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The immune response to infection with vaccinia virus in mice

II. Appearance of hypersensitivity, production of macrophage migration inhibitory factor and transformation of spleen cells in response to virus antigens

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SUMMARY

The appearance of specific hypersensitivity to virus antigens was examined in mice infected intravenously with vaccinia virus. Both immediate hypersensitivity, transferable by serum, and delayed-type hypersensitivity, transferable only by cells, were apparent 8 days after infection and demonstrable for at least a further 130 days. Production of macrophage migration inhibitory factor by lymphocytes from infected mice was measured directly in terms of inhibition of migration by antigen or indirectly by determining the effect of soluble factors elaborated by the stimulated lymphocytes. The irregular results may have been the resultants of antigen-mediated macrophage stimulation, toxicity and induction of migration inhibitory factor. Transformation of spleen cells – presumably lymphocytes – from infected mice could be induced *in vitro* by virus antigens for at least 139 days after infection. Virus/lymphocyte interaction appears to be a particularly fruitful area for further study.

INTRODUCTION

The importance of cell-mediated immunity in recovery from or immunity to viral infections, in particular poxvirus infections, has recently been emphasized (Boulter, 1969; Glasgow, 1970; Blanden, 1971) and the onset, duration and magnitude of such responses merits at least as much study as that given to the acquisition of specific humoral antibody. The classic test for cell-mediated immunity is the presence of a delayed-type skin hypersensitivity reaction to intradermally inoculated antigen but the recent surge of interest in cellular immunology has led to the development of several *in vitro* models for detailed analysis of cell-mediated reactions. Two tests most frequently used in work with non-viral antigens involve study of production of macrophage migration inhibitory factor (MIF) and the transformation of lymphocytes in response to challenge doses of antigen (George & Vaughan, 1962; Oppenheim, 1968). The following work is an examination of the

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cell-mediated responses developed after infection with vaccinia virus in terms of skin hypersensitivity and an evaluation of lymphocyte transformation and MIF tests for more detailed analysis of the response.

MATERIALS AND METHODS

Animals

Outbred specific-pathogen-free CFLP female albino mice were used and infected intravenously as described in the previous paper (Hutt, 1975) with $10^{3 \cdot 3}$ p.f.u. of virus. For some experiments outbred female CS1 mice were obtained from Scientific Products Farm, Ash, Kent and inbred C57/BL female mice from Animal Supplies (London) Ltd. The CS1 and the C57/BL mice were not specific-pathogen-free and were kept under conventional conditions.

Virus

The WR strain of vaccinia virus was grown in mouse brain as described previously (Hutt, 1975). In addition, virus was passaged in primary chick embryo fibroblasts for use as challenge material in hypersensitivity experiments. This decreased the possibility of eliciting sensitivity reactions to host tissue. Control material was prepared from normal mouse brain. Similar batches of test and control material were prepared in HeLa cells for use in transformation experiments. Crude homogenates were used to ensure inclusion of all virus antigens. Virus was titrated as described previously (Hutt, 1975). All tissue culture virus used in hypersensitivity, MIF and transformation studies was inactivated by irradiation with ultraviolet light.

Measurement of hypersensitivity

Each mouse was inoculated with 0·03 ml. test antigen in one footpad and 0·03 ml. control antigen in the other. The test antigen contained $10^{7 \cdot 5}$ p.f.u./ml. before inactivation. Increase in footpad thickness was measured with a dial caliper gauge in units of 0·1 mm. Five readings were taken for each foot and the thickness was expressed as the mean. Specific swelling was calculated as the increase in thickness of the foot inoculated with test antigen less the increase in thickness of the foot inoculated with control antigen. Measurements were made at 3, 6, and 24 hr. so as to detect both immediate and delayed reactions; the latter diminished somewhat in size after 28–30 hr. Groups usually consisted of eight mice, some of which were killed after challenge for histological examination.

Histology

Feet were fixed in 10 % buffered formalin and decalcified in Gooding and Stewart's decalcifying fluid. Decalcified tissue was left overnight in lithium sulphate, dehydrated and embedded in paraffin. Sections were cut at 7 μm. and stained with hematoxylin and eosin or toluidine blue.

