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The immune response to infection with vaccinia virus in mice

I. Infection and the production of antibody neutralizing cell-associated and cell-free virus

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SUMMARY

The onset, duration and magnitude of antibody responses to a poxvirus infection were examined. Mice were inoculated intravenously with the WR strain of vaccinia virus and developed pocks on their tails. The number of pocks was related to the size of the inoculum. Virus was detectable in the spleen and infected mice were subsequently immune to intravenous and intra-nasal challenge. Sera of infected animals neutralized both cell-free and cell-associated virus. Antibody against cell-free virus appeared first; maximum titres were reached sooner but were lower than those of antibody neutralizing cell-associated virus. Titres remained high for at least 100 days after infection.

INTRODUCTION

The mechanisms of immunity to poxviruses remain obscure despite the long history of successful vaccination against smallpox. Key roles in protection against infection have been variously ascribed to neutralizing antibody, to specific and non-specific cell-mediated immunity, and to interferon, which may perhaps be considered as a mediator of cell-mediated immunity (Bloom, 1971).

Passively transferred antibody has been successful in preventing or modifying disease in smallpox contacts (Pierce, Melville, Downie & Duckworth, 1958; Kempe, 1960; Kempe *et al.* 1969) but the vaccination reactions of people with antibody deficiencies (Kempe, 1960; Medical Research Council Working-Party, 1969; Fulginiti *et al.* 1968), deficiencies in cell-mediated immunity or lack of both types of response (Kempe, 1960; Medical Research Council Working-Party, 1969; Fulginiti *et al.* 1966) have indicated that the cell-mediated response is vital for the elimination of infection. The appearance of poxvirus antigens on cell surfaces within 2 hr. of infection (Hayashi, Rosenthal & Notkins, 1972) and the ability of the virus to spread from cell to cell without release into extracellular fluid (Ichihashi, Matsumoto & Dales, 1971) provide a rationale for the necessity

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either of a cytolytic antibody system (Brier *et al.* 1971) or for target-cell killing such as that important in tumour and transplantation immunity (Hellström & Hellström, 1969) and operative against mumps virus infections in tissue culture (Speel, Osborn & Walker, 1968).

Animal models have not as yet been entirely successful in clarifying the issue. While there is evidence that cell-mediated immunity is of paramount importance in elimination of vaccinia virus infections in monkeys (Fulginiti, 1970) and ectromelia infections in mice (Blanden, 1970; Blanden, 1971*a*, 1971*b*) and that it plays a definite role in protection of rabbits from vaccinia virus (Pincus, Flick & Ingalls, 1963), work with vaccinial infections of mice has produced equivocal results (Hirsch, Nahmias, Murphy & Karmer, 1968; Worthington, Rabson & Baron, 1972; Worthington, 1973*a*, *b*).

Two considerations led to the re-evaluation of immune responses following experimental infection. First, although several studies have been made of serum antibody directed against vaccinia virus much of this work was done with antibody titrated for its capacity to neutralize virus released from infected cells by mechanical disruption – so-called 'cell-associated' virus. And it has recently been demonstrated that this antibody is incapable of neutralizing the small proportion of virus which is released naturally from cells – so-called 'cell-free' virus (Appleyard, Hapel & Boulter, 1971). Secondly, studies with non-viral antigens have provided new techniques for the evaluation of cell-mediated immunity which are only beginning to be used for the study of immunity to viruses. These may be improvements on the classical skin test for delayed hypersensitivity which has been considered both unreliable for work with vaccinia virus (Worthington *et al.* 1972) and an insensitive measure of cell-mediated immunity (Hirsch *et al.* 1968).

The first paper describes a non-lethal infection of mice with vaccinia virus and the production of antibody neutralizing both cell-associated and cell-free virus. The second (Hutt, 1975) describes the concurrent induction of hypersensitivity to the virus and examines some of the *in vitro* techniques associated with cell-mediated immunity.

MATERIALS AND METHODS

Animals

Outbred female CFLP albino mice were obtained from Carworth Europe Ltd as specific-pathogen-free animals and were kept in air-filtered units under conditions designed to maintain their microbiological status. Only mice more than 6 weeks old and weighing more than 20 g. were used.

