Archives of Virology 64, 329—338 (1980)

Archives of Virology

Prepubertal Vaccination of Mice Against Experimental Infection of the Genital Tract with Type 2 Herpes Simplex Virus

By

G. R. B. Skinner, D. R. Williams, A. W. Moles, and A. Sargent

Department of Medical Microbiology, The Medical School, Birmingham, England

With 6 Figures

Accepted January 30, 1980

Summary

Pre-pubertal immunisation of mice with a formalin-inactivated type 1 and 2 herpes simplex virus vaccine conferred a level of life-long protection against primary type 2 genital infection. Protection levels were better with type 1 vaccine and strikingly influenced by vaccine dosage where a one-hundred-fold reduction from the standard vaccine dosage diminished protection to insignificant levels. Vaccine efficacy was not significantly affected by the method of virus inactivation, the number of immunisations or the age of the mouse at immunisation. Vaccination conferred better protection than previous type 2 genital infection; this may be a consequence of a higher antigenic dose, more acceptable antigenic presentation or to a perversion of the immune response in a latently infected animal to homologous virus challenge.

Introduction

The feasibility of immunisation against herpes simplex infections was first demonstrated in rabbits by Lipchutz (9). These early studies also provided evidence of the contribution of cell-mediated factors on host immunity and even hinted at possible antigenic distinctiveness between herpes simplex virus strains. More recently, successful immunisation against type 1 herpes virus has been demonstrated with live vaccine in mice (17) and with inactivated vaccine in mice and in rabbits (2, 8). Successful immunisation against type 2 herpes virus has been demonstrated in mice using a formalin-inactivated type 1 or 2 vaccine (4) and with live type 1 or 2 vaccine where, however, only homologous interactions were investigated (29). Other studies have suggested that mice immunised with type 1 vaccine were less likely to develop a latent ganglionic type 1, or indeed type 2 herpes virus infection (3, 11).

0304-8608/80/0064/0329/$ 02.00

There is evidence that herpetic cervicitis, a discommoding venereal disease of increasing prevalence, is associated with pre-invasive and invasive carcinoma of the cervix. This evidence is based on studies of *in vitro* cell transformation with type 2 herpes simplex virus (5, 12, 22) and on sero-epidemiological surveys which have indicated a higher prevalence of neutralizing antibody (13, 1) and type-specific complement-fixing antibody (26) to type 2 herpes virus in patients with pre-invasive and invasive cervical carcinoma; this latter study also indicated a significantly lower prevalence of type 1 specific antibody in the case group, suggesting that pre-adolescent exposure to type 1 infections may protect against primary type 2 herpes infection in later life.

Therefore there seems to be a good case for vaccination of female subjects particularly against primary type 2 herpes simplex virus infection. This study has investigated the efficacy of pre-adolescent immunisation of mice using formaldehyde-inactivated herpes simplex type 1 and 2 vaccine preparations. The influence of vaccine dosage, method virus inactivation, virus type and immunisation schedule on vaccine efficacy has been examined and compared with levels of protection following "natural" type 2 genital herpes simplex virus infections.

Materials and Methods

Cells

BHK-21 (clone 13), a stable line of cells derived from a single clone of baby hamster kidney cells (10), were used for virus propagation and vaccine production. The cells were maintained in supplemented Eagle's medium (28) containing 10 per cent v/v tryptose phosphate broth and 10 per cent v/v calf serum (ETC).

Prototype Virus Strains

The HFEM derivative of the Rockefeller strain HF (30) and strain 3345, a penile isolate, were used as prototype type 1 and type 2 strains for vaccine preparation and virus challenge (7, 16). Viruses were titrated by the plaque method of Russell (20).

Virus Antigen

BHK-21 cells (10) were infected at high multiplicity (10 PFU/cell) with type 1 herpes virus and incubated at 37° C for 24—36 hours. The infected cells were removed from the glass, washed, resuspended in saline at a concentration of 10^7 cells/ml, and disrupted by ultrasonic vibration. As a routine, antigen preparations were tested in immunodiffusion against homologous immune sera; under these circumstances they gave multiple precipitin lines. Antigen preparations were stored at −70° C.