Tests for migration inhibitory factor

CS1 mice were used. Although fewer pocks developed on their tails and the antibody titres of their blood were about tenfold less than those of CFLP mice, the infection followed a similar course in both strains and their hypersensitivity responses were similar. Peritoneal exudates were induced with 2 ml. Freund's incomplete adjuvant (Difco) 72 hr. before collection and contained 50% macrophages, 50% lymphocytes and less than 1% polymorphs as judged by differential staining and uptake of carbon particles. Peritoneal exudate cells (PEC) were washed and treated by a method similar to that of Sandok, Hinsdill & Albrecht (1971). They were resuspended in Eagle's medium with 10% heat-inactivated fetal calf serum with or without antigen, incubated for 30 min at 37° C. at a concentration of 10^5 cells/ml. and diluted to 10^6 cells/ml. for another 3 hr. incubation in siliconized roller bottles. The cells were then centrifuged at 350 *g* for 5 min., resuspended in a small volume of the supernatant, packed into plain capillary tubes and incubated in Mackaness-type chambers at 37° C. in medium with or without antigen. Areas of migration of cells were measured by projection onto constant weight paper; the projected areas were cut out of the paper and weighed. Replicates were never fewer than 4 or more than 16. As a routine, measurements were made after incubation for 24 hr. at 37° C. The difference in migration of cells with and without antigen did not subsequently change. Results were expressed as a migration index (MI), where

$$\text{MI} = \frac{\text{area of migration in presence of antigen}}{\text{area of migration in absence of antigen}} \times 100.$$

Comparison was made of migration of three samples of normal PEC which had been treated by the method described above but in the absence of antigen. The mean areas of migration for each set were 1·03, 0·97 and 1·02 respectively, and the ratio of variance between sets to variance within sets was not significant. Since the variance of test values differed significantly from those of controls, a modified *t*-test was used to evaluate results.

Lymphocyte transformation

Mouse spleens were chopped in heat-inactivated fetal calf serum and pushed through stainless-steel mesh. Larger fragments were allowed to settle and the supernatant cell suspension was centrifuged for 10 min. at 350 *g*. Pellets were resuspended in Eagle's medium with 10% heat-inactivated fetal calf serum and with or without antigen. The cell concentration was adjusted to $10^{6\cdot7}$ cells/ml. and the suspension was dispensed in 4 ml. volumes in polystyrene tissue culture tubes (115 × 15 mm.). Cultures were incubated at 33° C. in air containing 5% CO_2. Medium was changed after 24 hr. and 1 μc. of tritiated thymidine (^{3}HTdR; Radiochemical Centre Ltd, Amersham) was added to each culture for the last 24 hr. of incubation. Incorporation of ^{3}HTdR was measured by precipitation of trichloracetic acid insoluble material onto glass-fibre filter papers, bleaching and drying the filter papers with methanol and placing the dry filters in scintillant

(8 g./l. butyl PBD in toluene) for measurement of radioactivity in an Intertechnique liquid scintillation spectrometer model SL40. The average coefficients of variance determined for 40 sets of duplicate cultures with and 40 sets of duplicate cultures without antigen were only 5·5% and 6·9%, respectively. Results were expressed as counts per minute (c.p.m.) or as a stimulation index (SI), where

$$\text{SI} = \frac{\text{mean c.p.m. with mitogen}}{\text{mean c.p.m. without mitogen}}$$

and represented the mean results from duplicate cultures. Since outbred mice were used, individual spleens were kept separate to avoid transformation in response to histocompatibility antigens.

RESULTS

Hypersensitivity to virus antigens after infection with vaccinia virus

One hundred and four CFLP mice were inoculated intravenously with $10^{3\cdot3}$ p.f.u. of WR virus and developed an average of 19 pocks per mouse. They were tested for hypersensitivity at the intervals shown in Fig. 1 and histological sections were made from feet of mice challenged at 0, 6, 11, 21 and 30 days after infection.

Three-hour reactions (Fig. 1*a*) appeared by day 8 and increased slightly on challenge between 8 and 30 days. They could be elicited at least until day 60. There was no infiltration of cells after challenge on day 0 or day 6 but there was some mononuclear infiltration on days 8, 11, 21, and 30 and clumps of polymorphs were seen.