Virus

The WR strain of vaccinia virus was obtained from Dr G. Turner, Lister Institute, Elstree, Herts, England, and passaged twice in the mouse brain. It was harvested as a 20% suspension of brain homogenized in phosphate buffered saline, containing $10^{6\cdot9}$ p.f.u./ml., $10^{5\cdot9}$ intracerebral mouse LD50/ml. and $10^{3\cdot2}$ intra-nasal mouse LD50/ml.

For neutralization tests the virus was propagated in HeLa cells and prepared as cell-associated or cell-free virus by modifications of the methods of Appleyard and co-workers (1971). Cell-associated virus (CAV) was prepared by mechanically disrupting whole infected cultures with glass beads at 72 hr. after infection and filtering the resulting material through sterile gauze. The filtrate contained $10^{8 \cdot 0}$ p.f.u./ml. and probably more than 99% of the virus was CAV (Turner & Squires, 1971). Cell-free virus (FV) was prepared by adding excess rabbit anti-vaccinia serum prepared against inactivated cell-associated virus to cultures at 10 hr. after infection. At 72 hr. after infection when cell monolayers were still adherent, the maintenance medium was removed and centrifuged at 300 *g* for 10 min. The supernatant contained $10^{4 \cdot 3}$ p.f.u./ml. of live virus and comparison of supernatants from cultures with and without antiserum indicated that the ratio of neutralized: live virus was approximately 3:1.

All virus was stored at $-60°$ C. and before use was treated with ultrasound for 45 sec. in ice-cooled water in the high intensity cleaning tank of a Dawe Soniprobe type 1130/2 with a power output of 60 W. at 20 kHz.

Cell cultures

Primary chick embryo fibroblast cultures were prepared from minced and trypsinized 10-day chick embryos and were grown in medium 199 with 10% heat-inactivated fetal calf serum, 0·15% sodium bicarbonate, 100 i.u./ml. penicillin and 100 μg./ml. streptomycin. HeLa cells were grown in Eagle's MEM with the same supplements. For maintenance the serum content was reduced to 1%.

Virus assay

As a routine virus was assayed in terms of plaque-forming units in primary chick embryo fibroblasts (CEF). Suspensions of CEF in growth medium were stored at 4° C. and virus and cells were seeded simultaneously into Linbro Multidishes, FB-6-TC (Linbro Chemical Co. Ltd). Approximately 46 hr. later, monolayers were stained with 0·2% aqueous neutral red and plaques were counted. The cells retained their sensitivity to virus during 3 days storage and 1 p.f.u. was equivalent to less than 2 pk.f.u.

The virus content of spleens from infected mice was assayed by homogenizing chopped spleens in 3 times their volume of phosphate-buffered saline, treating the homogenate for 4 min. with ultrasound to complete disruption of cells and titrating material in the chorioallantoic membranes of 11-day eggs. High concentrations of the disrupted material were toxic for CEF. The extraction procedure was controlled by examining its effect on virus added to normal spleens and treated in the same way as infected material. The average pock counts in the same dilutions of untreated and treated virus were 23 and 22 respectively.

Some virus material was also titrated intranasally in mice by dropping $3 \times 0{\cdot}02$ ml. of various dilutions of virus into the nasal passages of anaesthetized animals. The LD50 was calculated according to the method of Reed & Muench (1938).

Neutralization tests

Blood was collected aseptically from the thoracic cavities of groups of five mice which had been asphyxiated with carbon dioxide. The sera were pooled, heat-inactivated at 56° C. for 30 min., stored at 4° C. and subsequently assayed for anti-vaccinia activity by a plaque reduction method. Samples of CAV or FV giving 30–70 p.f.u. per monolayer were incubated with dilutions of test serum or with the diluent – heat-inactivated fetal calf serum – for 2 hr. at 37° C. Each mixture was then assayed for p.f.u. The neutralization titre of serum was defined as the $\log_{10}$ of the reciprocal of the dilution which reduced the number of p.f.u. to 50 % of those produced by virus incubated with diluent alone.

A known reference serum was included in all tests as a control. The neutralization titre of serum did not alter during at least 139 days storage and the standard deviations of assays of anti-CAV and anti-FV were 0·2 and 0·3 $\log_{10}$ respectively. Anti-FV could not be measured by examination of 'anti-comet' activity in antisera (Appleyard *et al.* 1971) since WR virus did not produce such comets in CEF.

