Immunology 1976 **31** 481

Serological relatedness of herpes simplex viruses

TYPE-SPECIFICITY OF ANTIBODY RESPONSE

G. R. B. SKINNER, M. E. THOULESS, S. TRUEMAN, J. EDWARDS & A. J. GIBBS *Department of Virology, Medical School, University of Birmingham, Birmingham*

Received 19 *January* 1976; *accepted for publication* 11 *March* 1976

Summary. The serological relatedness of forty-seven strains of type 1 and type 2 herpes simplex virus was investigated by reciprocal and non-reciprocal neutralization kinetics. Early rabbit antisera divided the virus strains into two distinct groups where confident identification of virus type was possible. Hyperimmune mouse and rabbit antisera did not divide the two virus types into distinct non-overlapping groups. The extent of overlap varied with the particular attribute of the virus being studied. The virus types were best discriminated by their neutralizability by type 1 antisera and least well by their neutralizability by type 2 antisera. The results of reciprocal kinetic neutralization tests with hyperimmune mouse antisera were analysed by multidimensional cluster analysis.

Hyperimmune mouse or rabbit antisera could not be discriminated with respect to their immunogenic type by their absolute neutralization rate constants against either type 1 or type 2 virus, but could be distinguished on a group basis by their relative neutralizability against both virus types (antiserum specificity attribute); however, using this latter criterion, the type of immunogen could only be predicted in seven of the forty antisera under test. 'Early' mouse antisera could also be distinguished as groups by their absolute k-values against type 1 herpes virus. Thus, immunogenic identification, on other than a group basis, was unreliable. The specificity of a given serum was inversely related to its titre.

There was a positive correlation between the specificity of a given virus strain and of its corresponding antiserum.

Correspondence: Dr G. R. B. Skinner, Department of Virology, The Medical School, University of Birmingham, Birmingham B15 2TJ.

INTRODUCTION

Herpes simplex viruses can be divided into two distinct groups by a number of criteria; type 1 strains are usually isolated from oral, pharyngeal, conjunctival or upper limb lesions and type 2 strains from the genitalia or lower limbs. The virus types can be discriminated by plaque morphology (Schneweis, 1962; Munk & Donner, 1963), efficiency of plaquing in chick embryo fibroblasts (Figueroa & Rawls, 1969), pock morphology (Parker & Banatvala, 1968), thermorestriction of virus replication at temperatures above 39° (Ratcliffe, 1971) and thermostability of virus-induced thymidine kinase enzyme activity (Thouless & Skinner, 1971). Moreover, the densities of the DNA of the two virus types differ by 0·002 g/cm^3 (Goodheart, Plummer & Waner, 1968; Kieff, Bachenheimer & Roizman, 1971; Graham, Ludwig, Bronson, Benyesh-Melnick &

Biswal, 1972) with only 40% sequence homology as judged by DNA–RNA hybridization on nitrocellulose membrane filters (Bronson, Graham, Ludwig, Benyesh-Melnick & Biswal, 1972).

While there is general agreement that the virus types are serologically distinct but related, whether tested by immunodiffusion (Thouless, 1972), complement-fixation (Martin, Palmer & Kissling, 1972) or neutralization of infectivity (Schneweis, 1962), there is some disagreement over the precise degree of relatedness, Shubladze & Chzhu-Shan (1959) reporting complete antigenic distinctiveness, whereas infectivity neutralization tests suggest that the viruses are serologically related with asymmetric cross-reactivity (Schneweis, 1962; Plummer, 1964; Pauls & Dowdle, 1967).

In most studies, antisera have been prepared against one, perhaps two, well-established prototype type 1 or type 2 virus strains, and a number of isolates—sometimes a large number—(Dowdle, Nahmias, Harwell & Pauls, 1967) have been tested, usually by neutralization against these 'prototype' antisera. There are two theoretical objections to this approach.

(1) The results are inherently biased towards greater type-specificity by the selection of prototype virus strains for the preparation of antisera.

(2) The procedure assumes a 'unidimensional relatedness' between the items under investigation; in other words, it assumes that sero-groupings obtained with a given antiserum will coincide with groupings obtained on testing against antisera raised against every other virus strain in the group; thus, the greater the proportion of virus strains represented by their homologous antisera, the less likely are spurious clusterings of over-distinctiveness.

It is scarcely surprising that antibody typing—a question of paramount importance in the evaluation of sero-epidemiological studies—is even more problematical than virus typing. First, similar considerations as outlined above equally apply when only one, or perhaps two, prototype virus strains are used in the neutralization tests. A second problem concerns the interpretation of data where, in contrast to virus typing, the sero-distributions cannot be compared to independently derived biological or biochemical data (Plummer, Goodheart, Miyagi, Skinner, Thouless & Wildy, 1974). Thus, the reliability of antibody type prediction can only be assessed by less certain and more indirect methods, such as comparison of data derived from (a) immunization of experimental animals with virus of known 'type' (Nahmias, Josey, Naib, Luce & Duffy, 1970; Aurelian, Royston & Davis, 1970) or from (b) human populations that have probably been exposed to only one virus type, *e.g.* type 1 virus in children (Rawls, Tompkins & Melnick, 1969). The reverse situation, namely, a defined population where one can be reasonably confident of a negative history of type 1 herpes virus infection, is less easily obtained. A further difficulty in interpreting sero-epidemiological surveys is that most human sera, if they have antibody at all, will neutralize both virus types to a varying extent. Thus, on the evidence of immune animal sera or defined human sera, various workers have attempted to overcome this problem by defining various critical ratios of the relative type 1/type 2 neutralizing antibody (Nahmias *et al*, 1970; Rawls, Iwamoto, Adam & Melnick, 1970) and judging previous type exposure in terms of these ratios.

We have previously expressed reservation over the uncritical adoption of such ratios as indicators of immunogenic type (Skinner, Taylor & Edwards, 1974); we were concerned that, as both types of virus particles possess both type-common and type-specific antigenic determinants, such ratio indices will be influenced not only by the presence or absence of previous exposure to these viruses, but also by the relative magnitude of the immune response to each virus type.

In this study, the discrimination of virus types as individuals and as groups was investigated by reciprocal neutralization kinetics using forty-one hyperimmune and six 'early' antisera prepared in mice. The results were compared with the type discrimination obtained using random pairs of hyperimmune mouse antisera and the type-discrimination obtained using a pair of hyperimmune and 'early' antisera prepared in rabbits. This situation obtains in most studies of the serological relatedness of herpes simplex viruses. To permit easy comparison of the virus strains and their antisera, five arbitrary measurements of serological behaviour have been defined. In addition, the results of the serological tests were ordinated using Gower's Principal Coordinates method of analysis (Gower, 1966) and the clusterings obtained investigated.

The type-discrimination of the corresponding antisera was similarly investigated; in particular, the reliability of immunogenic type-prediction of individual antisera and groups of antisera was considered in terms of the various attributes of antiserum beha-

viour (as defined in the Materials and Methods section). In addition, the influence of hyperimmunity on antiserum specificity was investigated for hyperimmune mouse antisera and compared with results of 'early' non-hyperimmune antisera.