Virus antigen preparations were thawed and resonicated and formaldehyde was added to a final concentration of 0.04 per cent. The antigen preparation was then dialysed against phosphate-buffered saline for 72 hours at 4° C, with frequent changes of the dialysing medium. There was no residual virus infectivity following this procedure. For control purposes, a formaldehyde treated uninfected BHK21 cell extract was prepared ("control vaccine").

Vaccination Schedule

Unless otherwise indicated, Swiss mice at 3 to 4 weeks of age (which, in terms of sexual maturity, corresponds to "adolescence" in these animals) were given a "standard" dose containing the equivalent of 10^6 infected cells by the subcutaneous route. A second standard dose of vaccine was given after an interval of three weeks.

Virus Challenge

A cotton pledget was allowed to soak in the virus suspension, appropriately diluted in medium (ETC), and gently introduced into the mouse vagina using small surgical forceps. Mice were routinely challenged with 1×10^6 PFU of type 2 virus unless otherwise indicated. The pledgets were removed after 16 hours.

Investigation of Challenged Mice

Mice were investigated virologically and cytologically at one, two, three and seven days following virus challenge. Specimens for cytologic investigation were obtained by rotating a dry cotton pledget within the mouse vagina. Smears were made and immediately fixed in absolute ethanol.

Having made the cytologic spread, the same cotton pledget was then quickly wetted in sterile medium (ETC), re-inserted into the mouse vagina, twisted for three complete rotations, removed and dropped into 1 ml of medium. In this way, we attempted to standardize the virus yields obtained by the sampling technique. The pledget-containing suspension was frozen and then thawed, sonicated and titrated in BHK 21 cells.

Blood for neutralizing antibody studies was obtained by cardiac puncture of mice under deep ether anaesthesia. Residual ether in the serum was blown off with nitrogen. Sera were tested at a five-fold dilution by kinetic neutralisation and "k values" calculated as previously described (25).

Mortality rates were calculated in terms of mice with cytological or virological evidence of virus infection, thereby excluding animals which were never infected, for example, by immediately extruding the infecting intravaginal pledget. The criteria of virus infection in infected mice were those previously described (31).

Results

The efficacy of vaccination in pre-sexual maturity mice is shown in Table 1 and Figs. 1—4. In mice receiving two immunisations of our "standard vaccine", viz. 10^6 infected cells equivalent per dose, mortality was reduced by over 50 per cent and average virus yields per mouse were reduced tenfold by the 5th day and

Table 1. *Protection of mice immunized with different vaccine dosages*

Vaccine preparation	Number of mice	Mortality in provenly infected mice (%)	Specific cytopathic effect at 7th day (%)	Average virus yield per mouse at 7th day (PFU)
Standard vaccine				
Type 1	60	14/53 (26%)	27	0.48 ± 0.14
Type 2	60	19/54 (35%)	39	1.30 ± 0.29
Standard vaccine at 10^{-2} dilution				
Type 1	40	26/38 (67%)	38	1.95 ± 0.32
Type 2	40	32/39 (86%)	45	1.89 ± 0.22
Standard vaccine at 10^{-4} dilution				
Type 1	40	31/37 (84%)	44	1.68 ± 0.39
Type 2	40	28/37 (82%)	49	1.60 ± 0.30
Control vaccine	105	84/95 (88%)	65	2.66 ± 0.26

one-hundred-fold by the 7th day of infection (Table 1, Fig. 1). The proportion of mice with specific herpetic cytopathic effects (CPE) was consistently lower in mice immunised with the standard vaccine (Fig. 2) and, similar to virus yields, was most striking after 5 days of infection. The proportion of mice with a non-specific inflammatory response and the extent or degree of specific CPE, quantitated in terms of "plusses" *(vide supra)* was also lower in immunized mice, although with these criteria, the differences remained similar at the various times of testing following infection (Figs. 3 and 4, respectively).

