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Hybrids derived from the viruses of alastrim and rabbit pox

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INTRODUCTION

In the preceding paper (Dumbell & Bedson, 1964) an account was given of the isolation of presumptive hybrid clones from the viruses of alastrim and rabbit pox. We report here the results of a more detailed examination of the seven clones of virus in question. As with the variola major–cowpox hybrids considered in the succeeding paper (Bedson & Dumbell, 1964) the main object of the work has been the investigation of the ceiling temperature character and its possible relationship to virulence among the pox viruses.

TESTS FOR MARKER CHARACTERS

The virus strains used and the methods of inoculating eggs and preparing stock suspensions were those described in the preceding paper (Dumbell & Bedson, 1964). Where appropriate the tests for marker characters have been made using the methods developed by Fenner (1958).

(1) *Pock morphology*

The size, colour and degree of ulceration of discrete pocks was noted after 2 and after 3 days' incubation at 35° C. Those of rabbit pox were large, red and grey in colour and were ulcerated. The pocks of alastrim were smaller, white and not ulcerated.

(2) *Ceiling temperature*

Tests were made to determine the presence or absence of pocks at 40° C., using inocula which would produce 50–100 pocks at 35° C. Under these conditions rabbit pox produced obvious lesions and alastrim produced none.

(3) *Haemagglutinin*

Extracts of heavily infected chorioallantoic membranes (C.A.M.S) were diluted 1/5, 1/20 and 1/80 in saline and were tested for haemagglutinin with a 1% suspension of vaccinia-agglutinable fowl cells containing 1% normal rabbit serum. The mixtures were shaken and incubated at 37° C. for 20 min., shaken again and incubated for a further hour. Readings were taken after the cells had been allowed to settle for 10 min. at room temperature. Rabbit pox gave no haemagglutination, whereas alastrim did, at each of the dilutions tested. The ability to induce the production of anti-haemagglutinin in rabbits (Fenner, 1958) was not tested.

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(4) *Thermal stability*

Freshly prepared virus suspensions were sealed in thin glass ampoules and immersed in a water bath at 55° C. for 20 min. The reduction in titre of the heated suspension was determined by titration on the C.A.M. after storage at 4° C. overnight. With rabbit pox the fall in titre was 1·0 log unit or less, while the comparable figure for alastrim was in the region of 5·0 log units.

(5) *Type of plaque in chick embryo tissue culture*

Monolayers of chick embryo fibroblasts were prepared in 5 cm. Petri dishes using a modification of Dulbecco's method (Dulbecco, 1952). The growth medium was based on that of Porterfield & Allison (1960). It contained 5 % inactivated calf serum, 0·25 % lactalbumin hydrolysate, and was buffered with *tris* (hydroxymethyl) aminomethane. Each dish received approximately $10^{7\cdot7}$ cells suspended in 4 ml. of growth medium, and was incubated at 35° C. for 2 days before use.

Inoculations were made with virus diluted appropriately in Hanks's phosphate buffered saline, 0·2 ml. being applied to each dish after removal of the growth medium. After adsorption at room temperature for 1 hr., 4 ml. of Eagle's medium (Eagle, 1955) containing 1 % calf serum and triple strength bicarbonate was added. The dishes were incubated at 35° C. in a humidified atmosphere of 5 % CO_2 in air for periods of 2, 3 and 4 days. The medium was then removed and the cell sheets stained with strong carbol fuchsin (Postlethwaite, 1960). With rabbit pox, plaques were present at 2 days. In appearance they were trabeculated and similar to those of cowpox depicted by Bedson & Dumbell (1964; Plate 1A). With alastrim, the monolayers appeared normal at 2 days. The characteristic heavy-rimmed plaques were not present until 4 days. The appearances in this case were indistinguishable from those described for variola major (Bedson & Dumbell, 1964; Plate 1C, D).

