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INDUCTION AND *IN VITRO* DEMONSTRATION OF CELLULAR IMMUNITY TO DNA AND RNA VIRUSES IN GUINEA-PIGS

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SUMMARY

Guinea-pigs were immunized with different cells infected with vaccinia virus, herpes simplex virus type 1, herpesvirus saimiri, and the virus of vesicular stomatitis. Development of cellular immunity against these viruses was observed with transformation of blood and spleen lymphocytes and with the migration inhibition test using peritoneal exudate cells. Cellular immunity against vaccinia virus was first seen 6 days after the inoculation of cell-bound vaccinia virus by lymphocyte transformation.

The activation of the vaccinia virus specific cellular immune response could be induced with tissue culture cells as soon as 3 hr after their infection with vaccinia virus. Since infectious virus particles are not synthesized within this time period, it is likely that virus-induced antigens in the cell surface are active in production of cellular immunity.

Vaccines from heterologous host cells were more effective inducers of an immune response than syngeneic cell cultures. For *in vitro* testing of cellular immunity to viruses, viral antigens could be used in both infective and inactivated form. Delayed hypersensitivity to viral antigens was always accompanied by immune reactions to the host cells used for virus propagation.

INTRODUCTION

Usually viral infections induce the production of humoral antibodies, whose neutralizing activity can be shown by *in vitro* examination. Neutralization of viruses by specific antiviral antibodies plays a special role as a protective mechanism in virus infections like poliomyelitis. In this disease the degree of immunity can be tested by titrating the antibody response (Bodian, 1952). However, in some other virus infections the titre of neutralizing antibodies cannot serve as a standard of virus-specific immunity (Allison, 1966; Blanden, 1970). In these infections cell-mediated immunity (CMI) seems to play an essential role. Delayed

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type immunity can be checked by skin test reactivity. There are also *in vitro* assays which can be used for testing of CMI, for example lymphocyte transformation and the migration inhibition test (George & Vaughan, 1962). These two tests seem to be suitable to test CMI for insoluble, soluble and viral antigens (David *et al.*, 1964, Rosenberg *et al.*, 1972a). We were interested in correlating these methods, for the preparation of infectious and inactivated viral antigens suitable for sensitization and *in vitro* testing. Furthermore we investigated the influence of the cell species used for virus propagation on the course of immunization and the accompanying immune reaction to these host cells.

MATERIALS AND METHODS

Cell cultures, virus propagation and virus titration

Cell cultures were grown and maintained in Eagle's minimum essential medium supplemented with 15% inactivated calf serum, 100 units of penicillin/ml and streptomycin (100 μg/ml). Calculation of $TCID_{50}$/ml were performed according to the method of Reed & Muench (1938).

Virus were propagated as follows.

Virus strain	Propagated on:	$TCID_{50}$/ml in the preparations used for immunization
Vaccinia	Permanent monkey kidney cells (Vero) Permanent baby hamster kidney cell (BHK) Primary rabbit kidney cells (RK) Primary embryonal guinea-pig fibroblasts (GPF)	$10^{6.5}$
Herpes simplex Type 1	NMRI mice brain BHK Vero	$10^{5.3}$
Vesicular stomatitis virus, strain Indiana	Primary owl monkey kidney cells (OMK) BHK Vero	$10^{6.5}$
Herpesvirus saimiri*	OMK	$10^{5.0}$

* Herpesvirus saimiri virus was obtained by Professor Dr R. Laufs, Hygiene-Institut, Göttingen.

Antigens and sensitization

The infected cell cultures and the aseptically removed tissue material were disrupted by three cycles of freezing and thawing. The protein content of the homogenates was equilibrated to 1 mg/ml (Lowry *et al.*, 1951) with Eagle's medium. After titration of virus infectivity the homogenates were intramuscularily injected into guinea-pigs. The animals, weighing between 300 and 400 g, were immunized three times with an interval of 3 days between each injection and again 1 week after the last injection. One week later the animals were bled. In experiments to measure the primary immune response only one injection was performed. The volume of the first injection was 6 ml, the following times always 3 ml. The results from four guinea-pigs were pooled for every experiment.