Rabbit antiserum

Rabbit antiserum against CAV was kindly donated by Dr G. Turner and was prepared by inoculation of virus extracted from sheep dermal pulp and inactivated by dye and visible light. (Turner & Squires, 1971). The virus was emulsified with an equal volume of Freund's complete adjuvant and inoculated intradermally. Forty days later an intravenous dose of the same material was given without adjuvant. Rabbits were bled before immunization and at 10 days after this second dose and sera were separated and inactivated at 56° C. Preimmunization sera contained no antibodies to vaccinia virus.

RESULTS

Infection

Primary infection

Sixteen groups of 16 mice were inoculated intravenously with WR virus in twofold dilutions starting with $10^{3·6}$ p.f.u. and decreasing (Table 1). Mice inoculated with $10^{0·6}$ p.f.u. or more developed pocks along their tails. In most animals they appeared as faint red areas on day 5. By day 6 the areas were raised and some showed white centres; by day 8 scabs had formed on nearly all of them. The scabs fell off during the next 10 days, leaving healed but hairless areas of skin. At no time did the mice appear sick. Maximum pock counts for each mouse indicated that although the dose response was not linear, decreasing the inoculum resulted in a diminished number of pocks. Analysis of counts in groups in which all mice developed pocks and of pooled data from subsequent experiments indicated that they did not follow a Poissonian distribution (Table 2). There was a large variation in pock counts between animals. The mean of 176 counts derived from three experiments in which $10^{3·3}$ p.f.u. of WR virus were inoculated was 19 with a standard deviation of 10·4.

Table 1. *Appearance of pocks on tails of mice after intravenous inoculation of various amounts of WR virus*

Inoculum ($\log_{10}$ p.f.u.)	Pock counts: Mean	Pock counts: Mean for mice with pocks	No. of mice with maximum counts by this day: 7	8	9	10	Mice with pocks: No. inoculated	Ratio p.f.u.:pocks
3·6	24	24	2	14	0	0	16/16	167
3·3	18	18	3	10	1	1	15/15	111
3·0	9	9	5	7	3	0	15/15	111
2·7	8	8	6	5	1	3	15/15	63
2·4	6	6	7	5	3	1	16/16	42
2·1	4	4	3	2	7	1	13/16	31
1·8	2	3	2	3	3	3	11/16	31
1·5	2	3	1	1	5	5	12/16	15
1·2	1	3	3	0	2	1	6/16	15
0·9	0	1	1	0	1	0	2/16	—
0·6	0	3	3	0	0	1	4/16	—
0·3	0	0	0	0	0	0	0/16	—
0·0	0	0	0	0	0	0	0/16	—
<0·0 (0·50 p.f.u.)	0	0	0	0	0	0	0/16	—
<0·0 (0·25 p.f.u.)	0	0	0	0	0	0	0/16	—
Diluent	0	0	0	0	0	0	0/16	—

Table 2. *Analysis made with the Poisson heterogeneity test of pock counts (x) on tails of mice after inoculation of various amounts of WR virus*

No. of mice	Inoculum ($\log_{10}$ p.f.u.)	$\bar{x}$	$\Sigma (x-\bar{x})^2$	$\frac{\Sigma (x-\bar{x})^2}{\bar{x}} = \chi^{2*}$
176	3·3	18·6	13079·2	703·2
15	3·0	8·6	493·6	57·4
15	2·7	7·8	215·4	27·6
16	2·4	5·4	363·7	67·4

* Counts do not follow a Poissonian distribution if values of χ^2 for $P = 0{\cdot}05$ are greater than: 213·52 for 175 degrees of freedom, 26·12 for 14 degrees of freedom, 27·49 for 15 degrees of freedom.

Although pocks appeared only along the tails of infected animals the virus multiplied internally. Assays were made of virus in the pooled spleens of groups of five mice killed at various intervals after inoculation of $10^{3\cdot3}$ p.f.u. of WR virus. Virus was detectable 8 hr. after inoculation (Table 3) and again from 2 to 11 days later. Maximum titres were found at 7 days after infection. Spleens also increased in weight during this period.

Secondary infection

Eight mice from each of the 16 groups previously inoculated were reinoculated intravenously with $10^{3\cdot3}$ p.f.u. of WR virus, 31 days later. At the same time, some of the other eight mice from alternate dilutions were inoculated intranasally with $10^{4\cdot7}$ p.f.u., i.e. ten intranasal mouse LD50. The remaining mice were reinoculated intravenously with $10^{3\cdot3}$ p.f.u. 189 days later.

