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CELL SURFACE ANTIGENS INDUCED BY POXVIRUSES

I. EFFECTS OF ANTIMETABOLITES ON CELL SURFACE ANTIGENS

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SUMMARY A virus-induced cell surfare antigen (VICSA) of FL or RK1 cells infected with poxvirus was demonstrated by immune hemadsorption (IHAd) and the fluorescent antibody technique without fixation (uf FAT). Sera from rabbits immunized with Shope fibroma virus (SFV) OA strain, cowpox virus (CPV) LB red strain (CPVR) and FL cells were used.

VICSA was observed between antiviral sera and FL cells infected with homologous poxviruses. VICSA was detectable on the surface of cells infected with CPVR from 2 hr after infection, and in cells infected with SFV OA from 10 hr after infection by IHAd or uf FAT.

The cross reactions of VICSA between anti-CPVR rabbit serum and cells infected with two strains of vaccinia virus (Ikeda and Ecuador-Moscow 63), several strains of CPV (50, 51, 58 and Amsterdam), ectromelia virus (Hampstead) and variola virus (Harvey) were all positive by IHAd and uf FAT. But VICSA of cells infected with SFV OA only gave a weak positive reaction by IHAd.

The surface antigens of the host cells themselves were not affected appreciably by virus infection, and may overlap virus induced antigens after infection, since uninfected FL cells and FL cells infected with CPVR or SFV OA both gave strongly positive IHAd with anti-FL cell serum.

Formation of VICSA on cells infected with CPVR was not inhibited by the presence of cytosine arabinoside or 5′-2′-fluorodeoxyuridine. This indicates that the production of VICSA is independent of the replication of viral DNA. When actinomycin S or puromycin was added within 1 hr or 3 hr, respectively, after infection, no VICSA could be detected. This suggested that the VICSA is a protein synthesized within 3 hr after infection, and that its synthesis is mediated by messenger RNA transcribed within 1 hr after infection.

Cells infected with CPVR showed simple hemadsorption with chick red cells mediated by hemagglutinin induced by virus infection. Cells infected with CPVR in the presence of cytosine arabinoside did not show simple hemadsorption but still produced VICSA. VICSA could be demonstrated in cells infected with SFV OA which does not induce hemagglutinin. These results suggest that VICSA of poxvirus is not identical with hemagglutinin.

INTRODUCTION

A virus-induced cell surface antigen (VICSA) has been demonstrated by several different immunological methods on transformed cells after infection with several viruses. The method used were the complement fixation test (Black et al., 1963; Huebner et al., 1963; Sabin and Koch., 1964; Habel et al., 1965; Defendi et al., 1964), the fluorescent antibody technique (FAT) (Tevethia et al., 1965a b; 1968; Irlin, 1967; Kluchareva et al., 1967; Malmgren et al., 1968; Diamandopoulos et al., 1968), the transplantation immunity test (Khera et al., 1963; Habel and Eddy, 1963; Defendi, 1963; Deichman and Kluchareva, 1964; Eddy et al., 1964; Rapp et al., 1966) and the colony inhibition test (Hellström and Sjögren, 1967; Hellström and Hellström, 1969). Hamada and Uetake (1968) showed that the appearance of VICSA on cells infected with several highly oncogenic adenoviruses (Type 12, 18 and 7) was popular phenomen regardless of the abortive infection occasionally resulting in cellular transformate or productive. VICSA has also been demonstrated in the cytolytic cycle of infection with Polyoma virus and SV40 (Irlin, 1967; Kluchareva et al., 1967).

VISCA in cultured cells infected with various nononcogenic viruses has been demonstrated by immune hemadsorption (IHAd) (Fagraeus and Espmark, 1961). Positive results were obtained with cells infected with vaccinia, measles and canine distemper viruses (Fagraeus and Espmark, 1961). However, the possibility that the hemagglutinin of these viruses on the surface of virus-infected cells is involved has not been excluded.