Formalin inactivation

Virus inactivation was performed by addition of 1:4000 diluted formaldehyde solutions (Merck number 4003 Darmstadt) at 37°C for 24 hr. After the incubation the formalin was bound by sodium pyrosulphide in an equimolar concentration. Decrease of infectivity was tested on Vero cells at 4-hr intervals.

Inhibition of virus growth by interferon

Interferon (kindly supplied by Dr G. Neumann, Hygiene-Institut, Göttingen) was produced in human lymphocytes using Sendai virus as inducer. Test tubes containing Vero cells were inoculated with arithmetic dilutions of interferon to assay the antiviral activity. After 24 hr stationary incubation the monolayers were washed with Eagle's medium and superinfected with 100 $TCID_{50}$/ml VSV. The interferon activity was read when cytopathic effects could be seen in control tubes without interferon. The titre of an interferon preparation was the highest dilution in which a 50% inhibition of cytopathic effects was obtained. Interferon was incubated with a titre of 1:64 for 60 min at a temperature of 37°C with OMK cells, grown in Roux flasks. Incubation was continued for 24 hr after adding a 5-fold volume of tissue culture medium. VSV (100 $TCID/_{50}$ml) was added and after 2 hr adsorption washed twice with medium. After a period of 36 hr the incubation at 37°C of the tissue culture was stopped by repeated freezing and thawing. The antigens for sensitization were prepared as described above.

Peritoneal macrophage migration

Five days after the last antigen injection the animals received 20 ml of 3% thioglycolate i.p. The test was performed in a modification of the method of David & David (1971). The animals were bled 48 hr later by heart puncture. Twenty millilitres of Hanks's balanced salt solution (HBSS) supplemented with 50 u of heparin (Thrombovetren, Promonta) were injected intraperitoneally and the peritoneal cells were aseptically removed. The cells were packed by centrifugation and washed three times in HBSS containing 5% homologous serum. After 1:10 dilution in culture medium, containing TCM 199 with 15% homologous, kaolin-absorbed, inactivated serum, 100 u of penicillin and 100 μg of streptomycin, the cells were incubated at 37°C with 20% antigen (1 mg protein/ml).

After 1 hr of incubation small capillary tubes (1·2 mm, inside diameter) were filled with the cell suspension, sealed at one end and after centrifugation at 25 *g* for 5 min the tubes were cut at the cell–fluid interface. The cell-containing portions were mounted with silicon grease

in leucocyte migration chambers (Greiner, Nürtingen, number 25S). After filling with medium containing 10% antigen, the chambers were closed with greased cover glasses. Twenty-four hours later the areas of migration were projected on plain paper, cut out and weighed. The results were expressed as percentage migration. The migration of virus-free controls was taken as 100% migration. Every assay for every animal was run in triplicate. Chambers with antigen-free medium and chambers containing medium with non-infected tissue cell antigen acted as controls. Nonspecific effects were excluded by testing the cells from an unsensitized animal in every investigation. The infectious virus cell homogenate and the tissue antigen control used for investigation had no migration inhibitory activity on unsensitized peritoneal cells.

The percentage of migration of antigen-incubated cell cultures was calculated as [(migration in medium containing virus infected cells)/(migration in medium containing uninfected cells used for propagation of test antigen)] × 100.