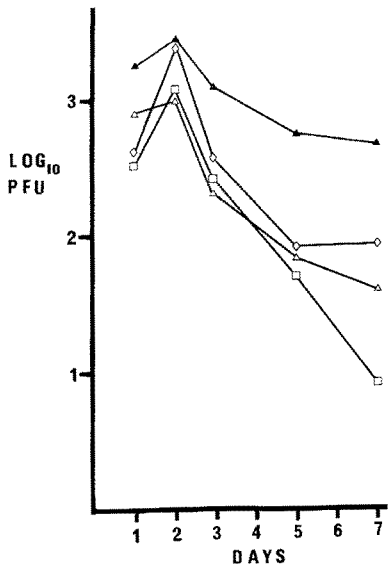

Fig. 1

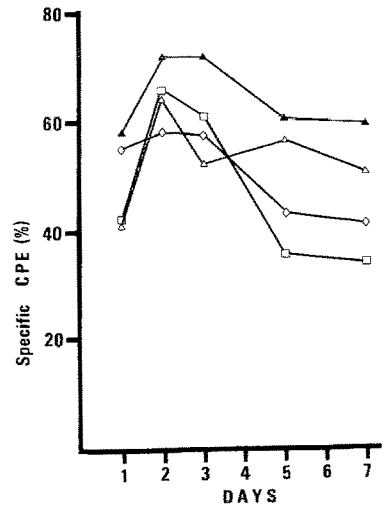

Fig. 2

Fig. 1. Virus yields (PFU) per mouse following challenge with 10^6 PFU of type 2 herpes virus in mice immunized with standard vaccine (—□—□—□—), vaccine at one-hundredfold dilution (—◇—◇—◇—), vaccine at one ten-thousandfold dilution (—△—△—△—) and control vaccine (—▲—▲—▲—)

Fig. 2. Prevalence (per cent) of mice with specific viral cytopathic effect (CPE) following challenge with 10^6 PFU of type 2 herpes virus in mice immunized with standard vaccine (—□—□—□—), vaccine at one-hundredfold dilution (—◇—◇—◇—), vaccine at one ten-thousandfold dilution (—△—△—△—) and control vaccine (—▲—▲—▲—)

Vaccine at 10^{-2} and 10^{-4} dosage resulted in intermediate levels of protection in terms of virus yields and to a lesser extent in terms of the proportion of mice with evidence of specific CPE (Table 1, Fig. 2) and with non-specific inflammatory response (Fig. 3). In terms of mortality, however, there was significant protection with only the type 1 vaccine at 10^{-2} dosage ($p \simeq 0.01$; Table 1).

Protection levels provided by virus antigen inactivated by methanol or acetone was not significantly different from formaldehyde-inactivated antigen (data not presented).

Duration of Immunity

Mice were protected for at least 2 years following vaccination. The percentage mortalities in mice immunised with standard type 1 and 2 vaccine were significantly less than in control mice ($p < 0.01$; Fig. 5). Virus yields from mice immunized with standard type 1 vaccine were significantly less than in control mice at all times ($p < 0.01$); however, with the standard type 2 vaccine, differences were significant at only 3, 6 and 12 months ($p < 0.05$). Minor degrees of protection were observed following vaccination with the lower vaccine dosages (Figs. 5 and 6).

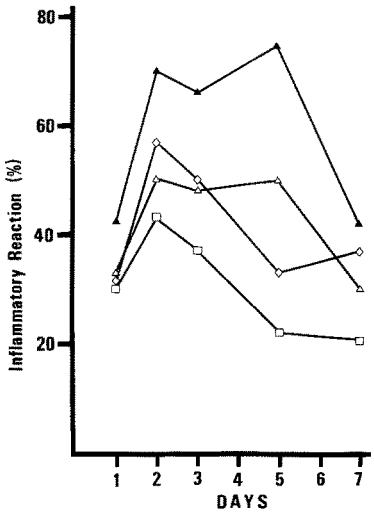

Fig. 3

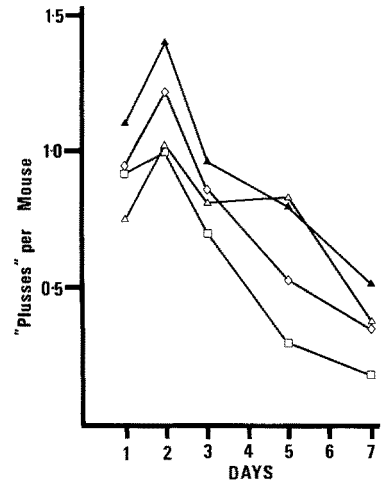

Fig. 4

Fig. 3. Prevalence (per cent) of mice with non-specific inflammatory reaction following challenge with 10^6 PFU of type 2 herpes virus in mice immunized with standard vaccine (—□—□—□—), vaccine at one-hundredfold dilution (—◇—◇—◇—), vaccine at one ten-thousandfold dilution (—△—△—△—) and control vaccine (—▲—▲—▲—)