MATERIALS AND METHODS

Cell culture

BHK 21 (C13) cells, a stable cell line derived from a single clone (MacPherson & Stoker, 1962), were used for virus propagation, antigen production and virus titrations. RK 13 cells (Beale, Christofinis & Furminger, 1963) were used to prepare antigen for immunizing rabbits. Cell cultures were routinely checked for bacterial, fungal or mycoplasmal contamination.

Viruses—virus strains

The virus strains, their passage history, site of isolation and virus type according to a number of independent criteria, have been previously indicated (Plummer *et al.*, 1974). Ten strains were recently isolated and thirty-three strains, also recently isolated, were kindly donated from other virus laboratories. Several strains were 'classical' laboratory viruses: Lovelace ('LOV'), obtained from Dr A. H. Nahmias; 'HFEM', a derivative of the Rockefeller strain 'HF' (Wildy, 1955); 'MP', the macroplaque strain of Hoggan & Roizman (1959); 'Nash', isolated from labial lesions in 1958 (Bedson & Gostling, 1958); 'PDK' and a thymidine kinaseless mutant termed 'MDK' in this study (Dubbs & Kit, 1964).

The virus isolates were not routinely cloned before testing or before inoculation into animals for antisera preparation. Virus infectivity was titrated by the suspension method of Russell (1962).

Neutralization tests

Sera were heated to 56° for 30 min to inactivate complement. 'Early' sera were diluted 1 in 10 in saline (0·15 M NaCl) and hyperimmune sera 1 in 30 in saline and tested in a kinetic neutralization test at room temperature. The serum dilution was mixed with an equal volume of virus suspension containing 4×10^4 plaque-forming units (p.f.u.) of virus and samples taken at 15 and 30 min, diluted 1 in 100 and assayed on BHK 21 cells by the plaque method of Russell (1962). The k-value for each virus was calculated according to the formula:

$$k = \frac{2{\cdot}3}{ct} \log\left(\frac{V_0}{V_t}\right)$$

where c = volume of antiserum in test mixture divided by total volume of test mixture; t = time in minutes; V_0 = initial virus concentration (plaque-forming units); V_t = virus concentration at time 't'.

The discrimination of virus strains was considered in terms of the following five measurements: (1) mean k-value of a strain against all antisera (VI) raised by type 1 virus inoculation; (2) mean k-value of a strain against all antisera (V2) raised by type 2 virus inoculation; (3) 'mean specificity'*, i.e. the mean k-value of a strain against all type 1 antisera divided by the mean k-value of this strain against all type 2 antisera (VS); for brevity the term 'specificity' is used instead of 'mean specificity'. throughout this text; (4) virus 'inherent neutralizability', i.e. the sum of the mean k-value of a given virus strain against all type 1 antisera plus the mean k-value for this strain against all type 2 antisera (VN), all divided by a factor of 2, and (5) Archetti–Horsfall criterion of antigenic relatedness (Archetti & Horsfall, 1950) which is a square root measure of the ratio of the relative specificity of two virus strains, viz:

$$\text{'AH'} = \sqrt{\frac{\text{VS for type 2 strains}}{\text{VS for type 1 strains}}} \times 100.$$

Antisera were analysed in similar fashion where, of course, 'antisera' should replace 'virus strain' and *vice versa* in the above description. The corresponding attribute suffixes are respectively A1, A2, AS, and AN and AH; the latter is, of course, identical to the corresponding value for virus strains for this criterion. Attribute AN is referred to an 'antiserum neutralizing potency'. It should be noted that the attribute 'mean specificity' is the mean of the individual VS values and not the ratio of the mean V1 and V2 values which is a different function, viz:

$$\underset{\text{(VS)}}{\frac{\frac{V1}{V2}}{n}} \text{ for n items} \neq \frac{\text{V1 for n items}/_n}{\text{V2 for n items}/_n}$$

* The term 'specificity' is really a misnomer and is, in a sense, only applicable to strains whose mean k-value corresponds with the numerator in the V1/V2 ratio, i.e. type 1 strains in our system. For type 2 strains, this ratio is really a measure of 'non-specificity' or 'unspecificity', as the ratio will clearly decrease as the strains or group of strains became more efficiently neutralized by type 2 antisera, (V2), the denominator.

where n = number of isolates or antisera under consideration.

Similar considerations apply to the specificity values for antisera.

The test error of each attribute was investigated by duplicate reciprocal testing of a randomly selected type 1 and type 2 virus strain against a randomly selected type 1 and type 2 antiserum. The reproducibility of the results was examined by conducting each test on a different day, using different virus suspensions of the same virus strain and independently prepared 'dilutions' of the same antiserum and enjoining the assistance of other personnel.

The variation coefficients were approximately 20% for each attribute with the exception of the AH criterion which was significantly lower (7·4%) ($P < 0.05$). Thus, the latter appeared a reproducible index of sero-relatedness between viruses or antisera.

Preparation of antisera

Virus antigen for immunization of mice. BHK 21 cells at a concentration of 5×10^7 cells/ml were infected in suspension with 10 to 20 p.f.u. per cell. Virus adsorption was continued for 1 h at 37° after which the infected cells were dispensed into a 500 ml bottle and incubated at 37° for 24 h. The cells were removed from the glass, resuspended in an appropriate volume of supernatant medium, disrupted ultrasonically and stored at −70° in 1-ml amounts.

Immunization schedules

(1) *Early mouse antisera.* Adult inbred mice were inoculated subcutaneously with a dose of antigen diluted in saline to contain 10^4 p.f.u. of virus. The procedure was repeated after 14 days and sera were withdrawn by cardiopuncture after a further 10 days. Five mice were given the same virus inoculations and their sera pooled.

(2) *Hyperimmune mouse antisera.* One millilitre of diluted antigen (in saline), containing 10^7 p.f.u., was inactivated by addition of 0·1 ml of 4% formaldehyde and incubated for 1 h at 37°. It was established that this procedure destroyed all virus infectivity. Groups of five mice were first inoculated subcutaneously with 0·2 ml of this formalized virus preparation and thereafter were given a course of five inoculations at 3-weekly intervals, with an equivalent dose of unformalized virus antigen. Sera were removed from the five mice 10 days after the last inoculation and pooled.

As the mice were inoculated with virus given in baby hamster kidney cells, the antisera contained anti-BHK and anti-calf serum reactivity; this was demonstrated by gel diffusion and complement fixation. However, equivalent doses of uninfected BHK cell extract did not stimulate any virus neutralizing antibody activity in the immune animal's serum.

Virus antigen for immunization of rabbits. This was prepared in rabbit kidney cells as described by Watson *et al.* (1966). Prior to infection of these cells, the virus was passaged twice in rabbit kidney cells at low multiplicity. The cells were maintained in medium containing rabbit serum instead of calf serum and the infected cells were washed with PBS before ultrasonic disruption and freeze-drying.

(1) *'Early' rabbit antisera.* Rabbits were immunized according to the schedule as described for 'early' mouse antisera; in this case, however, the dosage was increased to 10^6 p.f.u. of virus per immunization.