Six-hour reactions (Fig. 1*b*) were elicited by day 8 and subsequently for at least 60 days. The intensity fell slightly at day 35 but otherwise altered little between days 8 and 60. There was no infiltration on challenge on day 0 or day 6 but on days 8, 11, 21 and 30 there were clumps of polymorphs and mononuclear infiltration which was more intense than at 3 hr.

Twenty-four-hour reactions (Fig. 1*c*) appeared by day 8 and increased slightly in intensity on challenge up to day 60; other tests on similar batches of mice showed that a response of this intensity was maintained for at least a further 130 days. Such responses were always larger than 3 or 6 h reactions. Infiltration was apparent by 8 days and was greater than that at 3 or 6 hr. No more cells were seen in 11-, 21- and 30-day sections than in those at 8 days. A few isolated mast cells were seen in sections stained with toluidine blue. No correlation was found between the intensity of hypersensitivity reactions and the number of pocks on individual animals.

Transfer of hypersensitivity

The nature of the reactions measured at 3, 6, and 24 hr. was verified by transfer experiments. Donor animals were taken from a batch of mice inoculated intravenously 166 days previously with $10^{3\cdot3}$ p.f.u. and reinoculated with the same material 30 days later. Eight of the mice were footpad tested on the day before the transfer experiments (Table 1). Another eight mice were bled and their sera