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Table 3. *Weights and virus content of spleens of mice after intravenous inoculation of* $10^{3\cdot3}$ *p.f.u. of WR virus*

Day after inoculation	Spleen weights		pk.f.u./ spleen
	Mean (mg.)	% increase	
0 (before inoculation)	109	0	<2
5 hr.	105	0	<2
8 hr.	104	0	3
1	124	13	<2
2	100	0	4
3	114	4	15
4	169	55	30
5	141	29	40
6	189	73	88
7	208	91	193
8	207	90	74
9	221	102	11
10	264	142	5
11	240	120	5
12	269	147	<2
13	235	116	<2
14	255	134	<2
15	229	110	<2
16	261	139	<2
18	216	98	<2
20	204	87	<2
22	186	70	<2
24	211	93	<2
28	183	68	<2
36	184	69	<2
56	191	75	<2
12 (controls)	113	4	<2
56 (controls)	123	13	<2

Except for one mouse originally receiving $10^{1\cdot8}$ p.f.u., no pocks appeared on the tails of mice receiving more than $10^{0\cdot9}$ p.f.u. originally or in several of those receiving $10^{0\cdot9}$ or $10^{0\cdot6}$ when challenged at 31 days (Table 4). Many of these mice had developed no pocks after the original inoculation. There was no significant reduction in the numbers of pocks on individual mice that developed them but only in the number of mice that developed any at all. Immunity was still demonstrable in most mice at 189 days (Table 5) but had apparently waned in the animals originally receiving $10^{0\cdot9}$ p.f.u. Mice originally inoculated with $10^{1\cdot8}$ p.f.u. were not killed by intranasal challenge with 10 LD50 at 31 days and there was a reduction in mortality in those originally receiving $10^{0\cdot0}$–$10^{1\cdot8}$ p.f.u. (Fig. 1).

Attempts to establish when immunity developed by reinoculation of virus intravenously at 4–10 days after infection were unsuccessful. At no time did the second inoculation increase the number of tail pocks (Table 6).

Table 4. *Pocks on tails of mice intravenously inoculated with various amounts of WR virus and challenged intravenously* 31 *days later with* $10^{3\cdot3}$ *p.f.u. of WR virus*

Original inoculum ($\log_{10}$ p.f.u.)	Pock counts: After second inoculation								Mean	Mean for mice with pocks
3·6	0	0	0	0	0	0	0	0	0	0
3·3	0	0	0	0	0	0	0	0	0	0
3·0	0	0	0	0	0	0	0	0	0	0
2·7	0	0	0	0	0	0	0	0	0	0
2·4	0	0	0	0	0	0	0	0	0	0
2·1	0	0	0	0	0	0	0	0	0	0
1·8	0	1	0	0	0	0	0	0	0	1
1·5	0	0	0	0	0	0	0	0	0	0
1·2	0	0	0	0	0	0	0	0	0	0
0·9	0	0	46	0	0	40	0	0	11	43
0·6	0	30	31	0	0	24	0	0	11	28
0·3	11	56	49	61	18	46	26	20	36	36
0·0	47	43	0	13	15	14	18	7	20	22
<0·0 (0·50 p.f.u.)	32	10	22	15	14	27	37	14	21	21
<0·0 (0·25 p.f.u.)	28	20	22	16	18	42	8	13	21	21
Diluent	32	17	45	35	50	33	35	19	33	33

Table 5. *Pocks on tails of mice inoculated intravenously with various amounts of WR virus and challenged intravenously* 189 *days later with* $10^{3\cdot3}$ *p.f.u. of WR virus*

Original inoculum ($\log_{10}$ p.f.u.)	Pock counts: After second inoculation								Mean	Mean for mice with pocks
2·3	0	0	0	0	0	0	0	0	0	0
2·7	0	0	0	0	0	0	0	0	0	0
1·5	0	0	0	0	0	0	0	0	0	0
0·9	36	22	6	9	7	31	0	—	16	19
<0·0 (0·25 p.f.u.)	14	6	23	19	—	—	—	—	16	16
Diluent	21	25	2	18	19	25	7	3	15	15

Antibody

Definition of virus used in antibody assays

Definition of challenge virus is crucial for comparison of activity against CAV and FV in mouse sera taken after infection. Since the distinction between CAV and FV was initially recognized in terms of the behaviour of the viruses in neutralization tests (Boulter, 1969; Appleyard *et al.* 1971) the behaviour in such tests of the two pools prepared as and designated CAV and FV was examined.