(6) *Rabbit virulence*

Virulence for the rabbit was assessed by the type of lesion which resulted from the intradermal inoculation of a standard dose of 10^5 pock forming units (p.f.u.) of virus. Inspections were made daily in the week following inoculation. By the 5th day, rabbit pox lesions showed extensive haemorrhage and necrosis with surrounding oedema. Alastrim produced only trivial lesions, which, by the 5th day, had almost completely regressed.

(7) *Mouse virulence*

The virulence of a virus for the mouse was determined from the mortality rates in 1-month-old mice inoculated by the intracerebral route. A group of five mice was used for each virus. Inocula were 0·01 ml. and contained 10^5 p.f.u. of virus. Under these conditions, rabbit pox was uniformly lethal, the mice dying within 5 days of inoculation, whereas alastrim produced no deaths.

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ANALYSIS OF THE ALASTRIM-RABBIT POX CLONES

Seven clones of virus developed from the alastrim–rabbit pox system were subjected to characterization in detail. In the following account they will be referred to as AR viruses 1–7. The results of applying tests for the seven marker characters to these AR viruses are presented in Table 1. The characters of the parent viruses are included in the table for ease of comparison. In most instances the individual characters of the AR viruses were of one or other parental type but some intermediate results were obtained. None of the viruses had all the characters of alastrim but there was one (AR 5) which was indistinguishable from rabbit pox. The other six differed from the parent viruses and also amongst themselves. Each had its own distinct combination of parental characters. There were thus six separate new types of virus.

The genetic stability of these new viruses has not been investigated specifically. In the course of the tests for marker characters the virus clones have been passed on the C.A.M. at 35° C., and several hundred pocks have been examined in tests for ceiling temperature and thermal stability. In all these experiments each of the six clones has maintained the uniformity of its pock type. It was felt that this evidence was sufficient for the purposes of the present study. A more detailed investigation has been made of the stability of the variola major–cowpox hybrids (Bedson & Dumbell, 1964).

Although the AR viruses had been derived from mixed infections and had been shown to exhibit combinations of the appropriate parental characters, it is important to consider in greater detail the question of whether they may be accepted as hybrid viruses. Upon this point rests the validity not only of the claim, made in the preceding paper (Dumbell & Bedson, 1964), that temperature may be used selectively to facilitate the recovery of hybrids, but also of some of the deductions made in that paper about the properties of 'heat-tethered' virus.

The only reasonable alternative explanation is that the AR viruses are mutants derived from the reactivated rabbit pox virus. This virus is known to produce a wide variety of white mutants (Gemmell & Fenner, 1960), but the frequency with which it does this, although appreciable, would not seem to be nearly as great as in our reactivation experiments. In particular, it is to be noted that the six new AR viruses came from only two pocks.

A further point against their being mutants is the fact that practically all the characters of the other virus concerned (alastrim) have been found amongst them. Although this argument has *general* validity, it should be noted that it is much less convincing for an *individual* virus, because of the very wide range of characters shown by the white variants of rabbit pox (Gemmell & Fenner, 1960).

There remains the possibility that the heat-inactivation of rabbit pox may have damaged the genetic material and led to an increase in the frequency of mutation. This suggestion runs counter to current concepts of heat-inactivation and reactivation which, for the pox viruses, is regarded as a 'non-genetic' process (Fenner, 1962). In any case a high frequency of mutation does not seem to have been observed when reactivation has been carried out with an active virus of another

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Table 1. *Characters of rabbit pox, alastrim and hybrids developed from them (AR 1–7)*