In preliminary work (Miyamoto and Kato, 1968), VICSA was demonstrated on cells infected with several cowpox viruses (hemagglutinin +) and with Shope fibroma virus (hemagglutinin —) using IHAd and the fluorescent antibody technique without fixation (uf FAT). Furthermore, in the presence of cytosine arabinoside, cells infected with poxvirus failed to show viral DNA synthesis or simple hemadsorption with chick red blood cells but still produced VICSA.

Ueda et al. (1969) also demonstrated VICSA on cells infected with vaccinia virus by uf FAT.

This paper reports the effects of antimetabolites on the induction of VICSA by oncogenic and nononcogenic poxviruses.

MATERIALS AND METHODS

1. *Cells and media*

A cloned line of FL cells (derived from human amnion cells) and BSC1 cells (derived from African green monkey kidney cells) were grown in Eagle's minimum essential medium (Eagle's MEM) (Eagle, 1959) with 5% heat inactivated calf serum. RK1 cells (derived from Rabbit kidney cells) were grown in Ham F 12 medium (Ham, 1965) with 10% heat inactivated calf serum. All media contained 100 units/ml of penicillin and 100 μg/ml of streptomycin.

FL cells were removed from the bottle with phosphate buffer saline without Mg^{++} and Ca^{++} (PBS —) (Merchant et al., 1960) with 0.02% EDTA and RK1 or BSC1 cells were removed with PBS (—) with 0.25% trypsin (Difco 1 : 250).

Cells were dispersed in Leighton tubes each containing a coverslip (11 × 42 mm). Monolayer cultures of FL or RK1 were infected with virus. Each Leighton tube contained about 5×10^5 cells. Monolayer cultures of BSC1 cells in plaque bottles were used for assay of plaque forming units of CPVR.

2. *Viruses*

Shope fibroma virus (SFV) OA strain (hemagglutin —) from the American Type Collection Center was passaged intracutaneously in rabbits 4 times, and in primary rabbit kidney cells 8 times. Then it was passaged RK1 cells. Virus at the 4th passage level was used as the stock virus solution.

Cowpox virus (CPV) LB red strain (CPVR) has been serially passaged in FL cells in this laboratory since 1958 (Kato et al., 1959). Other strains of CPV (50, 51, 58 and Amsterdam) originally obtained from Dr. R. Gispen (NIH Utrecht, Netherlands) have been maintained in FL cells in this laboratory since 1963.

Vaccinia virus (Ikeda and Ecuador-Moscow 63 strain), variola virus (Harvey strain) and ectromelia virus (Hampstead strain) were also used.

Stock solutions of these viruses were obtained as

follows. Twenty-four hr after infection with these viruses, cells were centrifuged and suspended in Eagle's MEM or Ham F 12 without serum and disrupted by sonic vibration at 1,000 cycles per second for 5 min with a Toyo 200 watt 20 Kc magnetostrictive oscillator. The sonicated samples were centrifuged at 3,000 rev/min and the supernatants were stored in a Reveco at −120 C as virus stock preparations.

3. *Assay procedures*

SFV was titrated as described previously by Kato et al. (1963 c). The SFV index was also used for titration of SFV as follows. Serial dilutions of stock virus were made with Ham F 12. Aliquots of 0.5 ml of viral suspension were inoculated into monolayer cultures of RK1 cells in Leighton tubes. The number of cells bearing " B " type inclusion bodies, indicating viral multiplication at a cellular level, was counted after incubation for 24 hr (Kato et al., 1963 b), and the percentage of inclusion bearing cells instead of tumor forming units is expressed as the SFV index.

Infectivity of CPVR was assayed on monolayer cultures of BSC1 cells and titers are expressed as plaque forming units (PFU).

4. *Infection*

An inoculum of 0.5 ml of virus solution was introduced into each Leighton tube. The inoculum of SFV OA had an SFV index of about 100. The multiplicity of infection of CPVR was 1 to 10. Infected cultures were washed three times with 3 ml volumes of Hanks' salt solution after 1 hr and 3 hr incubation periods for adsorption of CPVR and SFV OA, respectively, and fresh medium was added.

5. *Immune sera*

Domestic rabbits, weighing 3 to 4 Kg, were used to obtain hyperimmune sera.