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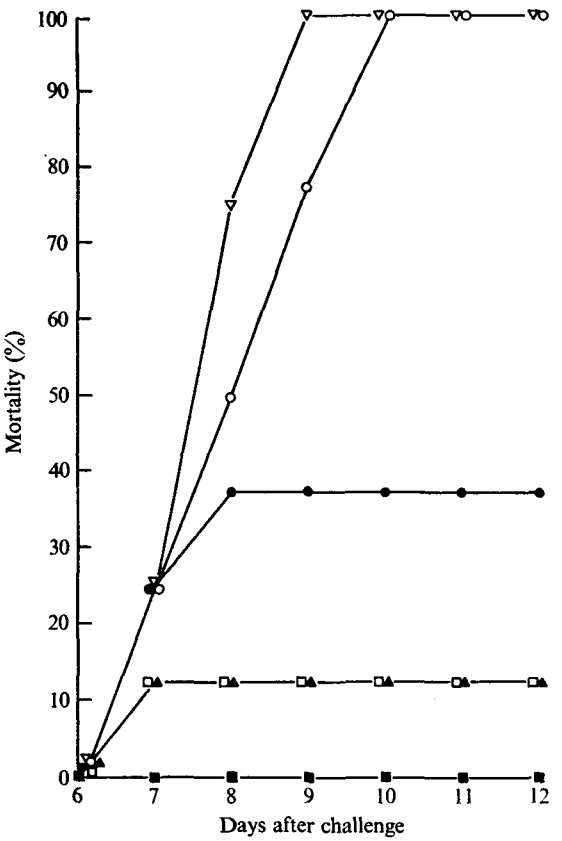

Fig. 1. Deaths in mice inoculated intravenously with various amounts of WR virus and challenged intranasally 31 days later with 10 INLD50. Mice intravenously inoculated with: ▽, PBS; ○, $<10^{0\cdot0}$ p.f.u. (0·25 p.f.u.); ●, $10^{0\cdot0}$ p.f.u.; ▲, $10^{0\cdot6}$ p.f.u.; □, $10^{1\cdot2}$ p.f.u.; ■, $10^{1\cdot8}$, $10^{2\cdot4}$, $10^{3\cdot6}$ p.f.u.

Table 6. *Tail pocks on mice after 2 intravenous inoculations of* $10^{3\cdot3}$ *p.f.u. of WR virus*

Day of second inoculation	Pock counts					Mean
4	17	38	8	26		22
	17	28	13	20		
11	12	8	11	25	43	21
	12	10	16	34	35	
13	8	5	20	8	27	21
	17	8	36	20	41	
15	17	27	16	32	14	19
	30	26	21	4	6	
No second inoculation	31	9	8	14	25	20
	17	31	13	16	35	

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Table 7. *Activity of antisera prepared against CAV or CAV + FV against different preparations of challenge virus*

Source of challenge virus (designation)	Neutralization titre	
	Rabbit anti-CAV	Mouse anti-CAV + FV
Culture medium of infected cells (FV + CAV)	3·4	2·1
Culture medium of infected cells with excess anti-CAV (FV)	< 1·0	2·1
Mechanically disrupted infected cells (CAV)	4·5	2·3

Two antisera were used:

(i) Rabbit anti-vaccinia serum designated anti-CAV and prepared against inactivated virus which was obtained by mechanical disruption of infected cells; such immunizing virus would be expected to be almost entirely CAV before inactivation and inactivation would probably destroy any antigenic activity associated with FV (Turner & Squires, 1971).

(ii) Mouse anti-vaccinia serum designated anti-CAV + FV and taken 11 days after infection with vaccinia virus; since the virus undergoes replication in the mouse both CAV and FV are probably released and the antibodies to both types of virus would be expected (Appleyard *et al.* 1971).

Three virus pools were included in the assay:

(i) The pool designated CAV which was prepared from mechanically disrupted cells.