(2) *'Late' rabbit antisera.* Rabbits were injected intramuscularly with 100 mg of freeze-dried formalin-inactivated antigen and then injected six times at monthly intervals with equivalent doses of 'live' antigen. Thereafter, the rabbits were given booster immunizations at three-monthly intervals. All immunizations were given with Freund's incomplete adjuvant. Rabbits were bled 14 days following each booster immunization.

Statistical methods

Where numerical data were consistent with a normal or Gaussian distribution, mean values were compared by Student's *t*-test or by analysis of variance. The testing errors between attributes (*vide supra* p 4) were compared by calculating the standard error of the difference between coefficients of variation.

The correlation between two sets of values was calculated as the 'product moment correlation coefficient' or '*r*' value and the significance of the '*r*' value judged by Student's *t*-test.

Probability levels of greater than $P = 0.05$ were considered not significant.

Cluster analysis

The relatedness of each strain to every other strain was calculated from the k values and expressed as a 'similarity coefficient' which might be considered as units of similarity or distances in multidimensional space; these distances were then statistically sorted into the fewest most representative dimensions. In

Table 1. Discrimination of groups of virus strains: correlation of attributes

	Number of strains tested	Type 1 antisera (V1)	Type 2 antisera (V2)	Specificity (VS)	Neutralizability (VN)	AH	Correlation of attributes r (*P*) (V1 *vs* V2)
Hyperimmune mouse antisera							
Type 1 strains	21	4·56(±0·20)	4·29(±0·21)	1·08(±0·06)	4·25(±0·19)		+0·50($P<0·05$)
Type 2 strains	20	2·58(±0·14)	2·79(±0·17)	0·91(±0·04)	2·67(±0·16)		+0·73($P<0·001$)
Index ratio		1·77	1·54	1·19	1·58		
Significance of difference in means		<0·0001	n.s.	<0·01	<0·0001	92	
Distribution overlap*		9/44	26/40	36/40	19/44		
'Early' non-hyperimmune mouse antisera							
Type 1 strains	3	0·35(±0·08)	0·09(±0·01)	3·81(±1·4)	0·22(±0·02)		n.d.
Type 2 strains	3	0·21(±0·07)	0·16(±0·09)	1·60(±0·56)	0·18(±0·01)		n.d.
Index ratio		1·66	0·56	2·38	1·22		
Significance of difference in means		n.s.	n.s.	n.s.	n.s.	57	
Hyperimmune rabbit antisera							
Type 1 strains	20	1·92(±0·17)	0·32(±0·04)	7·6(±0·81)	1·12(±0·09)		+0·6 ($P<0·001$)
Type 2 strains	17	0·88(±0·09)	0·48(±0·05)	2·1(±0·2)	0·68(±0·05)		+0·52($P\sim0·05$)
Index ratio		2·18	0·66	3·62	1·65		
Significance of difference in means		<0·001	<0·01	<0·001	<0·001	55	
Distribution overlap*		23/37	33/37	11/37	22/37		
'Early' rabbit antisera							
Type 1 strains	17	0·74(±0·02)	0·23(±0·03)	2·31(±0·2)	0·55(±0·03)		+0·7 (<0·01)
Type 2 strains	13	0·31(±0·04)	0·55(±0·05)	0·48(±0·05)	0·46(±0·05)		+0·91(<0·001)
Index ratio		2·39	0·42	4·81	1·20		
Significance of difference in means		<0·0001	<0·001	<0·0001	n.s.	42	
Distribution overlap*		0/30	21/30	5/30	23/30		

n.s. = Not significant; n.d. = not determined.
The results are expressed as mean *k* values (standard error in brackets).
* Number of type 1 and type 2 strains lying within 2 s.d. of heterotypic mean.

analyses of our data, over 90% of the information was contained in the first, second and third dimensions or vectors (76·4%, 11·1% and 5·9%, respectively) which thus permitted a three-dimensional pictorial representation of the relatedness of the strains under investigation.

RESULTS

Reciprocal kinetics of neutralization

Hyperimmune antisera prepared in mice

The discrimination of the virus types was assessed for groups and individuals by the five attributes of serological relatedness defined in the Materials and Methods section.

Type 1 strains were most powerfully differentiated from type 2 strains by their neutralizability by type 1 antisera (VI) (Table 1). The difference in the two means, 4·56 for type 1 isolates and 2·58 for type 2 isolates, was significant ($P<0·0001$) (Table 1). However, as Fig. 1 shows, five type 1 isolates lay within 2 s.d. from the mean of the type 2 isolates and four type 2 isolates lay within 2 s.d. from the mean of

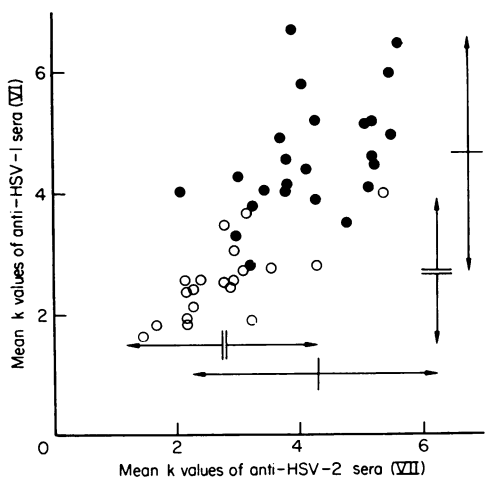

Figure 1. Distribution of k values of virus strains with hyperimmune mouse antisera. (●) Type 1 virus strains; (○) type 2 virus strains; (—) mean k values for type 1 virus strains; (=) mean k values for type 2 virus strains; (↕) denotes 2 s.d.

the type 1 isolates. In other words, for nine of the forty-one isolates, one could not be confident of correct identification by this criterion alone.

Strains were not correctly 'typed' by type 2 antisera (V2) (Fig. 1) and, as will be discussed later, the difference in the means, 4·29 and 2·79, which was, of course, the reverse of what one would have expected in a homologous versus heterologous virus antisera system, could be attributed to the increased inherent neutralizability of the type 1 isolates. This point will be dealt with more fully later.

On the criterion of specificity the virus types have remarkably similar mean values, viz. 1·08 and 0·91 for type 1 and type 2 isolates, respectively (Table 1). However, the scatter of values for this attribute was small and the mean values were thus significantly different ($P<0·01$). The virus distributions showed considerable overlap with not one type 2 strain lying more than 2 s.d. from the mean of the type 1 strains, and only five type 1 strains lying more than 2 s.d. from the mean of the type 2 strains. The relative specificity index (AH criterion) was 92%.

Cluster analysis

Using the k value of the reactions of individual strains against different antisera as attributes, forty-seven of the strains were ordinated by the principal co-ordinates method (Gower, 1966, 1967); the strains did not form two distinct clusters but formed two rather indistinct zones, one zone occupied predominantly by type 1 strains and the other by type 2 strains. There was considerable overlap, some type 1 strains being closer to type 2 strains than other type 1 strains. Consistent with the distributions shown in Figs 1–8, the type 2 virus strains formed a more close-knit group than the type 1 strains.