1) Anti-SFV immune rabbit serum

Rabbits were inoculated intracutaneously with stock solution of SFV OA. The tumor grew progressively at the site of virus inoculation. About ten days later, the tumors ceased to increase in size, and began to degenerate showing crust formation. About one month later, the crust desquamated and finally disappeared completely (Kato et al., 1963 c). Then a stock solution of SFV OA was inoculated intravenously two or three times at weekly intervals. Ten to 15 days after the last inoculation, blood was collected. The titer in the complement fixing antibody reaction was × 512.

2) Anti-CPVR immune rabbit serum

One milliliter of diluted stock solution of CPVR (10^4 PFU/ml) was injected into the skin of the back of a rabbit. An inflammatory reaction occurred at the site of virus inoculation, which persisted for a month and then ceased leaving a scar. Two months after virus inoculation immune serum was obtained. The titer of the serum in the complement fixation antibody reaction was × 1024.

3) Anti-FL cell immune rabbit serum

A suspension of washed FL cells from a fully grown culture bottle was mixed with an equal volume of complete Freund's adjuvant to give an emulsion of cell antigen. One milliliter of the emulsion, containing 1×10^8 cells, was inoculated into rabbits intracutaneously 3 to 4 times at intervals of 2 weeks.

One month after the last inoculation, blood was collected after the serum had been tested by IHAd. The IHAd titer of the anti-FL cell serum was 1×10^5. No reaction of the serum against RK1 cells was detected by IHAd.

4) Other anti-viral sera

Anti-SV 40 and -polyoma virus immune rabbit sera were kindly supplied by Dr. A. Hakura of this Institute.

Anti-chikungunya virus (Ch V) and -Japanese B encephalitis virus immune rabbit sera were also supplied by Dr. M. Mantani of this laboratory and by Dr. K. Takaku (Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kagawa).

5) Other antisera

Anti-sheep erythrocyte immune rabbit serum (Hemolysin) with a titer of × 4000 was purchased from Midori Jūji, Osaka.

Anti-rabbit γ-glubulin immune goat serum (Coombs' serum) was purchased from Hyland Laboratories, U.S.A., and kindly supplied by Dr. K. Kano (State University of New York, Baffalo) and Dr. S. Tanabe of this Institute.

Normal rabbit serum was obtained from newly purchased animals.

6. *Immune hemadsorption procedure*

The immune hemadsorption procedure, originally described by Milgrom et al. (1964), was carried out as described previously (Miyamoto and Kato, 1968). Volumes of 1 ml of antisera were put into a series of tubes with monolayers. The tubes were kept for

1 hr at room temperature. Then the monolayers were washed three times with Hanks' salt solution.

In this experiment, indicator cells were prepared as follows. Sheep red blood cells (SR) were washed with PBS (−) until the supernatant fluid became clear, and then 4% suspension of cells in PBS (−) was prepared. Sensitized sheep red blood cells (SSR) were prepared by mixing the SR suspension with an equal volume of hemolysin diluted sufficiently not to cause agglutination (usually 1 : 320). The mixture was incubated for 30 min at 37 C. Then the cells were washed three times with PBS (−) and resuspended in PBS (−) at a concentration of 2%. The SSR suspension was then mixed with an equal volume of 10-fold diluted Coombs' serum and stood for 1 hr at room temperature. Under these conditions, sensitized red blood cells showed strong agglutination with 10-fold diluted Coombs' serum. The agglutinated erythrocytes were washed by shaking them vigorously three times with PBS (−). Then they were resuspended in culture medium without calf serum at a concentration of 0.5%, as complete indicator cells (CIC).

As controls throughout these experiments simple indicator cells were used, namely 0.5% suspensions of SR or SSR.

Each cell culture was mixed with 1.0 ml of indicator cells and kept for 1 hr at room temperature in a horizontal position with the monolayer at the bottom to permit the erythrocytes to settle on the surface of the monolayers. Then the tube was gently shaken, and inverted (monolayer up), and the adherence of the erythrocytes to the monolayers was examined under the microscope.