Lymphocyte transformation

Blood was aseptically removed by heart puncture and 25 u of heparin/ml were added The erythrocytes were sedimented for 45 min by the addition of 1 ml of 5% Dextran (molecular weight 250,000, Pharmacia, Uppsala) to 9 ml of blood and incubation at 37°C in tubes held at an angle of 45°. The cells in the supernatant were washed twice in HBSS containing 2% homologous serum and 5 u of heparin and then suspended in the same culture medium as were the peritoneal exudate cells, in a concentration of 1×10^6 cells/ml. In the preparation of spleen lymphocytes the spleens were aseptically removed, cut with scissors and cell suspension obtained by repeated pipetting. The cells were then suspended in culture medium containing 30% homologous serum. After 1 hr of sedimentation at 4°C the phase containing single cells was withdrawn and after washing in HBSS the cells were suspended in the same culture medium as the blood lymphocytes. Tests were carried out by the addition of 0·2 ml amounts of: (a) viral antigen; (b) non-infective tissue homogenate from cells used for the propagation of the sensitizing viral material; (c) non-infective tissue homogenate from cells used for the propagation of viral material used for the *in vitro* tests, to 1-ml amounts of lymphocyte suspension in plastic tubes (number 160 S, Greiner, Nürtingen). All these antigens were of equal protein content (1 mg/ml). Other controls were set up with tissue culture medium alone. Further controls with phytohaemagglutinin (Wellcome) were run to test the ability of stimulating cells *in vitro*. The tubes were closed with loose caps and incubated at 37°C for 5 days in an atmosphere of 5% CO_2 in air. Every culture was set up at least in triplicate.

Twelve hours before the stopping of cultures 1·5 μCi of [^{3}H]thymidine (specific activity 23 mCi/mmol, Radiochemicals Amersham, Buchler, Braunschweig) was added to every tube. DNA extraction was performed after the cultures were terminated (Havemann, 1969). Incorporation of radioactivity was counted in a liquid scintillation spectrometer (Packard, Model 3380) and calculated as disintegrations per minute (d/min) or as a transformation ratio (ratio of incorporation in antigen-transformed cultures to control cultures).

RESULTS

Induction of cellular immunity to viral antigens

Sensitization of guinea-pigs with vaccinia, VSV and herpesvirus saimiri resulted in cell-

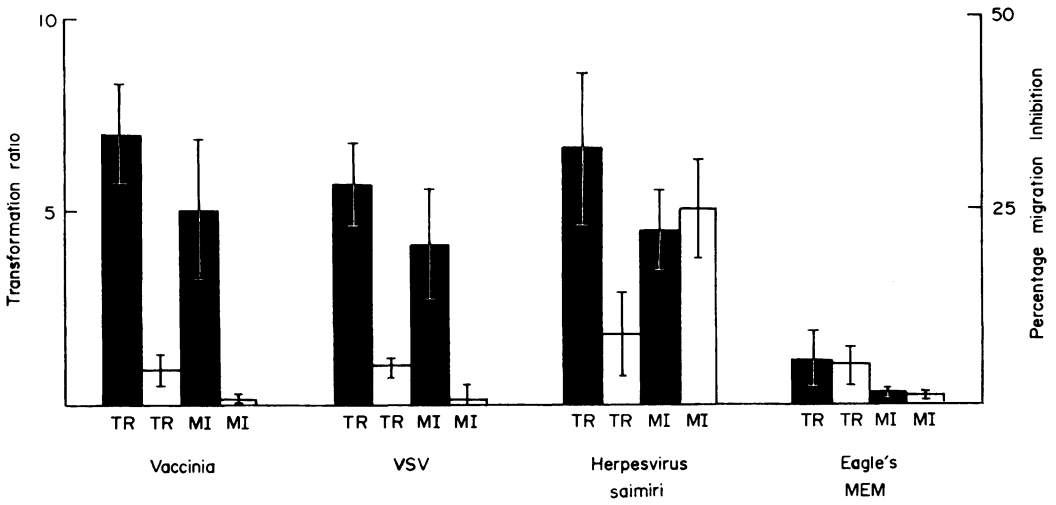

FIG. 1. Comparison of lymphocyte transformation with migration inhibition of the guinea-pigs injected with vaccinia virus, virus of vesicular stomatitis and herpesvirus saimiri. Vaccinia virus and VSV used for sensitization were propagated in BHK cells, herpesvirus saimiri in OMK cells. Antigens for *in vitro* testing were propagated, if possible, in different host cells, vaccinia in Vero cells, VSV in OMK cells, herpesvirus saimiri in OMK cells. (■) Virus-infected cells. (□) Uninfected cell controls. The columns show the transformation ratio (TR) or percentage migration inhibition (MI) of cells from animals injected with vaccinia virus, VSV or herpesvirus saimiri. Controls were run with animals injected only with medium; the depicted results with vaccinia are representative for all viruses used in this experiment.