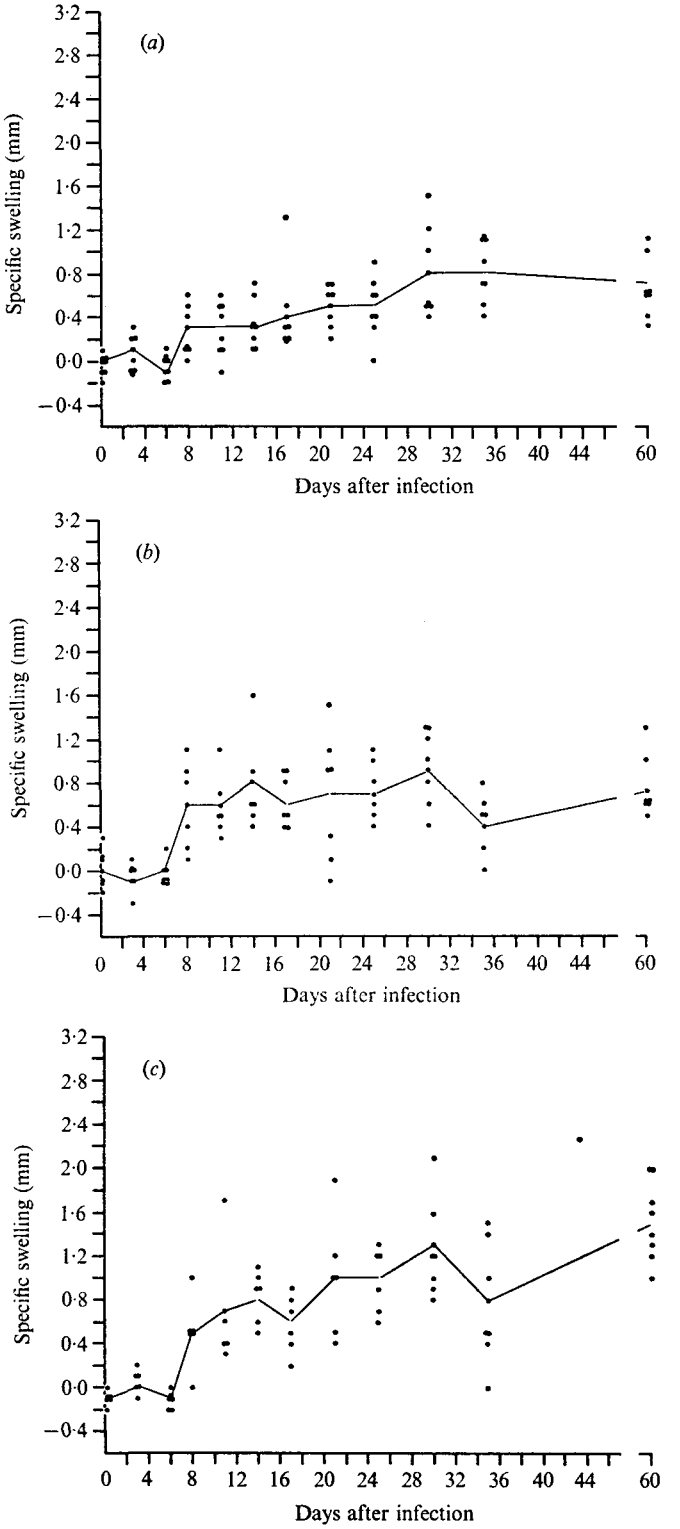

Fig. 1. Specific swelling of feet of mice in response to inoculation of inactivated virus into the footpad. Inoculations were made at intervals after infection with vaccinia virus and the swelling was measured at (*a*) 3 hr., (*b*) 6 hr. and (*c*) 24 hr. after inoculation. ——, Line fitted to mean values for each day; ●, readings on individual mice.

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Table 1. *Specific swelling of feet of normal recipient mice and infected mice after* 3, 6 *and* 24 *hr. in response to inoculation of inactivated virus into the footpad at* 0 *hr.*

Mice	No. in group	Mean specific swelling (mm.) at: 3 hr.	6 hr.	24 hr.
From same group as donors	8	1·3	1·2	1·2
Recipients of serum	3	0·9	0·2	0·1
Recipients of peritoneal cells	1	0·0	0·5	0·7
Recipients of spleen cells	8	0·0	0·5	0·4

Mice were inoculated with cells or serum from infected donors 1 hr. before footpad challenge.

Table 2. *Effect of inactivated virus on the migration of PEC from infected mice*

Day after infection	Antigen ($\log_{10}$ p.f.u./ml. before inactivation)	Migration indices* Normal mice	Infected mice	P†
13	6·5	97	54	< 0·001
	5·5	42	59	< 0·010
14	6·5	128	93	NS‡
	5·5	152	82	< 0·001
	5·4	154	112	NS
29	6·5	63	80	< 0·001
	5·5	89	57	< 0·001
31	5·5	83	117	< 0·001

* Migration indices calculated as $\frac{\text{migration in presence of antigen}}{\text{migration in absence of antigen}} \times 100$.

† P is the significance of difference between values for normal and infected mice.

‡ NS = not significant.

were pooled; their peritoneal cells were collected and pooled in Hanks' basal salt solution and a suspension of their spleen cells was prepared in the same fluid. Twelve normal mice from the same batch as the donors were inoculated with either 0·5 ml. of the spleen cell suspension containing 10^8 cells, 0·5 ml. of the peritoneal cell suspension containing 10^8 cells, or 0·5 ml. serum. These animals were footpad tested 1 hr. later. Three-hour reactions were apparent in mice receiving serum but there were no 6 or 24 hr. responses (Table 1). Spleen and peritoneal cells transferred only 6 and 24 hr. reactions.

Production of MIF by cells from infected animals

Migration of peritoneal cells from infected mice not previously inoculated with Freund's incomplete adjuvant was regular but greater than that of cells from normal animals. MIF tests therefore were performed with peritoneal exudate cells, which migrated similarly whether they were from normal or infected

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Table 3. *Effect of culture medium of spleen cells from mice infected* 13 *days previously on the migration of PEC from a normal guinea-pig*

Duration of migration (hr.)	Day of culture on which medium was collected	Migration indices with medium of cultures from:* Normal mice	Infected mice	P
24	1	145	104	$<0{\cdot}010$
	2	119	88	$<0{\cdot}050$
	3	106	105	NS
48	1	127	101	$<0{\cdot}020$
	2	110	95	NS
	3	94	89	NS

* Spleen cells were incubated with and without inactivated WR virus and indices were calculated as:

$$\frac{\text{migration in medium from cultures incubated with virus}}{\text{migration in medium from cultures incubated without virus}} \times 100.$$

animals. Tests were initially attempted with inactivated virus as the challenge antigen (Table 2). Antigen induced both enhancement and inhibition of migration of cells from normal and infected mice. No consistent response was evident in this or subsequent similar experiments despite the fact that mice from the same infected group as those killed for MIF tests demonstrated delayed hypersensitivity reactions to the inactivated virus.