In a negative reaction (−), no cells adhered to the monolayer or only a few single red blood cells or small clumps of erythrocytes were visible on the monolayer. In a weakly positive reactions (+), 10 to 30% of the monolayer surface was covered with erythrocytes. In a moderately positive reaction (2+), 30 to 70% of the surface was covered and in a strongly positive reaction (3+) from 70% to the whole surface was heavily covered with red blood cells and the outlines of the cultured cells were hardly recognizable. The reaction titer is expressed as the reciprocal of the highest antiserum dilution that gave at least a 2+ reaction.

7. *Absorption procedure of sera*

All anti-viral sera were absorbed with a wet precipitate of washed SR and FL cells to eliminate nonspecific factors on adhesion to the tested cells. Two parts of antiserum diluted 1 : 10, were mixed with one part of SR and stood at room temperature for 30 min and then at 4C for 30 min. Then the mixture was centrifuged at 3,000 rev/min to remove SR. Two parts of the supernatant were mixed with one part of wet packed cells, treated again as described above and then centrifuged at 8,000 rev/min to remove cells.

8. *Fluorescent antibody technique*

1) Preparation of fluorescein conjugated antibody

The method described by Kawamura (1969) was slightly modified as follows. Ammonium sulfate was added to antiserum to give 50% saturation. The mixture was stirred at room temperature for 1 hr and then centrifuged at 10,000× g for 20 min. The precipitate was dissolved in the original volume of PBS (−). Ammonium sulfate was added to give 33% saturation and the mixture was stirred at room temperature for 1 hr. The precipitate was dissolved in PBS (−) as described above. This procedure was repeated three times. Then the ammonium sulfate was removed by chromatography on Sephadex G25. The rabbit γ-globulin obtained was conjugated with fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratories) by adding 0.01 mg of FITC per mg of protein in solution adjusted to pH 9.5 with 0.5 M carbonate buffer solution before mixing. The mixture was stirred gently at 10C for 18 hr and then chromatographed on Sephadex G25 to remove unconjugated FITC. The effluent was fractionated by DEAE-cellulose chromatography. Fractions with which a) uninfected cell were not stained and b) specific fluorescence was blocked by preincubation of a coverslip preparation with rabbit antiserum, were used.

2) Procedure for staining with fluorescent antibody

For staining, fluorescent antibody was diluted 4-fold in all experiments. The staining titer of FITC conjugated γ-globulin was ×32.

a) Fixed preparations

Cells on coverslips were dried and fixed with acetone at room temperature for 10 min. Then each preparation was covered with FITC conjugated γ-globulin, and left for 1 hr at 37 C in a moist chamber. Then the samples were washed repeatedly with PBS (−) and coered with a drop of mounting medium (glycerol diluted with PBS (−)).

b) Unfixed preparations

Cell cultures infected with virus were washed with

PBS (−), and immediately covered with FITC conjugated γ-globulin. After incubation for 45 min at 37 C in a moist chamber, the samples were washed with PBS (−), and mounted as described above.

Fluorescence was observed under a Nikon fluorescent microscope with a dark field condenser.

9. *Chemicals*

5′-2′-Fluorodeoxyuridine (FUdR) was obtained from Dr. F. W. Stahl (Institute of Molecular Biology, University of Oregon) through the courtesy of Dr. A. Nakata of this Institute. Actinomycin (AM) S and cytosine arabinoside (CA) were gifts from Dr. J. Kawamata and Dr. M. Takahashi, respectively. Puromycin (PM) was purchased from Nutritional Biochemical Corporation, U.S.A.

Unless otherwise mentioned, FUdR was used at a concentration of 10^{-5} M, in Eagle's MEM without calf serum. CA was used at a concentration of 10 μg/ml, AM at 5 μg/ml and PM at 20 μg/ml in Eagle's MEM without calf serum.

10. *Autoradiography*

^{3}H-Thymidine (SA. 5.0 c/mM) and ^{3}H-uridine (SA. 6.7 c/mM) were purchased from the Radiochemical Centre, Amersham, England.

Autoradiography was carried out by the method of Kato et al. (1963a).