(ii) The pool designated FV which was prepared from culture medium of infected cells in the presence of excess anti-CAV.

(iii) A pool prepared in parallel with the pool FV but which contained 3 times as much virus as the FV pool since it had been prepared in the absence of antiserum in the supernatant medium; this pool was designated FV + CAV.

The rabbit anti-CAV neutralized the pool designated CAV and the pool CAV + FV; it did not neutralize the pool designated FV (Table 7). The mouse anti-CAV + FV neutralized all three pools. This indicated that the pools designated FV and CAV contained virus which behaved in the expected manner. The third pool appeared to be a mixture of virus behaving as FV and CAV with more than 50 % behaving as CAV. It confirms the finding of Appleyard and his colleagues, that some virus is released into extracellular fluid which behaves as CAV. This virus had been successfully neutralized in the preparation of the FV pool and did not constitute challenge virus in the tests using the FV pool. The titre of the anti-CAV was lower against pool FV + CAV than against pool CAV. This suggested that though FV was not neutralized by the anti-CAV it did adsorb the antibody and reduce the amount available for neutralization of CAV. Some damage to the outer

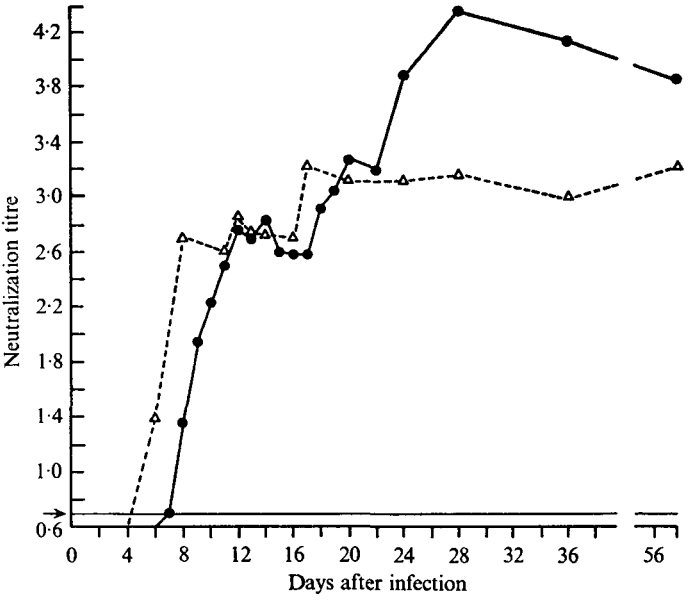

Fig. 2. Appearance of neutralizing antibody in the sera of mice after infection with vaccinia virus. ●——●, Antibody neutralizing CAV; △– – –△, antibody neutralizing FV; ⟶ limit of sensitivity.

envelopes of FV particles (Appleyard *et al.* 1971) may have led to exposure of enough of the antigenic sites found on CAV particles to adsorb antisera but not enough to result in neutralization.

Antibody production after infection

Assays were made of activity against CAV or FV in the pooled sera of groups of five mice killed at various intervals after inoculation of $10^{3\cdot3}$ p.f.u. of WR virus. These were the same mice as those from which spleens were removed for infectivity assays.

Control sera were negative at a dilution of 1/5. Antibody that neutralized CAV appeared on day 7 and reached its highest titre on day 28 (Fig. 2). There were apparently peaks of antibody production, at day 14 and at day 28, the second being higher.

Antibody to FV was detectable earlier at day 6. Until day 10 there was greater activity against FV than CAV; between days 10 and 18 activity was similar to that against CAV. A second peak of anti-FV became apparent on day 17 just before the second rise in anti-CAV. This pattern of antibody production could be demonstrated repeatedly.

DISCUSSION

The course of infection after intravenous inoculation of vaccinia virus into mice was similar to that described by Boyle, Haff & Stewart (1966) except that pocks appeared over a longer period. The reason for the localization of pocks on the tail

is not clear. Virus may lodge in the tail tissues during inoculation but the finding that pocks developed no more frequently at the site of inoculation than elsewhere on the tail argues against this possibility. Because infectious virus can be detected in the tail 5 or 6 days after infection it has been suggested that it is a primary site of multiplication (Allen & Mudd, 1973) but it seems equally likely that virus arrives at the tail after replication in the viscera and secondary dissemination via the bloodstream; ectromelia infections in mice follow this pattern (Fenner, 1948). A large proportion of virus is probably removed rapidly from the blood by macrophages in the spleen and liver (Mims, 1964). Skin infection may in part be initiated by the small amount of virus – possibly associated with cells – that is not cleared from the blood (Mims, 1964), but I detected small amounts of virus in the spleen 8 hr. after inoculation which suggests primary localization in this organ. Increased amounts were present by 3–7 days. If virus began to leak into the blood at 2–3 days (Worthington *et al.* 1972) then as with cowpox virus (Mims, 1968) visible tail pocks might be expected about 6 days after infection.