Fig. 4. "Average extent" of local infection in mice challenged with 10^6 PFU of type 2 herpes virus in mice immunized with standard vaccine (—□—□—□—), vaccine at one-hundredfold dilution (—◇—◇—◇—), vaccine at one ten-thousandfold dilution (—△—△—△—) and control vaccine (—▲—▲—▲—)

Number of Immunisations

There was no evidence that three doses of any vaccine provided better protection than the usual regime of two vaccinations. Indeed, virus yields by the 7th day of infection were tenfold greater following three standard immunisations (Table 2). With one standard immunisation, while the level of protection was less than obtained with two standard immunisations, particularly in terms of average virus yields, it remained, on all criteria, significantly greater than in control mice (Table 2).

Age of Vaccination

The influence of age or, more specifically sexual maturity on the efficacy of vaccination was investigated by comparing the results with a group of 20 mice vaccinated in adulthood according to our usual immunization regime. There was

no evidence of improved protection; mortality was 38 per cent, 20 per cent of mice had evidence of specific viral CPE by the 7th day of infection and average virus yields were 3 PFU per mouse (cf. Tables 1—3).

Protection in Relation to Previous Vaccination and/or Type 2 Herpes Virus Genital Infection

Vaccinated and unvaccinated mice which had survived previous challenge with type 2 herpes virus were re-challenged. Vaccination was clearly the most important factor in determining the level of protection. Previous type 2 genital infection did not improve the degree of protection in vaccinated mice and offered little or no protection at all in unvaccinated mice (Table 3).

Neutralizing Antibody in Vaccinated Mice

Twenty four-week old mice were investigated for neutralizing antibody against herpes simplex viruses following two standard type 1 vaccinations. Every mouse developed neutralizing antibody with a mean k value of 0.35 ± 0.02 against type 1 virus and 0.19 ± 0.014 against type 2 virus.

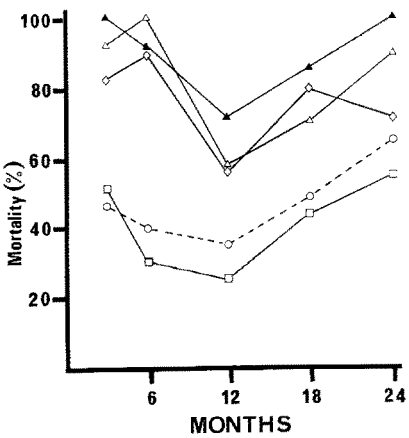

Fig. 5

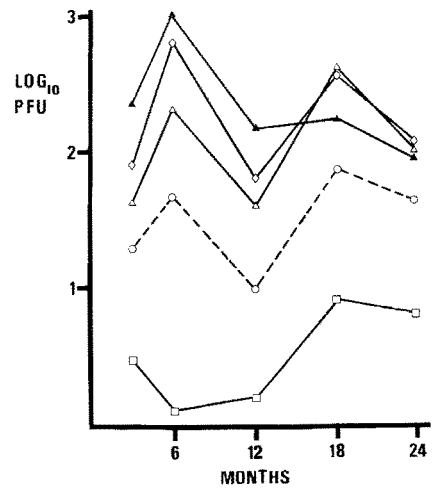

Fig. 6

Fig. 5. Mortality (percentage) in mice challenged at various time intervals with 10^6 PFU of type 2 herpes virus following immunization with standard type 1 vaccine (—□—□—□—), standard type 2 vaccine (– – o – – o – – o – –), vaccine at one-hundred-fold dilution (—◇—◇—◇—), vaccine at one ten-thousandfold dilution (—△—△—△—) and control vaccine (—▲—▲—▲—). Percentages at 3 months were calculated from groups of mice containing 40—60 animals and all other percentages from groups containing 20—30 animals