11. *Hemadsorption procedure*

The procedure used was described by Fujio (1962). A monolayer culture on a coverslip was washed with PBS (−) and incubated with a 0.2% suspension of chick erythrocytes in PBS (−) for 30 min at room temperature. The coverslips were taken out of the tubes after washing out the chicken erythrocytes and cells were fixed and stained with Giemsa solution.

RESULTS

1. *Appearance of virus induced cell surface antigen on cells infected with either cowpox virus or Shope fibroma virus using IHAd and uf-FAT*

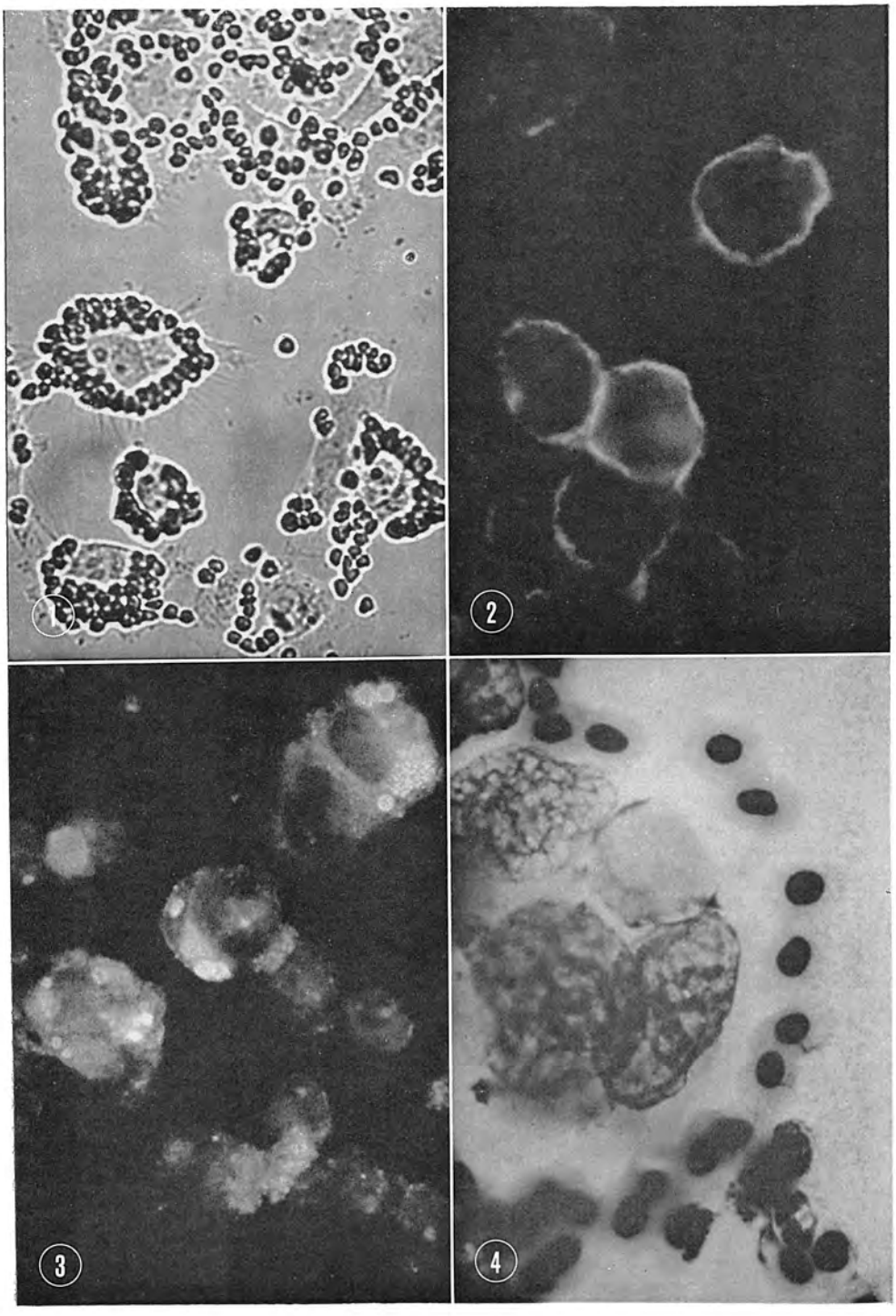
Cells infected with either SFV or CPVR were washed three times with Hanks' salt solution. Then they were treated with homologous antiviral serum, and indicator cells were added. The results are shown in Table 1. Positive reactions were obtained when SFV-infected RK1 cells, SFV-infected FL cells and CPVR-infected FL cells were treated with homologous antiviral serum, and then with CIC (Fig. 1). No HAd was observed on poxvirus-infected cells which had been treated with homologous antiviral serum and then with either SR or SSR only. Moreover, no IHAd was seen on infected cells which had been treated with normal rabbit serum, instead of antipoxvirus sera. Furthermore nc HAd was seen, when uninfected cells were treated with either normal rabbit serum or antiviral serum and then with either SR, SSR or CIC.