TABLE 1. Specificity of *in vitro* stimulation of immune lymphocytes by vesicular stomatitis virus and vaccinia virus

Antigens used for *in vitro* transformation* ($TCID_{50}$/ml $10^{6\cdot5}$)	[^{3}H]thymidine transformation of lymphocytes from guinea-pigs immunized with VSV†		[^{3}H]thymidine transformation of lymphocytes from guinea-pigs immunized with vaccinia	
	Uptake (d/min)	Ratio ± s.d.	Uptake (d/min)	Ratio ± s.d.
VSV	1864	3·46 ± 0·33	656	0·90 ± 0·36
Vaccinia	496	0·92 ± 0·22	2256	3·13 ± 0·88
Vero cell control	539	– –	721	– –

* Virus antigens used for *in vitro* test were propagated on Vero cells, while immunization was performed with virus material, which was grown on BHK cells.

† Lymphocytes were obtained from four guinea-pigs in each group 7 days after immunization, the values are the mean of three assays with at least triplicate cultures per animal for each antigen.

mediated immunity, as shown by lymphocyte transformation and migration inhibition. Both tests showed a good correlation of the results. The immune reaction was virus-specific and cross-reactions between different viruses were not observed (Fig. 1, Table 1). After a single injection of viral antigens the lymphocyte transformation test and migration inhibition test were performed every 3rd day post-inoculation. Specific cellular immune

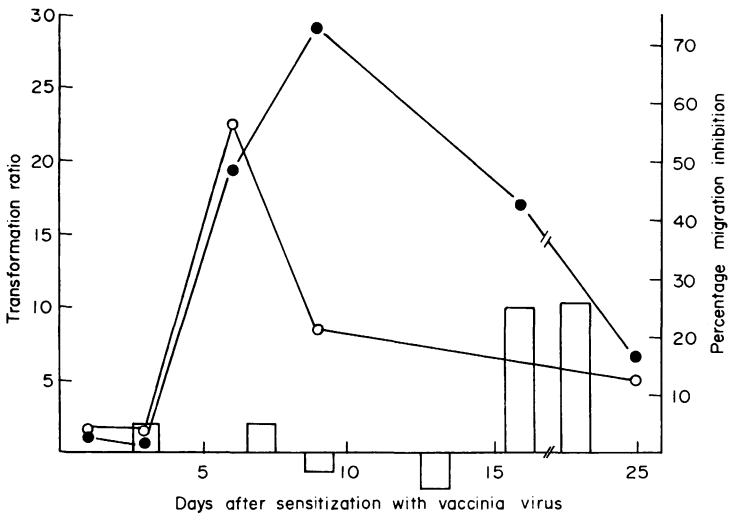

FIG. 2. Relationship of lymphocyte transformation to migration inhibition after sensitizing with vaccinia virus. Thirty-two guinea-pigs were injected intramuscularly with 6 ml of vaccinia virus $TCID_{50}$/ml $10^{6.5}$ propagated on BHK cells. Virus propagated in Vero cells was used for *in vitro* testing. In 3- or 4-day intervals animals were killed and transformation with peripheral blood lymphocytes (●) and spleen lymphocytes (○) as well as migration inhibition tests (□) with peritoneal exudate cells were performed.

reactions could be demonstrated in lymphocyte transformation 6 days after the application of vaccinia virus, while significant migration inhibition was first seen 10 days later (Fig. 2).

Infectious tissue homogenates of vaccinia virus were used as antigen for the lymphocyte transformation test and for the migration inhibition test and compared with formalin-inactivated vaccinia virus. Both the non-inactivated and the inactivated vaccinia virus gave the same results. In the comparison of formaldehyde-inactivated herpes simplex virus (HSV) to live HSV the results were different. While inactivated HSV gave only positive results with lymphocytes from HSV-immunized guinea-pigs, the live HSV also stimulated lymphocytes derived from animals not sensitized with HSV. Active and inactivated herpesvirus saimiri gave positive results only with lymphocytes of animals injected with herpesvirus saimiri (Table 2).