Fig. 6. Virus yields (PFU) per mouse following challenge at various time intervals with 10^6 PFU of type 2 herpes virus following immunization with standard type 1 vaccine (—□—□—□—), standard type 2 vaccine (– – o – – o – – o – –), vaccine at one-hundredfold dilution (—◇—◇—◇—), vaccine at one ten-thousandfold dilution —△—△—△—), and control vaccine (—▲—▲—▲—). Average virus yields at 3 months were calculated from groups of mice containing 40—60 animals and other virus yields from groups containing 20—30 animals

Table 2. *Protection of mice in relation to number of standard vaccinations*

	Number of mice	Mortality in provenly infected mice (%)	Specific cytopathic effect at 7th day (%)	Average virus yield per mouse at 7th day (PFU)
One immunization				
Standard	20	8/17 (47%)	47	1.94±0.21
Two immunizations				
Standard	105	27/94 (29%)	34	0.95±0.26
At 10^{-2} dilution	80	58/75 (76%)	42	1.92±0.24
At 10^{-4} dilution	80	59/74 (83%)	47	1.64±0.27
Three immunizations				
Standard	20	6/18 (33%)	40	1.96±0.16
At 10^{-2} dilution	20	15/18 (83%)	23	1.98±0.36
At 10^{-4} dilution	20	9/15 (60%)	29	1.69±0.33
Three immunizations with control vaccines	20	16/16 (100%)	88	2.82±0.44

Table 3. *Protection of mice in relation to previous vaccinations and/or type 2 genital infection*

	Number of mice	Mortality in provenly infected mice (%)	Specific cytopathic effect at 7th day (%)	Average virus yield per mouse at 7th day (PFU)
Mice with a history of:				
Standard vaccination only	28	4/25 (16%)	25	1.49±0.23
Standard vaccination followed by type 2 genital infection	60	5/50 (10%)	27	1.04±0.18
Type 2 genital infection only	13	10/13 (77%)	77	2.58±0.56
No previous vaccination or genital infection	12	11/12 (91%)	82	2.62±0.64

Discussion

The results indicate that mice can be protected from primary type 2 genital infection by pre-pubertal vaccination with a formalin-activated type 1 vaccine (Table 1, Figs. 1—4). This finding is generally consistent with sero-epidemiological studies by RAWLS *et al.* (19) and NAHMIAS *et al.* (13) in which pre-adolescent type 1 infection appeared to impart a measure of protection against primary type 2 genital infections. It was encouraging that protection, which in general terms is considered to be more short-lived with inactivated than live virus vaccines, continued until two years following vaccination (Figs. 5 and 6). Investigation of longer vaccination-challenge intervals was precluded by the natural average two-year life span of our strain of mice.

Vaccination with one-hundredfold and the ten-thousand-fold vaccine dosages, 10^4 and 10^2 infected cells equivalent respectively, reduced the degree of protection by all criteria (Table 1, Figs. 1—4). This was particularly apparent following long vaccination-challenge intervals where no significant protection was apparent with these lower vaccine dosages (Figs. 5 and 6). The dosage is clearly critical and, in the longer term, the vaccination of human subjects emphasises the necessity to investigate dose-protection relationships in larger animals, for example, primate species. Similar considerations obtain with respect to the number of vaccinations where there seemed, at least in our mouse experimental system, no indication to exceed two standard vaccinations (Table 2).

There is little doubt that type 1 standard vaccine offered a higher level of protection than type 2 standard vaccine (Tables 1 and 2, Fig. 6). While this is unexpected from the "direction" of the one-way cross-neutralisation reaction between type 1 and 2 herpes simplex virus (14, 15, 16), *in vitro* neutralization tests do not measure cytolytic or opsonic humoral antibody or indeed the variety of cell-mediated immune factors which may be of paramount importance in protection from herpetic infections (18, 6).