'Early' antisera prepared in mice

Equal discrimination was achieved by the attributes of specificity (VS) and neutralizability by type 1 antisera (V1) (Table 1) although the difference in mean values for both attributes was not significant. As with late antisera, type 1 virus strains were more readily neutralized than type 2 virus strains (attribute VN) but the difference between types was not significant. However, in contrast to the hyperimmune antisera, the early type 2 antisera neutralized the type 2 isolates better than the type 1 isolates, although the difference in mean values, 0·16 and 0·09, respectively, was again not significant. The relative specificity index (AH criterion) for early mouse antisera was 57%.

Non-reciprocal kinetics of neutralization

Discrimination of strains using random pairs of hyperimmune antisera prepared in mice

The values shown in Table 1 are, of course, com-

Table 2. Archetti–Horsfall (AH) criterion for random virus pairs

Intra-type 1 virus pairs		Intra-type 2 virus pairs		Inter-type 1-type 2 virus pairs	
1716–S3	106	3889–2219	80	894–2248	86
HFEM–894	170	7427–7514	81	JA–2219	85
5516–MS	223	484–25766	81	64179–8065	63
2830–Nash	128	8065–Bry	107	Duffy–Par	78
S2–S4	111	2248–2037	76	MS–1034	91
JA–64179	95	2a–Fay	191	HF–3345	104
Duffy–Wal	64	3345–Par	78	Hil–2037	110
2913–5007	81	1034–LOV	115	1716–Par	136
Hil–Kir	101	5289–E304	98	5516–3889	91
Bil–Kan	75	E294–E194	103	Nash–E194	91
Mean value	114·9		101·1		93·5
s.e.	±15·2		±10·9		±5·56
Coefficient of variation (%)	39·6		32·1		17·7

pound means and thus embrace the variance of the values generating each mean. It is possible therefore that high levels of discrimination might obtain with certain pairs of antisera and this was investigated by two analyses.

(a) The relative specificity index (AH criterion) was calculated for random heterologous and homologous virus–antiserum pairs for the hyperimmune mouse antisera data (Table 2).

It is clear that there were no notably low AH values (i.e. good discrimination), the lowest—63% for inter-type virus pair 64179–8065 being comparable to the intra-type value of 64% for virus Duffy–Wal.

(b) The distribution of the virus strains according to their neutralization by randomly chosen pairs of hyperimmune mouse antisera was investigated (Fig. 2a, b, c). Comparison of these distributions with the distributions of virus isolates using their mean behaviour against all the antisera (Fig. 1) indicates that, for these selected antisera pairs at least, the degree of type-distinctiveness was not improved. It appeared, therefore, that analysis of discrimination in terms of grand mean values was not obscuring unsuspected more clear-cut differences. To investigate whether these observations would obtain with antisera raised in another animal species,

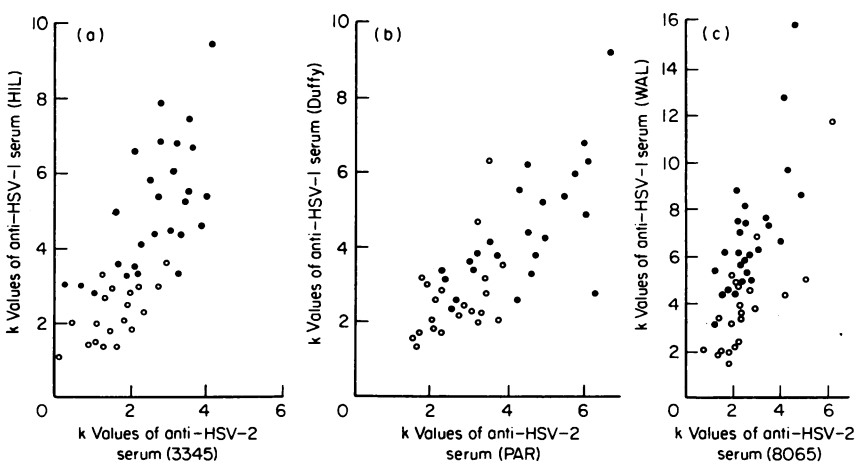

Figure 2. Distribution of k values of virus strains on testing with three pairs of hyperimmune mouse antisera. (●) Type 1 virus strains; (○) type 2 virus strains.

the isolates were tested against hyperimmune and early antisera prepared in rabbits against strain HFEM (type 1) and strain 3345 (type 2). These viruses are well-established prototype laboratory strains.

Hyperimmune antisera prepared in rabbits

The virus types were most powerfully discriminated by their inherent neutralizability (VN) and neutralizability by type 1 antisera (V1), the mean values for these attributes differing between virus types with a probability of $P < 0{\cdot}001$ (Table 1). The virus types were also powerfully discriminated by their specificity ($P < 0{\cdot}001$) and, of particular interest, were also discriminated by their neutralizability by type 2 antisera ($P < 0{\cdot}01$). This was the only hyperimmune antisera system in which the latter attribute distinguished the virus types. The AH value for this single antisera–virus group system was 55% which was considerably lower than the overall value for the hyperimmune mouse sera system (92%) and lower than any value derived from a single pair of virus-antiserum comparisons (Table 2). True AH values were not calculable in this system where only one type 1 and one type 2 antiserum were represented.

The distributions of the virus strains, irrespective of which criterion was investigated, showed considerable overlap (Figs 3 and 4). This was least apparent with the attribute of specificity (VS) and most apparent with attribute V2 (Figs 3 and 4, Table 1). Thus, the virus types could be discriminated as groups

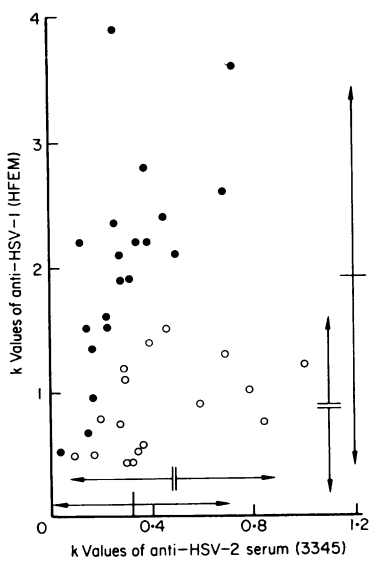

Figure 3. Distribution of k values of virus strains with hyperimmune rabbit antisera. (●) Type 1 virus strains; (○) type 2 virus strains; (—) mean k values for type 1 virus strains; (=) mean k values for type 2 virus strains; (↕) denotes 2 s.d.

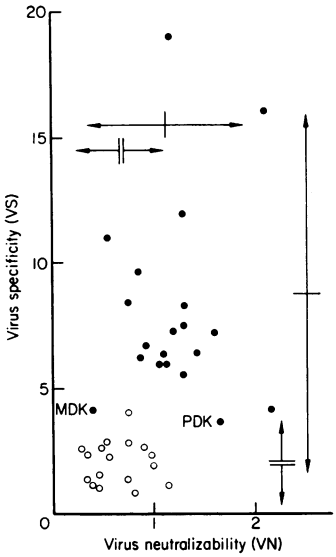

Figure 4. Distribution of VS and VN values for virus strains with hyperimmune rabbit antisera. (●) Type 1 virus strains; (○) type 2 virus strains; (—) mean value for type 1 virus strains; (=) mean value for type 2 virus strains; (↕) denotes 2 s.d. Strain MDK is the thymidine kinaseless mutant of type 1 herpes simplex virus strain PDK (Dubbs & Kit, 1964).