The relationship between the induction of cellular immunity and the duration of virus replication in tissue culture was investigated. Lymphocyte transformation and migration inhibition test could be correlated, showing that cellular immunity to vaccinia virus can be induced by tissue cultures infected with vaccinia virus for 3–4 hr (Fig. 3). During this period of time there is no production of new virus particles. Production of complete new virus is not necessary to induce cellular antiviral immunity. However, the production of viral antigens by the infected cells is a prerequisite for the induction of a virus-specific cellular immune response. When the propagation of VSV on OMK cells was inhibited by interferon, the interferon-treated tissue cultures could not induce VSV-specific cellular immunity. As was expected, cellular immunity to VSV developed only in the animals that had been immunized with VSV-infected, non-interferon-treated cell cultures (Table 3).

TABLE 2. *In vitro* reactions of cell-mediated immunity after immunization with different viruses. Virus specificity and actions of viral and inactivated vaccines on lymphocyte transformation and migration inhibition

Antigen used for:		Transformation ratio (±s.d.) of lymphocytes from:		Percentage migration inhibition
Immunization	*In vitro* test	Spleen	Blood	
Vaccinia	Vaccinia (BHK)	n.t.	6·95±0·71	20
(Vero)	Vaccinia (BHK) (formalin-inactivated)	8·43±1·50	n.t.	55
	HSV (BHK)	n.t.	9·75±0·78	2
	HSV (BHK) (formalin-inactivated)	n.t.	1·08±0·32	+4
	BHK cell antigen	1·01±0·22	1·25±0·14	1
HSV	HSV (BHK)	n.t.	5·83±0·95	24
(Vero)	HSV (BHK) (formalin-inactivated)	n.t.	8·43±2·07	72
	HV saimiri (OMK)	1·27±0·33	0·56±0·16	1
	HV saimiri (OMK) (Tween-ether-inactivated)	1·50±0·76	1·26±0·32	n.t.
	BHK cell antigen	0·83±0·21	1·44±0·35	n.t.
HV saimiri	HV saimiri (OMK)	n.t.	6·63±2·73	n.t.
(OMK)	HV saimiri (OMK) (Tween-ether-inactivated)	1·65±0·42	2·72±1·20	n.t.
	OMK cell antigen	0·73±0·29	1·42±0·64	25
	HSV (BHK)	6·79±0·44	n.t.	n.t.
	HSV (BHK) (formalin-inactivated)	0·93±0·34	n.t.	6

n.t. = Not tested.

Importance of the host cell for in vitro antiviral immune reactions

Vaccinia virus was propagated *in vitro* in Vero, BHK, RK and GPF cells and *in vivo* in NMRI mice brain. After sensitization of guinea-pigs with these infected cells, *in vitro* tests were performed. Viral antigens used for sensitization and for *in vitro* tests were propagated in host cells different from those used for the antigen propagation which were used for animal inoculation. Non-infected tissue cultures served as controls. In both tests strong immune responses were observed after sensitization with viral antigens grown on heterologous host cells. Propagation in isologous cells induced weak *in vitro* reactions (Table 4).

Simultaneously with cellular immunity to viral antigens a clear immune response to the host cell antigens, used for the propagation of sensitizing virus, was observed, reaching transformation ratios up to 5:39 (Table 4).