Similarly inconsistent results were obtained when the following variations in method were made:

(i) The soluble complement-fixing LS antigens of vaccinia prepared according to the method of Ueda & Nozima (1969) were used to induce MIF instead of inactivated virus; although the lack of good controls makes their experiments difficult to interpret, Ueda and Nozima claim that this antigen is most important in stimulating MIF production.

(ii) Inbred C57/BL mice were substituted for the outbred CS1 animals; contradictory evidence has been published as to whether histoincompatibility of participating cells influences MIF tests (Al-Askari, David, Lawrence & Thomas, 1965; Phillips, Carpenter & Merrill, 1972; Nieburger & Youmans, 1973).

(iii) The challenge virus was purified according to the method of Robinson & Kaplan (1969), to reduce any non-viral toxic material.

More consistent results were obtained in an indirect test. The supernatant media of cultures of 10^8 spleen cells in 4 ml. medium with or without antigen were collected over a 3-day period, stored at $-60°$ C. and assayed as culture medium for migrating guinea pig PEC (Table 3). Migration in medium of cultures from normal and infected mice was significantly different after incubation for 24 hr. in 1- and 2-day medium. Differences were largely due to increased migration in antigen-containing medium from control cultures. Migration of PEC in antigen-free medium from mouse spleen cultures was not significantly different from migration in fresh medium ($P > 0{\cdot}1$ by the Student t test performed on figures for areas of

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Table 4. *Stimulation indices of spleen cells from infected mice after culture with inactivated WR virus*

Mice	Antigen (log_{10} p.f.u./ml. before inactivation)	Incubation time (days) 2	3	4	5	6	7
Infected	6·0	1·0	1·1	2·6	1·0	3·7	0·6
	5·0	0·9	1·3	2·8	1·2	4·8	0·9
	6·0	1·4	1·4	13·0	8·4	1·1	7·8
	5·0	1·0	1·5	10·3	6·6	0·6	8·0
	6·0	1·1	1·2	3·1	9·2	7·5	2·5
	5·0	1·0	1·4	3·5	6·5	7·3	4·7
Uninfected controls	6·0	—	1·0	0·1	6·1	—	0·4
	5·0	—	1·0	1·0	0·7	0·7	0·7
	6·0	—	0·8	0·8	0·2	1·7	0·5
	5·0	—	0·8	0·8	0·5	1·2	0·6
	6·0	—	0·8	0·8	4·3	0·2	0·8
	5·0	—	—	1·3	2·7	0·8	0·7

migration transformed to log_{10} values to reduce the difference in variance between groups). The increased migration was therefore not due to toxicity of the antigen for the spleen cells, failure to utilize medium and consequent preservation of nutrients for the PEC.

Transformation of mouse spleen cells

Preliminary experiments were performed with the non-specific mitogen phytohaemagglutinin (PHA) to ensure that culture conditions were suitable for transformation and to determine the optimum concentrations of ^{3}HTdR and unlabelled thymidine. The responses of cells from infected mice to inactivated virus material were then measured in cultures prepared from spleens of normal mice or animals killed 27 days after infection. Different spleens were incubated for different lengths of time and ^{3}HTdR was added for the last 24 hr. of culture in each case. Stimulation of cells from infected mice was first clearly apparent after 4 days of culture (Table 4). The stimulation index was greater than 1 in 87% of the 30 cultures from infected mice harvested on days 3–7 and in 22% of the 28 cultures from uninfected mice harvested over the same period. Although the two highest stimulation indices were obtained on days 4 and 5, high values could be sustained for at least 7 days and all subsequent cultures were examined for ^{3}HTdR incorporation after incubation for 5 days. Transformation indices were reduced if fewer than $10^{7·3}$ cells were used and it was impossible to use more without reducing replicates; the optimal amount of challenge virus varied slightly from virus pool to virus pool and from spleen to spleen but no significant increases were found by use of more than the equivalent of 10^6 p.f.u./ml. and amounts greater than 10^8 p.f.u./ml. were inhibitory. All virus pools were individually titrated for optimal activity in transformation tests.

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Table 5. *Effect of temperature on the stimulation indices of spleen cells from infected mice after stimulation with inactivated WR virus*

Antigen (log_{10} p.f.u./ml. before inactivation)	Incubation temperature	
	33° C.	37° C.
	5·0	1·4
	4·2	3·3
	3·1	0·9
Mean	4·1	1·9
4·7	2·0	0·9
	1·4	0·8
	1·5	1·0
Mean	1·6	0·9

Indices in the same positions in columns at 33° C. as at 37° C. referto cultures from the same spleens.