It was possibly surprising that vaccination conferred a significantly greater level of protection against challenge with type 2 virus than did a previous episode of homologous type 2 genital infection where local antibody factors might also contribute to protection against intravaginal virus challenge (Table 3, Figs. 5 and 6). While it is impossible to quantify the precise virus immunogenic dose to the mouse following contraction and recovery from a type 2 genital infection, it is probably less than is contained in two subcutaneous formaldehyde stabilised vaccinations each containing the antigenic content equivalent of 10^6 infected cells per mouse; indeed, it was quite clear that reduction of vaccine dosages by one-hundredfold dramatically reduced protection levels (Tables 1 and 3, Figs. 1—6). A second explanation merits speculation, namely, that establishment of latent ganglionic virus involvement following a live type 2 genital infection inhibits further immunological response to challenge with the (particularly) homologous virus on the basis that "familiarity breeds immunological contempt". This concept is presently under investigation.

The vaccine preparation investigated in this study is a "crude" virus antigen preparation and is not under consideration for vaccination of human subjects. However, the operative antigen stimulating protective immunity in human subjects is probably the monoprecipitin antigen "Band II" (24, 21) and this antigen will necessarily be represented in even the most refined prospective vaccine for human subjects against herpes simplex infections. On this account, as far as can be judged from experiments in a rodent species, pre-pubertal vaccination would seem a feasible undertaking and the development of an inactivated virus particle and virus DNA free sub-unit vaccine is presently under investigation (27, 23).

Acknowledgments

We wish to thank Professor J. Newton, Department of Obstetrics and Gynaecology, University of Birmingham, for generous provision of facilities, and Mr. Kim Ward for technical assistance in the preparation of cytologic material. Mr. A. Moles was in receipt of MRC Grant G 975/935.

References

1. Adam, E., Kaufman, R. H., Melnick, R. H., Levy, A. H., Rawls, W. E.: Seroepidemiologic studies of herpes virus type 2 and carcinoma of the cervix IV. Amer. J. Epidemiol. **98**, 77 (1974).
2. Anderson, W. A., Kilbourne, E. D.: Immunization of mice with inactivated herpes simplex virus. Proc. Soc. exp. Biol. Med. **107**, 518 (1961).
3. Asher, L. V. S., Walz, M. A., Notkins, A. L.: Effect of immunization on the development of latent ganglionic infection in mice challenged intravaginally with herpes simplex virus types 1 and 2. Am. J. Obstet. Gynecol. **131**, 788 (1978).
4. Benda, R., Dbaly, V.: Immunogenic properties of formalin herpes antigen prepared from cell cultures. J. Hyg., Epidemiol., Microbiol., Immunol. **17**, 237 (1973).
5. Duff, R., Rapp, F.: Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. Nature (New Biol.) **233**, 48 (1971).
6. Ennis, F. A.: Host defense mechanisms against herpes simplex virus. II. Protection conferred by sensitized spleen cells. J. inf. Dis. **127**, 632 (1973).
7. Geder, L., Skinner, G. R. B.: Differentiation between type 1 and type 2 strains of herpes simplex virus by an indirect immunofluorescent technique. J. gen. Virol. **12**, 179 (1971).
8. Kitces, E. N., Morahan, P. S., Tew, J. G., Murray, B. K.: Protection from oral herpes simplex virus infection by a nucleic acid-free virus vaccine. Infect. Immun. **16**, 955 (1977).
9. Lipchutz, B.: Untersuchungen über die Ätiologie der Krankheiten der Herpesgruppe. Arch. Derm. Syph. **136**, 428 (1921).
10. MacPherson, I., Stoker, M.: Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. Virology **16**, 147 (1962).
11. McKendall, R. R.: Efficacy of herpes simplex virus type 1 immunization in protecting against acute and latent infection by herpes simplex virus type 2 in mice. Infect. Immun. **16**, 717 (1977).
12. McNab, J. C. M.: Transformation of rat embryo cells by temperature sensitive mutants of herpes simplex virus. J. gen. Virol. **24**, 143 (1974).
13. Nahmias, A. J., Josey, W. E., Naib, Z. M., Lucé, C. F., Duffey, A.: Antibodies to *Herpesvirus hominis* types 1 and 2 in humans. I. Patients with genital infections. Amer. J. Epidemiol. **91**, 539 (1970).
14. Nahmias, A. J., Roizman, B.: Infection with herpes simplex viruses 1 and 2. N. Engl. J. Med. **289**, 719 (1973).
15. Pauls, F. P., Dowdle, W. R.: A serological study of *Herpesvirus hominis* strains by microneutralisation tests. J. Immunol. **98**, 941 (1967).
16. Plummer, G., Goodheart, C. R., Miyagi, M., Skinner, G. R. B., Thouless, M. E., Wildy, P.: Herpes simplex viruses: Discrimination of types and correlation between different characteristics. Virology **60**, 206 (1974).
17. Price, R. W., Walz, M. A., Wohlenberg, C., Notkins, A. L.: Latent infection of sensory ganglia with herpes simplex virus: efficacy of immunisation. Science **188**, 938 (1975).
18. Rager-Zisman, B., Allison, A. C.: Mechanism of immunologic resistance to herpes simplex virus 1 (HSV-1) infection. J. Immunol. **116**, 35 (1976).
19. Rawls, W. E., Gardner, H. L., Flanders, R. W., Lowry, S. P., Kaufman, R. H., Melnick, J. L.: Genital herpes in two social groups. Amer. J. Obstet. Gynecol. **110**, 682 (1971).
20. Russell, W. C.: A sensitive and precise plaque assay for herpes virus. Nature **195**, 1028 (1962).
21. Sim, C., Watson, D. H.: The role of type-specific and cross-reacting structural antigens in the neutralisation of herpes simplex virus types 1 and 2. J. gen. Virol. **19**, 217 (1973).
22. Skinner, G. R. B.: Transformation of primary hamster embryo fibroblasts by type 2 simplex virus: evidence for a 'hit-and-run' mechanism. Brit. J. exp. Pathol. **57**, 361 (1976).