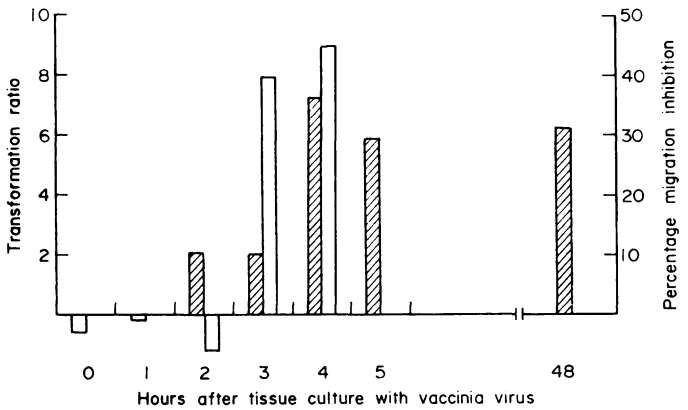

FIG. 3. Induction of an immune response dependent on time of virus propagation in sensitizing tissues. Vero cells were infected with $TCID_{50}$/ml $10^{3\cdot5}$ vaccinia virus. After 2 hr of adsorption monolayers were washed six times with medium. Thereafter no virus was found in the supernatants. Propagation of virus was stopped at hourly intervals. Logarithmic increase of infective virus in the supernatants began after 10 (10 hr = $10^{1\cdot5}$, 20 hr = $10^{2\cdot5}$, 30 hr = $10^{3\cdot5}$ and 40 hr = $10^{4\cdot4}$ $TCID_{50}$/ml). The infected tissue homogenates were injected into different groups of animals. Lymphocyte transformation and migration inhibition test to vaccinia virus were performed with cells collected 1 week after the last injection. (■) Transformation ratio. (□) Percentage migration inhibition.

TABLE 3. Sensitization of guinea-pigs with VSV-infected cell cultures. Inhibition of sensitization by prevention of virus propagation with interferon

Antigen used for:		Transformation ratio (± s.d.) lymphocytes from:		Percentage migration inhibition
Sensitization	*In vitro* test	Spleen	Blood	
OMK cell control	VSV (BHK)	1·38±0·62	1·25±0·24	+2
OMK+interferon +VSV	VSV (BHK)	1·13±0·56	0·95±0·36	−1
OMK+interferon controls	VSV (BHK)	1·54±0·58	1·69±0·78	n.t.
OMK+VSV	VSV (BHK)	6·24±0·53	5·59±1·72	−22

n.t. = Not tested.

TABLE 4. Importance of host cell for *in vitro* immune response to viral antigens. Increased cell-mediated immune response to tissue antigens, in which the sensitizing virus had been propagated

Antigen used for:		Transformation ratio of blood lymphocytes	Percentage migration inhibition
Sensitization	*In vitro* test	$\bar{X}$ s.d.	$\bar{X} \pm$ s.d
Vaccinia (BHK)	Vaccinia (Vero)	37·51 ± 22·49	n.t.
	BHK-cell antigen not infected	4·20 ± 1·08	n.t.
Vaccinia (RK)	Vaccinia (Vero)	20·88 ± 12·23	−22 ± 5
	RK-cell antigen not infected	5·39 ± 4·17	−19 ± 4
Vaccinia (mouse brain)	Vaccinia (Vero)	22·41 ± 14·69	−32 ± 8
	Brain-antigen not infected	4·24 ± 2·13	−35 ± 12
Vaccinia (GPF)	Vaccinia (Vero)	2·36 ± 0·94	−14 ± 2
	GPF-cell antigen not infected	1·76 ± 0·69	+8 ± 3

n.t. = Not tested.

DISCUSSION

Our knowledge of immunity to viral infections in man is substantially based on the determination of humoral antibodies. The existence of antibodies gives evidence of a former contact with viral antigens. Humoral antibodies can serve as markers for immunity. However, the immune status is not only dependent on humoral antibodies. Persons with an antibody-deficient syndrome can resist to some of the viral infections (Good & Varco, 1955), on the other hand disturbances of the immune system, which include impaired CMI, can effect the fate of the disease in some of the viral infections (Seligman *et al.*, 1968; Hirsch *et al.*, 1968; Blanden, 1970; Fulginiti, 1971). This points to the role of cellular immune mechanisms in viral infections.

Demonstration of cell-mediated immunity by skin reactions is possible (Noren *et al.*, 1966) but soluble viral antigens used for testing, even after a single cutaneous application, can induce virus-specific immune reactions (Rogers *et al.*, 1972). Therefore follow-up with *in vivo* test methods are limited. The investigation of cellular immunity to viral antigens in man, by means of *in vitro* testing, solves this problem.