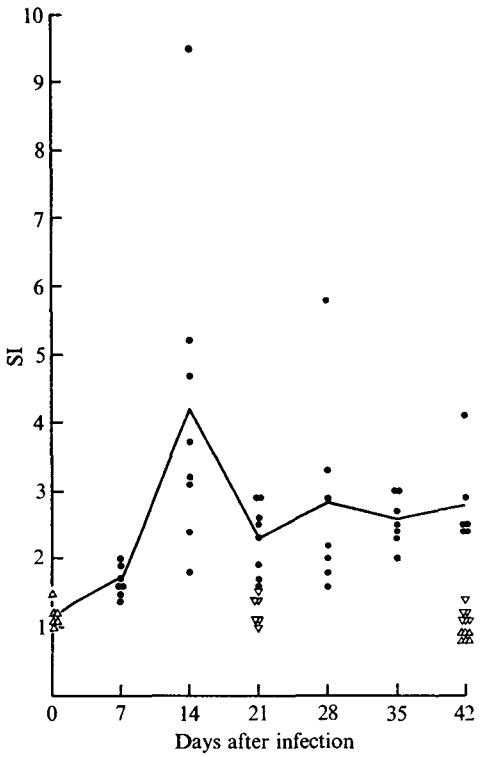

Fig. 2. Transformation of spleen cells from normal mice and from infected mice at various times after infection, in response to virus and control antigens. Transformation is expressed as a stimulation index (SI) where

$$SI = \frac{\text{incorporation of thymidine in presence of antigen}}{\text{incorporation of thymidine in absence of antigen}}.$$

●, Infected mice with virus antigen; ▽, infected mice with control antigen; △ normal mice with virus antigen; ——, line joining mean values for infected mice with virus antigen.

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Transformation of cultures was compared at 37° C. and at 33° C. Although spleen cells continued to respond to PHA at 37° C., there was less transformation in response to vaccinia virus at 37° C. than in replicate cultures incubated at 33° C. (Table 5). All cultures were subsequently incubated at 33° C.

The development of the ability of spleen cells from infected mice to transform in response to inactivated virus was followed in groups of 6–8 mice killed at various times after infection. The responses of cultures of spleens from these infected animals and from groups of normal controls were examined to irradiated virus material and to control material prepared from normal mouse brain which had been passaged and irradiated in the same manner (Fig. 2). Stimulation indices of cultures from infected mice with control antigen and normal mice with virus material were usually slightly greater than 1 but always less than the values obtained with cells from infected mice and virus material by 7 days after infection. Highest indices with the greatest scatter were seen in tests done on day 14. Values fell at day 21 but rose again slightly at day 28 and remained well above the day 7 values for at least another 4 weeks. The average number of pocks on the infected mice was 24.

DISCUSSION

Despite a contention that 'no reliable test for cell-mediated immunity in mice is available' (Worthington, Rabson & Baron, 1972), little difficulty was found in eliciting specific hypersensitivity to vaccinia or vaccinia associated antigens in these animals for at least several months after infection. Responses were first apparent on challenge at 8 days after infection. This is after the appearance of neutralizing antibody in the blood (Hutt, 1975) but preliminary experiments with different lots of mice and several similarly prepared batches of antigen suggest that whereas results with one antigen batch are reproducible in numerous tests, the sequence of inducibility of immediate and delayed hypersensitivity varies with different antigen batches.

Cellular infiltration at 24 hr. basically resembled in cellular composition that described by Collins & Mackaness (1968) although more cells were present; occasional clumps of polymorphonuclear cells were an unusual feature and may have been micro-abscesses due to necrosis or mild infection. Few basophilic cells were seen and there was no resemblance to the cutaneous basophil hypersensitivity which can be induced by vaccinia virus in guinea-pigs (Dvorak & Hirsch, 1971). Infiltration at 3 h was puzzling in that almost no polymorphonuclear cells were seen. The predominant cells were mononuclear and those polymorphs that were present were not distributed throughout the epidermis as described by others (Collins & Mackaness, 1968) but concentrated in dense clumps. Transfer studies did, however, confirm that 3 hr. responses were antibody-mediated and that 6 and 24 hr. responses were largely cell-mediated. With more information about the effects of variation in challenge dose there seems no reason why footpad tests should not prove as useful in studies of hypersensitivity to vaccinia virus in mice as they have done for other species and other antigens.