23. Skinner, G. R. B., Hartley, C. E., Moles, A. W., Williams, D. R., Buchan, A.: The preparation, efficacy and safety of a herpes simplex vaccine for human subjects. (In preparation.)
24. Skinner, G. R. B., Taylor, J., Edwards, J.: Precipitating antibodies to herpes simplex virus in human sera: a prevalence of antibody to common antigen (Band II). Intervirology **4**, 320 (1975).
25. Skinner, G. R. B., Thouless, M. E., Edwards, J., Trueman, S., Gibbs, A. J.: Sero-relatedness of type 1 and type 2 herpes simplex virus; type-specificity of antibody response. Immunology **31**, 481 (1976).
26. Skinner, G. R. B., Whitney, J. E., Hartley, C.: Prevalence of type-specific antibody against type 1 and type 2 herpes simplex virus in women with abnormal cervical cytology; evidence towards pre-pubertal vaccination of sero-negative female subjects. Arch. Virol. **54**, 211 (1977).
27. Skinner, G. R. B., Williams, D. R., Buchan, A., Whitney, J., Harding, M., Bodfish, K.: Preparation and efficacy of an inactivated sub-unit vaccine (NFU BHK) against type 2 herpes simplex virus infection. Med. Microbiol. Immunol. **166**, 119 (1978).
28. Vantsis, J. F., Wildy, P.: Interaction of herpes virus and HeLa cells: comparison of cell killing and infective centre formation. Virology **17**, 225 (1962).
29. Walz, M. A., Price, R. W., Hayashi, K. *et al.*: Effect of immunization on acute and latent infection of vagino-uterine tissue with herpes simplex virus types 1 and 2. J. inf. Dis. **135**, 744 (1977).
30. Wildy, P.: Recombination with herpes simplex virus. J. gen. Microbiol. **13**, 340 (1955).
31. Williams, D. R., Whitney, J., Harding, M., Bodfish, K., Skinner, G. R. B.: Cytological evaluation of experimental type 2 herpes simplex infection in mice. Acta Cytologica **22**, 41 (1978).

Authors' address: Dr. G. R. B. Skinner, Department of Medical Microbiology, The Medical School, Birmingham B15 2TJ, England.

Received October 22, 1979