The confinement of pocks to a particular area resembles the localization of lesions seen after inoculation of cowpox into mice (Mims, 1968) and rabbits (Downie, 1939) and the peripheral distribution of lesions in smallpox. Mims (1968) claims that only poxviruses giving red pocks on the CAM induce skin lesions and argues that action on blood vessels must be important. In preliminary experiments I found that Lister virus induced tail lesions although it gives white pocks on the CAM. Perhaps differences in skin temperature affect the distribution of lesions.

Pocks were clearly related to the size of the initial inoculum, but the relation was not linear over the range of virus used. The number of pocks was not limited by tail size since a similar dose response with a smaller maximal pock count was demonstrated in a different, less susceptible strain of mice (unpublished results). Larger inocula may have induced immune responses more rapidly.

Virus inoculated intravenously induced immunity both to intravenous and to intranasal challenge. The immunizing dose was less than that needed to induce pock formation. Immunity to intravenous challenge at 31 days was an all or nothing response and inocula as small as 1 p.f.u. protected as efficiently as larger doses; but the proportion of mice protected by such small doses was less at 189 days than at 31 days after infection. More mice were protected against death from intranasal infection by small immunizing doses than were protected against pock formation. Since much more multiplication is presumably needed to kill than to induce pocks this might be expected.

Multiplication of virus in the spleen diminished after 7–8 days and no pocks appeared after 9–10 days; reinoculation after 4 days failed to induce additional lesions so that the capacity to cope with primary and secondary infection obviously developed rapidly. The inability to reinfect mice as early as 4 days after infection may have been due to induction of interferon (Baron & Buckler, 1963; De Clercq & De Somer, 1968). However, by day 6 antibody which behaved as anti-FV was present in serum as the first evidence of a specific immune response. This finding presumably implies that there was no blood-borne spread of this form of virus after 5 days and possibly – since undiluted serum was not used in neutralization

tests – for some time before this. The earlier detection of anti-FV may be explained in terms of response to the progeny of the first cycle of infection rather than to the inoculum which probably contained mainly CAV; FV may be released earlier from cells than CAV. It cannot be ruled out, however, that more virus behaving as CAV may have been present in the blood to adsorb anti-CAV and render it undetectable. Yields were too low for total particle counts to be performed on FV pools and the ratio total particles: p.f.u. may have been much lower for FV and CAV and adsorption of antibody by inactive particles may have been less. It is unlikely that the anti-CAV added to the pool of FV during preparation might have adsorbed to FV to make it more sensitive to neutralization by anti-FV. 'Non-neutralizable' fractions were greater in titrations with FV than CAV suggesting that any such adsorbed antiserum obscured sites normally available to anti-FV in a manner analogous to that thought to account for the non-neutralizable fraction found in so many tests with poxviruses (Majer & Link, 1970).

The apparent 2-step production of antibody – both anti-CAV and anti-FV – may have represented sequential production of IgM and IgG. In mice IgM induced during the primary response diminishes rapidly, at least after stimulation by a non-replicating antigen (Torrigiani, 1971). The 2-step response may also of course have reflected persistance of viraemia since virus was detectable in the spleen for 11 days and may have been present after this time.

Antibody with the characteristics ascribed to that of anti-FV has been demonstrated as of prime importance in passive protection against early spread of infection (Boulter, Zwartouw, Titmuss & Maber, 1971). The greater amount of activity found here against CAV and the finding that a preponderance of virus released from HeLa cells was sensitive to anti-CAV suggest, however, that this form of virus must also be important in spread of infection. If antibody or cell-mediated lysis of cells bearing virus antigens takes place then increased release of CAV is more likely than leakage of FV and the anti-CAV component of antiserum becomes of great importance in the later stages of infection.

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