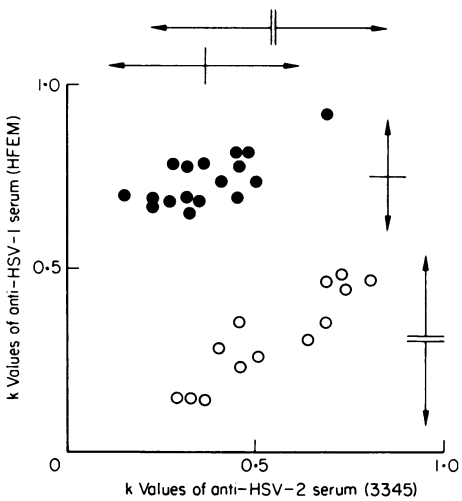

Figure 5. Distribution of k values of virus strains with early rabbit antisera. (●) Type 1 virus strains; (○) type 2 virus strains; (—) mean value for type 1 virus strains; (=) mean value for type 2 virus strains; (↕) Denotes 2 s.d.

by all four criteria, at least with this pair of hyperimmune antisera.

Early antisera prepared in rabbits

Thirty virus isolates were tested against early type 1 and type 2 rabbit antisera (Table 1, Fig. 5). Maximum discrimination of virus types was obtained with this system. The relative discriminatory value of each attribute reflected the findings with late hyperimmune rabbit antisera; they were most powerfully discriminated, respectively, by their neutralizability by type 1 antisera (V1), and by their specificity, by their neutralizability by type 2 antisera (V2) (Table 1). The virus types were not discriminated by their inherent neutralizability (VN).

In summary, it is clear from Table 1 that with hyperimmune antisera the viruses were best discriminated by attributes V1, VN and VS while with early antisera discrimination was most clear-cut with attributes V1, VS and V2; only with hyperimmune rabbit antisera were the viruses significantly discriminated as groups by all four attributes.

Correlation of attributes

In Figs 1, 3 and 5, and Table 1, the mean k value of each strain against type 1 antisera (V1) is compared with the corresponding k value against type 2 antisera (V2). Irrespective of the virus–antiserum system under investigation, there was a significant positive correlation between these attributes for both virus types.

There was no correlation between the specificity (VS) and neutralizability (VN) for type 1 or type 2 virus strains.

Discrimination of antisera

Hyperimmune antisera

The behaviour of groups of antisera and individual antisera in terms of attributes A1, A2, AS and AN is shown in Table 3 and Figs 6 and 7. Neither type 1 nor type 2 antisera could be distinguished by their mean behaviour against the homologous virus strains and could only be distinguished as significantly different groups when considered in terms of their specificity (AS) ($P < 0{\cdot}001$). Thus antisera discrimination, even on a group basis, required simultaneous testing against both type 1 and type 2 virus strains. The distribution of antisera in terms of their specificity is displayed for both type 1 and type 2 antisera on the vertical axis of Fig. 7. While certain members of each group seemed quite distinct, the distributions clearly overlap, with seven of the antisera lying outwith two standard deviations of the heterotypic

Table 3. Discrimination of antisera: correlation of attributes

	Number of antisera tested	Type 1 strains (A1)	Type 2 strains (A2)	Specificity (AS)	Neutralizing titre (AN)
Hyperimmune mouse antisera					
Type 1 antisera	20	4·56(±0·22)	2·58(±0·14)	1·86(±0·07)	3·57(±0·31)
Type 2 antisera	20	4·29(±0·21)	2·79(±0·17)	1·45(±0·07)	3·54(±0·34)
Index ratio		1·06	0·92	1·28	1·02
Significance of difference in means	(p)	n.s.	n.s.	<0·001	n.s.
Distribution overlap		32/40	37/40	33/40	40/40
'Early' non hyperimmune mouse antisera					
Type 1 antisera	3	0·35(±0·08)	0·21(±0·07)	2·10(±0·34)	0·28(±0·15)
Type 2 antisera	3	0·09(±0·01)	0·16(±0·09)	0·68(±0·17)	0·12(±0·06)
Index ratio	3	3·89	1·31	3·39	2·33
Significance of difference in means		<0·01	n.s.	<0·01	n.s.

n.s. = Not significant.

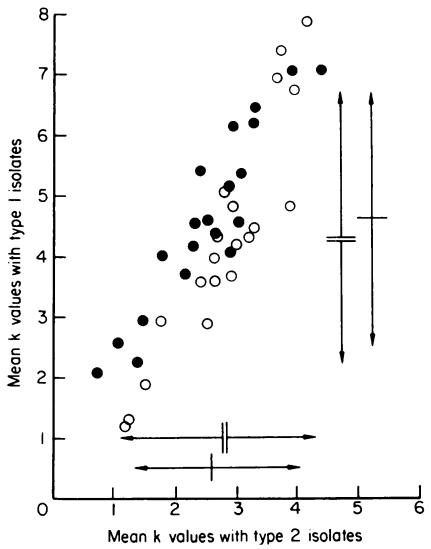

Figure 6. Distribution of k values of hyperimmune mouse antisera. (●) Type 1 antisera; (○) type 2 antisera; (—) mean k values for type 1 antisera; (=) mean k values for type 2 antisera; (↕) denotes 2 s.d.

mean. One can thus predict, with 95% confidence, that an unknown serum with a specificity value of under 1·2 has been raised in response to a type 2 virus strain, and that a specificity value of over 2·1 represents a serum that has been raised in response to a type 1 virus strain. However, it is salutary that only seven of the forty antisera could be thus identified. On these data, therefore, prediction of the immunizing virus type of a given hyperimmune antiserum may be unwise while group comparisons may be possible.

The neutralization titre of both groups of antisera was remarkably similar (AN), 3·57 versus 3·54, and is of no discriminatory value.

Cluster analysis of antisera

An ordination (Gower, 1966, 1967) of the relatedness of the antisera indicated that, while there were no distinct clusters, antisera raised in response to type 1 strains clustered around a different centre of gravity than antisera raised in response to type 2 strains. However, the degree of overlap was such that, while group comparisons may be possible, prediction of the immunizing virus type for a single individual antiserum was clearly impossible.

Early antisera

Consistent with the data for hyperimmune antisera, early type 1 antisera were most clearly discriminated from type 2 antisera by the attribute of specificity (AS) (Table 3), the AS value for the former being more than thrice the value of the latter ($P = <0{\cdot}01$). In addition, the early type 1 and type 2 antisera could also be discriminated by their neutralizing activity against type 1 virus isolates (attribute A1, $P = <0{\cdot}01$). The antisera could not be discriminated by their neutralizing activity against type 2 isolates (attribute A2) or by their neutralizing titre (attribute AN).