Virus	Pock type	Pocks at 40° C.	Haem-agglutinin	Thermal stability*	Plaque type	Plaques appear (days)	Rabbit virulence†	Mouse virulence‡
Rabbit pox	Large, red, ulcerated	+	0	0·8	Trabeculated	2	++	+
Alastrim	Small, white, non-ulcerated	0	+	5·0	Heavy-rimmed	4	0	0
AR 1	Small, white, ulcerated	+	+	0·8	Heavy-rimmed	3	+	0
AR 2	Small, white, ulcerated	+	+	0·9	Trabeculated	2	+	±
AR 3	Large, white, ulcerated	+	+	0·7	Heavy-rimmed	3	±	0
AR 4	Small, white, non-ulcerated	0	0	3·9	Trabeculated	4	0	0
AR 5	Large, red, ulcerated	+	0	1·1	Trabeculated	2	++	+
AR 6	Small, white, non-ulcerated	0	0	0·7	Trabeculated	4	0	+
AR 7	Large, white, ulcerated	+	0	1·0	Trabeculated	3	0	+

* Figures represent the reduction in titre ($\log_{10}$ units) after heating at 55° C. for 20 min.

† ++, Large lesion with haemorrhage and necrosis; +, papule with central necrosis but no haemorrhage; ±, papule without haemorrhage or necrosis; 0, insignificant lesion.

‡ +, 100% mortality; ±, some mortality; 0, no mortality.

Table 2. *Analysis of pairwise crosses of individual marker characters amongst the AR clones*

	Rabbit pox markers								
	Pocks on C.A.M.					Plaques			
Alastrim markers	Large	Ulcerated	At 40° C.	No haem-agglutinin	High thermal stability	Trabecular	2 days	Rabbit virulent	Mouse virulent
Pocks small	−	(+)	(+)	+	(+)	+	+	+	+
Pocks non-ulcerated	0	−	0	+	(+)	+	0	0	+
No pocks at 40° C.	0	0	−	+	(+)	+	0	0	+
Haemagglutinin	+	+	+	−	+	(+)	+	+	+
Low thermal stability	0	0	0	+	−	+	0	0	0
Plaques rimmed	+	+	+	0	+	−	0	+	0
No plaque 2 days	+	(+)	(+)	+	(+)	(+)	−	(+)	(+)
Not rabbit virulent	+	(+)	(+)	+	(+)	+	0	−	+
Not mouse virulent	+	+	+	+	(+)	(+)	0	+	−

+, Pairwise cross and its reciprocal present; (+), pairwise cross but not reciprocal present; 0, pairwise cross absent.

subgroup (Fenner & Woodroofe, 1960). Had white mutants occurred with increased frequency it would hardly have been possible for these workers to have made the observation that hybrids do not occur in this type of system.

It seems therefore extremely probable from all points of view that the six new AR viruses are hybrids. If this is accepted, it becomes of interest to examine the behaviour of the individual marker characters in segregation. The pairwise crosses between markers that have been found among the AR viruses are shown in Table 2. From the table it will be seen that, where necessary, markers have been arbitrarily redefined in such a way as to class intermediate results with one or other parent. It will also be seen that the number of markers considered has been increased to 9 by regarding pock size, degree of ulceration of pocks, plaque type and speed of plaque formation, all as separate characters. The colour of the pocks has been omitted from the analysis because selection of potential hybrids was based on this character and clones with red pocks were in general avoided. With nine characters, the number of possible pairings is 72 and 53 of these were shown by the six AR hybrids. In 18 instances the pairwise cross and its reciprocal cross were present and in only one case was neither of the two possible crosses for a pair of markers found. Considering the very few hybrids which have been examined the proportion of pairwise crosses encountered is surprisingly high. The evidence strongly suggests, therefore, that each of the markers used is capable of segregating independently. This point has, of course, a particular significance in relation to virulence characters, but a more extensive discussion of this aspect of the work will be made in the following paper after the properties of the variola–cowpox hybrids have been described (Bedson & Dumbell, 1964).

SUMMARY

The characters of seven clones of virus derived from mixed infections with alastrim and rabbit pox have been described. One clone was shown to behave as rabbit pox in respect of all its markers. The other six were found to be distinct new types of virus, each having a different combination of the parental characters. The reasons for accepting these viruses as hybrids have been discussed. An analysis of the pairwise crosses between individual markers suggested that each of the nine markers was capable of segregating independently.

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