & BLYTH, W. A. (1981). Acute and recurrent herpes simplex in several strains of mice. *Journal of General Virology* **55**, 31–40.

HILL, T. J., FIELD, H. J. & BLYTH, W. A. (1975). Acute and recurrent infection with herpes simplex virus in the mouse: a model for studying latency and recurrent disease. *Journal of General Virology* **28**, 341–353.

LOPEZ, C. (1975). Genetics of natural resistance to herpesvirus infections in mice. *Nature* **258**, 152–153.

LOPEZ, C. (1978). Immunological nature of genetic resistance of mice to herpes simplex virus type 1 infection. *IARC Science Publications* **24**, 775–781.

NASH, A. A., FIELD, H. J. & QUARTEY-PAPAFIO, R. (1980). Cell-mediated immunity in herpes simplex virus-infected mice: induction, characterization and antiviral effects of delayed type hypersensitivity. *Journal of General Virology* **48**, 351–357.

STEVENS, J. G. & COOK, M. K. (1971). Latent herpes simplex virus in spinal ganglia of mice. *Science, New York* **173**, 843–845.

THOMPSON, W. R. (1947). Use of moving averages and interpolation to estimate median-effective dose. *Bacteriological Reviews* **11**, 115–145.