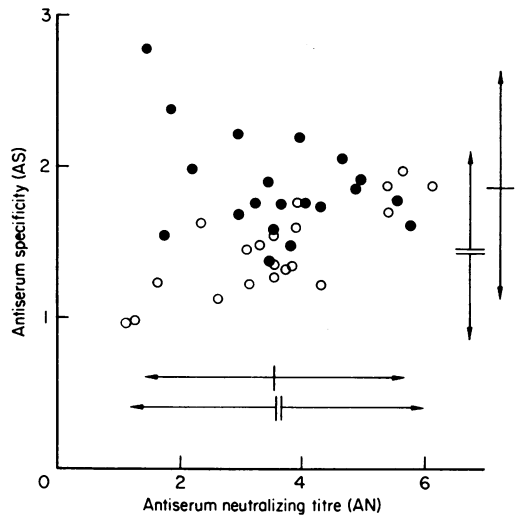

Figure 7. Distribution of AS and AN values for hyperimmune mouse antisera. (●) Type 1 antisera; (○) type 2 antisera; (—) mean value for type 1 antisera; (=) mean value for type 2 antisera; (↕) denotes 2 s.d.

Correlation of attributes; influence of antiserum titre

In Fig. 6 the mean k value of each hyperimmune mouse antiserum against all type 1 strains is compared with the mean k-value of that antiserum against all the type 2 strains. Both type 1 and type 2 antisera correlate to a high degree ($r = +0{\cdot}86$ and 0·85, respectively; $P = <0{\cdot}001$).

In Fig. 7 the specificity (AS) of each hyperimmune mouse antiserum is compared with its neutralizing titre (AN). For type 1 antisera the attributes correlated negatively but to an insignificant extent ($r = -0{\cdot}23$); with type 2 antisera the attributes positively correlated ($r = +0{\cdot}80$; $P = <0{\cdot}001$). These observations are not inconsistent as this latter correlation is essentially a negative correlation, the attribute of specificity, AS (= A1/A2), decreasing in value as the

antiserum becomes more efficient at neutralizing type 2 strains (attribute A2) (*vide supra*, page 3). It appears, therefore, that the specificity of antisera, particularly type 2 antisera, is inversely related to their titre. With only three antisera of each type, comparison of the attributes of specificity and potency was not feasible.

Correlation of virus versus serum attributes

In Fig. 8, the specificity of each virus strain is compared with the specificity of its corresponding antiserum. The attributes positively correlated for both

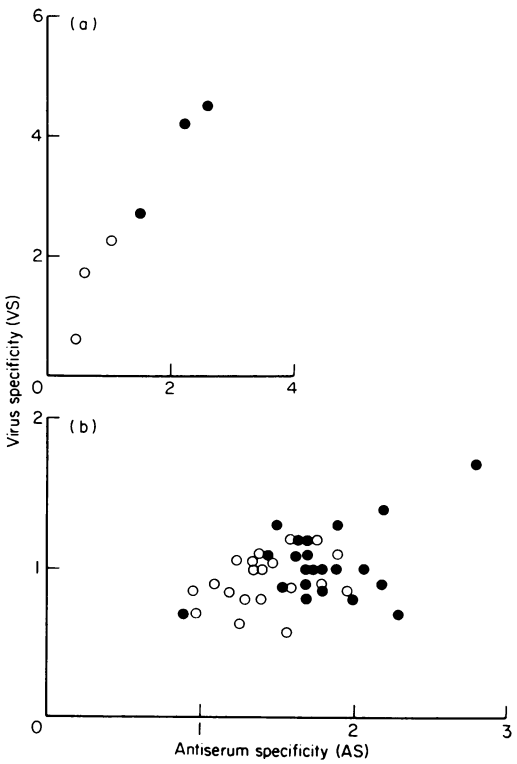

Figure 8. Correlation between virus and antiserum specificity on testing against 'early'. (A) and hyperimmune; (B) antisera. (●) Type 1 virus strains; (○) type 2 virus strains.

hyperimmune mouse antisera ($r = +0{\cdot}45$; $P = < 0{\cdot}05$) and 'early' mouse antisera ($r = +0{\cdot}96$; $P = < 0{\cdot}01$). As expected, there was no correlation between the neutralizability of a virus strain and the neutralizing titre of its corresponding antiserum.

DISCUSSION

This paper has investigated the serological relatedness of herpes simplex viruses and the type-specificity of the antibody response to a number of herpes simplex viruses in two species of experimental animal.

The serological relatedness of a group of organisms is a matter of definition. It is usually assumed, for example, that greater antigen–antibody reactivity bespeaks greater serological relatedness. However, this precept is not, in isolation, tenable; antiserum to herpes B virus of monkeys neutralizes type 1 herpes simplex virus to a greater extent than B virus (Watson *et al.*, 1967) and, in our study, certain type 1 herpes viruses were neutralized to a greater extent than type 2 herpes virus by antiserum prepared against type 2 herpes virus. Clearly, a virus is maximally related to itself and the above observations can only be accommodated by invoking the concept of inherent neutralizability. However, in this study, with due cognition of these considerations, it has been assumed that the neutralization rate between a virus and an antiserum (k value) is a measure of serological relatedness.

A second problem is that sero-relatedness is a relative rather than an absolute concept, being entirely a function of the specificity of the immune sera under test which is, again, a consequence of the method of antisera preparation and the technique of sero-testing. For this reason, both early and hyperimmune antisera have been prepared and the relatedness of the virus isolates measured by the kinetics of neutralization which, on the evidence of other studies of virus neutralization (Dulbecco, Vogt & Strickland, 1956; Lafferty, 1963) would seem to provide the most sensitive technique of virus discrimination.

A third problem arises when only a small proportion of virus strains under test are represented by their homologous antisera. This has been already considered in the Introduction where the importance of reciprocal sero-testing was emphasized. Thus, antisera were raised against forty-one of the forty-seven virus strains and each virus–antiserum reaction was investigated in reciprocal neutralization tests. The possibility that our methods of analysis, although incorporating for each strain the maximum information, might be obscuring a more clear-cut discrimination of virus isolates by given pairs of antisera was excluded by the analysis described in Results (Figs 2a, b and c; Table 2).

Type-distinctiveness of virus isolates

In a study of the immunological relatedness of adenoviruses, Rowe, Huebner, Hartley, Ward & Parrott (1956) defined a virus 'type' as showing 'immunological distinctiveness in reciprocal neutralization tests with established prototypes'. Whether or not 'complete distinctiveness' is required to satisfy this criterion is not clear. On this definition, we should be obliged to agree with Roizman, who prefers to consider type 1 and type 2 herpes viruses rather as 'subtypes' (Roizman, Keller, Spear, Terni, Nahmias & Dowdle, 1970) as, in our study with hyperimmune antisera, every strain was neutralized to some extent by every antiserum. The degree of distinctiveness thus resembles the situation which obtains with the sub-types of type 2 poliovirus or, indeed, the strain variation within sub-types of influenza A2 viruses (Pereira, 1969). It is relevant, however, that herpes simplex virus 'sub-type delineation' can be correlated with many other biological and biochemical parameters and with the site of isolation of the strains under investigation. On this account, it would seem most useful (usefulness being the ultimate criterion of any classification) to continue referring to herpes simplex viruses as 'type 1' and 'type 2'.