There have been investigations with animals on CMI to viruses using migration inhibition (Feinstone *et al.*, 1969; Tompkins *et al.*, 1970; Pilchard *et al.*, 1970) and lymphocyte transformation (Rosenberg *et al.*, 1972a, b). The T-cell cytotoxicity test was applied to demonstrate CMI to mumps virus (Speel *et al.*, 1968), lymphocytic choriomeningitis virus (Lundsted, 1969; Marker & Volkert, 1973; Zinkernagel & Doherty, 1974), ektromelia virus

(Gardner *et al.*, 1974), leukaemia virus (Profitt *et al.*, 1973; Wright *et al.*, 1973) and sindbis virus (McFarland, 1974).

We could induce cellular immunity to DNA and RNA viruses in guinea-pigs. Lymphocyte transformation and migration inhibition correlated very well. Induction of cellular immunity with vaccinia virus was observed 6 days after sensitization of the guinea-pigs using the lymphocyte transformation test. In migration inhibition the first positive reactions were found 10 days after injection, this could be due to a different sensitivity of the tests. The early peak of lymphocyte response has also been reported by others (Rosenberg *et al.*, 1972a; Gardner *et al.*, 1974). The early appearance of the maximal lymphocyte response, as measured *in vitro*, is followed by diminishing reactivity of the lymphocytes. Therefore high and low lymphocyte transformation ratios were observed in the same system with varying time intervals. Similar results were obtained by other authors using a modified migration inhibition test in rabbits with fibroma virus (Tompkins *et al.*, 1970). By skin testing in animals with herpes simplex virus (Rogers *et al.*, 1972) and vaccinia virus (Pilchard *et al.*, 1970), positive reactions could be demonstrated beginning 3–4 days after sensitization and reaching a maximum within 10 days. We observed that the virus-specific immune response of the guinea-pigs was accentuated after using heterologous host cells for the propagation of the immunizing virus.

During the procedure of sensitization to viral antigens the animals also develop an immune response to the tissue cell antigens in which the virus has been propagated. The immune response to injected tissue cell antigens is more accentuated when the cells are infected with vaccinia virus as compared to non-infected cells of equal protein content. The viral infection may act as an immunological adjuvant for tissue cell transplantation antigens (Bandlow *et al.*, 1972). Therefore viruses for sensitization and for *in vitro* testing should be propagated in different tissues, otherwise an immune response to tissue cell antigens may deceive a virus-specific immune reaction. Following such a procedure the purification of the test viruses can be omitted. Furthermore in lymphocyte transformation with DNA viruses, false positive reactions, caused by viral actions on lymphocytes with the incorporation of [^{3}H]thymidine, must be excluded (Bouroncle *et al.*, 1970). In our experiments we could observe [^{3}H]thymidine incorporation in lymphocytes of non-immune guinea-pigs by using infectious herpes simplex virus as antigen. This can be avoided by using inactivated viruses for *in vitro* testing. On the other hand, inactivation was accompanied by a certain loss of the stimulating activity of the virus preparations (Koszinowski *et al.*, 1974). Observing these limitations in *in vitro* reactions, cellular immunity can be demonstrated to be virus-specific and not caused by unspecific reactions.

Cell cultures, in which the propagation of virus was interrupted as early as 3 hr after infection, were shown to induce antiviral immunity. During these 3 hr there were no newly produced vaccinia virus particles; however, virus-specific antigens can be demonstrated on infected cell membranes (Ueda *et al.*, 1972). One could speculate that besides attached viral antigens, which are not removed by washing, early viral antigens on the cell surface may be equal to or even more effective than complete viral particles in the production of CMI to viral antigens. This finding is supported by the fact that viral surface antigens can act as an *in vivo* inducer of skin resistance (Ueda & Tagaya, 1973) and *in vitro* as antigens in the investigation of cellular immunity (Tompkins *et al.*, 1970). However, if virus growth is prevented by interferon, cellular immunity to viral antigen cannot be induced.

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