The results of tests for MIF were disappointing in their lack of reproducibility

especially since no similar difficulty was found in demonstrating MIF production by cells from guinea-pigs in direct or indirect tests with vaccinial antigens (unpublished results). The finding that unstimulated peritoneal cells from vaccinia infected mice migrated more than cells from uninfected animals was unexpected and contrary to findings of workers studying other viruses in mice (Feinstone, Beachey & Rytel, 1969; Tubergen & Oldstone, 1971). Perhaps macrophage activation of the type associated with increased macrophage mobility and phagocytic activity (North, 1969) might be effected by vaccinia virus. Activation of macrophages has already been suggested as a mechanism of the resistance to vaccinia virus shown by mice made allergic to certain bacteria (Allen & Mudd, 1973).

A very tentative hypothesis might be suggested to explain the anomalous results from MIF tests. Contact of mouse macrophages with vaccinia antigens may initially activate macrophages in terms of increased mobility and enhanced migration; sensitized lymphocytes may produce MIF. Production of MIF *in vitro* may be too slow for expression in a direct test in which there may be competition between the enhanced migration of activated macrophages and the inhibitory properties of MIF; toxic effects of antigen may add further complications. Combinations of these factors alone may account for the lack of obvious dose responses and poor reproducibility. The situation may be slightly simplified in an indirect system where MIF is present *ab initio* in PEC cultures. Perhaps variations in culture technique would increase the usefulness of the test in mice but at present the lack of reproducibility appears to render MIF tests of little value in analysing the cell-mediated response to vaccinia virus in this species.

The results of lymphocyte transformation tests were more encouraging. High indices were registered within the period found to be optimal for vaccinial stimulation in other species (Matsaniotis & Tsenghi, 1964; Rosenberg, Farber & Notkins, 1972); the optimal concentration that induced transformation varied slightly from animal to animal. Virus material stimulated cells from some uninfected mice; in one instance the stimulation index was 6·1 but this exceptional result may have been due to technical error. Tissue culture material without virus had similar effects on cells from infected mice. These have been reported by other workers (Smith, Chess & Mardiney, 1972); the reason for them is unclear.

The reduction in transformation when measured at 37° C. rather than at 33° C. was surprising. No direct comparisons of transformation at different temperatures were found in the literature but most workers incubate cultures at 37° C. The apparently intact PHA response at 37° C. suggests that the reduction in transformation at this temperature in response to vaccinia is a property of the virus; it may be more toxic at 37° C. Pinocytosis by lymphocytes may be more active at this temperature and facilitate entry of the virion into the cell.

Specific stimulation of cells from infected animals at intervals after infection followed the pattern described for cells from rabbits infected with vaccinia virus (Rosenberg *et al.* 1972) except that sensitivity persisted for much longer. The time course resembled that of delayed hypersensitivity rather than that of antibody (Hutt, 1975). It varied from that described for mice sensitized to PPD where

delayed hypersensitivity increased over a 12-week period while *in vitro* transformation markedly decreased (Phillips *et al.* 1972). The effects on transformation of antithymocyte sera, neonatal thymectomy, various regimens of immunization and different virus antigens should make a worthwhile study.

While doubt has been cast on the strict relationship between lymphocyte transformation and cell-mediated immunity (Bloom, 1971), the idea of a clear division of immune responses into cell-mediated and humoral has similarly become less acceptible and no information about cell/antigen interaction can be dismissed as necessarily irrelevant to either one or the other. Vaccinia virus does not multiply readily in normal leukocytes but can do so in those stimulated with PHA (Miller & Enders, 1968). If, like dengue virus (Halstead, Chow & Marchette, 1973) it also proves able to multiply in antigenically transformed lymphocytes, analysis of transformation reactions may open up a new approach to investigation of poxvirus infection and immunity.

This paper is based on a thesis accepted in October 1973 by the University of London for the degree of Ph.D.

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