Relative usefulness of virus sero-attributes

Hyperimmune mouse antisera most powerfully discriminated the viruses by their neutralizability by type 1 antisera (VI) and by their inherent neutralizability (VN), while hyperimmune rabbit antisera and 'early' rabbit antisera, in agreement with Plummer *et al.* (1974), most powerfully discriminated the virus types by their specificity (VS).

These findings may be due to the fact that the hyperimmune mouse antisera were prepared by immunizing small animals, namely mice, with, in relation to their body weight, rather large antigenic doses and in a situation where, for both virus types, there are fewer type-specific than type-common antigens (Sim & Watson, 1973); with repeated immunization, type-specific antibody may be swamped by type-common antibody. Thus, an antiserum to type 2 virus containing predominantly type-common antibodies may well neutralize type 1 virus better than type 2 virus on account of the greater inherent neutralizability of type 1 virus isolates; this may explain the apparently contradictory results (in terms of homologous *versus* heterologous virus–antiserum reactions) obtained by hyperimmune type 2 antisera (compare with Table 1). The precise molecular mechanisms involved in generating a state of increased inherent neutralizability, apparent to a greater or less extent in the published data of other workers (Dowdle *et al.*, 1967; Aurelain *et al.*, 1970; Wheeler, Briggaman & Henderson, 1970), but seldom particularly mentioned, is a fascinating but, as yet, unresolved problem.

Correlation between virus and antiserum specificity

The specificity of a virus and its corresponding antiserum correlated to a significant extent. This is at variance with Fazekas de St. Groth's (1969) data for influenza virus, where it was established that the strains demonstrating the least cross-reactivity generated immune sera which demonstrated the greatest breadth of reactivity or cross-reactiveness. Such strains were termed 'senior' and it was proposed that the amino acid residues of the relevant antigenic determinants created larger antigenic configurations into which antibodies elaborated in response to the 'junior' strain (with the smaller antigenic site) could not fit. Our data with herpes simplex virus, where there was a positive rather than a negative correlation between these attributes (Fig. 8) suggests that these considerations are not appropriate to herpes simplex viruses; indeed, the remarkable correlation obtained with early antisera suggests the possibility of unique strain specification by reciprocal neutralization kinetics using early antisera and employing this latter method of analysis. This investigation is under way.

Type-specificity of antibody response

Certain general difficulties associated with virus-typing of antisera have been considered in the Introduction to this communication. An additional problem is that antisera, unlike viruses, are imprecise biological entities, their properties being contingent on a wide variety of factors, e.g. the nature, dose and route of administration of the antigen preparations, the frequency and number of immunizations and the duration of time since the first and, of particular importance, the last immunization—thus determining the relative proportion of antibody isotype—which may well influence the specificity of the antiserum (Hampar, Mantos & Chakrabortyz, 1970). Moreover, the influence and interaction of these various factors may vary for different antigens of the same virus. It is theoretically unlikely, therefore, that measurement of the absolute level of antibody activity in a given antiserum against one herpes virus

type, attribute A1 or A2, for example, will provide any specific indication of previous exposure. This was apparent from Fig. 6 where there was gross overlap in the distribution of the antisera in terms of these attributes, and from Table 3 which indicates that the antisera are not discriminated even on a group basis by their behaviour against either type 1 or type 2 strains.

It is clear, therefore, that antisera discrimination requires simultaneous investigation of the relative neutralization of the antisera against both virus types which permits incorporation of both values into a single attribute, viz. attribute of specificity (AS). However, even with this ratio attribute, while the antisera could be distinguished on a group basis, type-prediction (with 95% confidence) was possible in less than 20% of the antisera under test. This was confirmed by cluster analysis where, again, the degree of overlap was such that prediction of the immunizing virus type for a given antiserum was not possible.

As previously suggested, the effect of hyperimmunity on antiserum specificity may be contingent on the relative proportion of type-common to type-specific antigenic sites on the immunizing virus. This was confirmed by our finding that the specificity (AS) of hyperimmune antisera was inversely proportional to their titre (AN) (Fig. 7). Moreover, if one accepts that type 2 virus strains have a greater proportion of type-common to type-specific antigenic sites than do type 1 strains (Rawls, 1968), one should expect a stronger inverse specificity to titre correlation for type 2 virus strains and, indeed, only with the type 2 strains did these attributes demonstrate a significant correlation ($P < 0{\cdot}01$). These observations are also consistent with the greater specificity of the early non-hyperimmune antisera which, in spite of there being only three representatives of each type, delineate into two significantly distinct groups in terms of their specificity.

It is apparent, therefore, that immunogenic prediction on a single given antiserum using any of the derived attributes would, in many cases, be accompanied by considerable uncertainty. This may be particularly relevant in human subjects who may have had long-term exposure to either virus type and may thereby correspond to our late, less specific antisera and, while theoretically the precision of virus typing of antibody in human subjects might be improved by application of discriminate weightings for this attribute, the fundamental inter-attribute relationships, if they exist, may vary for each population and will be confused by previous exposure to heterotypic virus infection.

It did seem possible, however, to compare average reactions and the following rule was evolved for sero-epidemiological group comparisons (Skinner, Thouless & Jordan, 1971): if two groups of human subjects demonstrated no significant difference in their mean type 1 neutralizing antibody activity but did demonstrate a significant difference in their mean type 2 neutralizing antibody activity, this was assumed to be a consequence of specific type 2 antibody activity. It is clear from Table 3 that if these conditions are fulfilled, the group of sera under investigation are at least exhibiting a greater degree of type specificity than both early and hyperimmune antisera of proven homotypy. This is scarcely a rigorous criterion but until type-specific antigens are available it does provide a reasonable rule-of-thumb for sero-epidemiological surveys.

ACKNOWLEDGMENTS

We are grateful to Mrs G. Powell for invaluable technical assistance and to Professor P. Wildy for much helpful discussion and advice. The work was supported by the United Birmingham Hospital Endowment Fund and the Medical Research Council.

REFERENCES

ARCHETTI I. & HORSFALL E.L. (1960) Persistent antigenic variation of influenza A viruses after incomplete neutralisation *in ovo* with heterologous serum. *J. exp. Med.* **92**, 441.

AURELIAN L., ROYSTON I. & DAVIS H.J. (1970) Antibody to genital herpes simplex virus; association with cervical atypia and carcinoma-in-situ. *J. nat. Cancer Inst.* **45**, 455.

BEALE A.J., CHRISTOFINIS G.C. & FURMINGER I.G.S. (1963) Rabbit cells susceptible to Rubella virus. *Lancet*, **ii**, 640.

BEDSON S.P. & GOSTLING J.V.T. (1958) Further observations on mode of multiplication of herpes viruses. *Brit. J. exp. Path.* **39**, 502.

BRONSON D.L., GRAHAM B.J., LUDWIG H.O., BENYESH-MELNICK M. & BISWAL N. (1972) Studies on the relatedness of herpes viruses through DNA–RNA hybridization. *Biochim. biophys. Acta (Amst.)*, **259**, 24.

DOWDLE W.R., NAHMIAS A.J., HARWELL R.W. & PAULS F.P. (1967) Association of antigenic type of herpes virus hominis with site of viral recovery. *J. Immunol.* **99**, 974.

DUBBS D.R. & KIT S. (1964) Mutant strains of herpes simplex deficient in thymidine kinase-inducing activity. *Virology*, **22**, 493.

DULBECCO R., VOGT M. & STRICKLAND A.G.R. (1956) A study of the basic aspects of neutralization of two

animal viruses, western equine encephalitis virus and poliomyelitis virus. *Virology*, **2**, 162.

FAZEKAS DE ST. GROTH S. (1969) The antigenic sub-units of influenza viruses. II. The spectrum of cross-reactions. *J. Immunol.* **103**, 1107.

FIGUEROA M.E. & RAWLS W.E. (1969) Biological markers for differentiation of herpes virus strains of origin. *J. gen. Virol.* **4**, 259.

GOODHEART C.R., PLUMMER G. & WANER J.L. (1968) Density difference of DNA of human simplex viruses types 1 and 2. *Virology*, **35**, 473.

GOWER J.G. (1966) Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika*, **53**, 325.

GRAHAM B.J., LUDWIG H., BRONSON D.L., BENYESH-MELNICK M. & BISWAL N. (1972) Physicochemical properties of the DNA of herpes viruses. *Biochim. biophys. Acta (Amst.)*, **259**, 13.

HAMPAR B., MARTOS L.M. & CHAKRABARTY M. (1970) Late 19S rabbit antibody neutralization test for differentiating herpes simplex virus types 1 and 2. *J. Immunol.* **104**, 593.

HOGGAN M.D. & ROIZMAN B. (1959) The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. *Amer. J. Hyg.* **70**, 208.

KIEFF E.D., BACHENHEIMER S.L. & ROIZMAN B. (1971) Size, composition and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. *J. Virol.* **8**, 125.

LAFFERTY K.J. (1963) The interaction between virus and antibody. I. Kinetic studies. *Virology*, **21**, 61.

MACPHERSON I. & STOKER M. (1962) Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. *Virology*, **16**, 147.

MARTIN M.L., PALMER E.L. & KISSLING R.E. (1972) Complement-fixing antigens of herpes simplex virus, types 1 and 2: reactivity of capsid, envelope and soluble antigens. *Infect. Immunol.* **5**, 248.

MUNK K. & DONNER D. (1963) Cytopathischer Effekt und Plaque-Morphologie verschiedener Herpes simplex Virus Stamme. *Arch. Virus-forsch.* **13**, 529.

NAHMIAS A.J., JOSEY W.E., NAIB Z.M., LUCE C.F. & DUFFY A. (1970) Antibodies to herpesvirus hominis types 1 and 2 in humans. I. Patients with genital herpetic infections. *Amer. J. Epidemiol.* **91**, 539.

PARKER J.D.J. & BANATVALA J.E. (1967) Herpes genitalis. Clinical and virological studies. *Brit. J. vener. Dis.* **43**, 212.

PAULS F.B. & DOWDLE W.R. (1967) A serologic study of herpes virus hominis strains by microneutralization tests. *J. Immunol.* **98**, 941.

PEREIRA H.G. (1969) Influenza: antigenic spectrum. *Progr. med. Virol.* **11**, 46.

PLUMMER G. (1964) Serological comparison of the herpes viruses. *Brit. J. exp. Path.* **45**, 135.

PLUMMER G., GOODHEART C.R., MIYAGI M., SKINNER G.R.B., THOULESS M.E. & WILDY P. (1974) Herpes simplex: discrimination of types and correlation between different characteristics. *Virology*, **60**, 206.

RATCLIFFE H. (1971) The differentiation of herpes simplex virus type 1 and 2 by temperature markers. *J. gen. Virol.* **13**, 181.

RAWLS W.E., IWAMOTO K., ADAM E. & MELNICK J.L. (1970) Measurement of antibodies to herpes virus types 1 and 2 in human serum. *J. Immunol.* **104**, 599.

RAWLS, W.E., LAUREL D., MELNICK J.L., GLICKSMAN J.M. & KAUFMAN, R.H. (1968b) A search for viruses in smegma, pre-malignant and early malignant cervical tissues, the isolation of herpesviruses with distinct antigenic properties. *Am. J. Epidemiol.* **87**, 647.

RAWLS W.E., TOMPKINS W.A.F. & MELNICK J.L. (1969) The association of herpes virus type 2 and carcinoma of the uterine cervix. *Amer. J. Epidemiol.* **89**, 547.

ROIZMAN B., KELLER J.M., SPEAR P.G., TERNI M., NAHMIAS A. & DOWDLE W. (1970) Variability, structural glycoproteins and classification of herpes simplex viruses. *Nature (Lond.)*, **227**, 1253.

ROWE W.P., HUEBNER R.J., HARTLEY J.W., WARD T.G. & PARROTT R.H. (1955) Studies of the adenoidal-pharyngeal-conjunctival (A.P.C.) group of viruses. *Amer. J. Hyg.* **61**, 197.

RUSSELL W.C. (1962) A sensitive and precise plaque assay for herpes virus. *Nature (Lond.)*, **195**, 1028.

SCHNEWEIS K.E. (1962) Serologische Untersuchungen zur type differenzierung des Herpes virus hominis. *Z. Immunforsch. exp. Ther.* **124**, 24.

SHUBLADZE A.K. & CHZHU-SHAN K. (1959) Study on the antigenic properties of herpes virus. *Vopr. virurol.* **4**, 80.

SKINNER G.R.B., TAYLOR J. & EDWARDS J. (1974) Precipitating antibodies to herpes simplex virus in human sera: prevalence of antibody to common antigen (Band II). *Intervirology*, **4**, 320.

SKINNER G.R.B., THOULESS M.E. & JORDAN J.A. (1971) Antibodies to type 1 and type 2 herpes virus in women with abnormal cervical cytology. *J. Obstet. Gynaec. Brit. Clth*, **78**, 1031.

SIM C. & WATSON D.H. (1973) 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.

THOULESS M.E. (1972) Serological properties of thymidine kinase produced in cells infected with type 1 or type 2 herpes virus. *J. gen. Virol.* **17**, 307.

THOULESS M.E. & SKINNER G.R.B. (1971) Differences in the properties of thymidine kinase produced in cells infected with type 1 or type 2 herpes virus. *J. gen. Virol.* **12**, 195.

WILDY P. (1955) Recombination with herpes simplex virus. *J. gen. Microbiol.* **13**, 346.

WHEELER C.E., BRIGGAMAN R.A. & HENDERSON R.R. (1969) Discriminating between two strains (types) of herpes simplex virus by various modifications of the neutralization test. *J. Immunol.* **102**, 1179.

WATSON D.H., SHEDDEN W.I.H., ELLIOT, A., TETSUKA T., WILDY P., BOURGAUX-RAMOISY D. & GOLD E. (1966) Virus specific antigens in mammalian cells infected with herpes simplex virus. *Immunology*, **11**, 399.

WATSON D.H., WILDY P. HARVEY B.A.M. & SHEDDEN W.I.H. (1967) Serological relationships among viruses of the herpes group. *J. gen. Virol.* **1**, 139.