To compare the results on VICSA by IHAd with those by FAT, cells infected with either CPVR or SFV were washed with PBS (−), and then immediately incubated with homologous antiviral serum conjugated with FITC. Annular or semiannular specific fluorescence along the margin of infected cells was clearly demonstrated (Fig. 2), but no fluorescence was observed on uninfected cells. These findings with uf-FAT are very different from the fluorescent areas seen on infected cells after fixation with acetone, and staining with fluorescent antibody (Fig. 3).

2. *Cross reactivity of antipoxvirus sera and antisera against viruses other than poxvirus with VICSA induced by various poxgroup viruses*

As shown in Table 2, VICSA of cells infected with either CPVR or SFV was not demonstrated with of antisera against SV 40, polyoma virus, Japanese B encephalitis virus or chikungunya virus. The antibody activities of these antisera were high enough to demonstrate homologous viral antigens by FAT.

The cross reactivity of anti-CPVR serum against VICSA of cells infected with various poxviruses was examined using IHAd and uf-FAT. As shown in Table 3, the VICSA's of poxviruses of the subgroup CPV reacted as strongly as VICSA of homologous virus. However VICSA of SFV gave a weak positive reaction with anti-CPVR serum which was only detectable by IHAd. VICSA of CPVR was also weakly positive with anti-SFV serum by IHAd (Table 1).


2
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◀

FIGURE 1. *Positive IHAd on CPVR-infected FL cells. CIC on infected FL cells is clearly seen.*

◀

FIGURE 2. *Specific fluorescence on the surface of FL cells infected with CPVR. Cells were harvested 24 hr after infection and allowed to react with FITC labeled rabbit γ-globulin without fixation. Note the annular fluorescent patterns at the surface.*

◀

FIGURE 3. *Specific fluorescent spot in FL cells infected with CPVR after fixation with acetone. The sample and original sera are the same as for Fig. 2.*

FIGURE 4. *CPVR infected FL cells with both "A" and "B" type inclusions and adsorbed chick erythrocytes stained with Giemsa solution.*

TABLE 1. *Immune hemadsorption with shope fibroma or cowpox virus infected cell cultures treated with different concentrations of rabbit sera*

Antisera	Dilution	Cell Culture														
		RK1-SFV OA			FL-SFV OA			FL-CPVR			RK1			FL		
		Indicator Cells														
		SR	SSR	CIC	SR	SSR	CIC	SR	SSR	CIC	SR	SSR	CIC	SR	SSR	CIC
Anti-SFV OA	10^{-1}	−	−	3+	−	−	3+	−	−	+	−	−	−	−	−	−
	10^{-2}	−	−	3+	−	−	3+	−	−	−	−	−	−	−	−	−
	10^{-3}	−	−	3+	−	−	3+	−	−	−	−	−	−	−	−	−
	10^{-4}	−	−	2+	−	−	3+	−	−	−	−	−	−	−	−	−
	10^{-5}	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−
	10^{-6}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Anti-CPVR	10^{-1}				−	−	+	−	−	+	−	−	−	−	−	−
	10^{-2}				−	−	+	−	−	2+	−	−	−	−	−	−
	10^{-3}				−	−	−	−	−	2+	−	−	−	−	−	−
	10^{-4}				−	−	−	−	−	3+	−	−	−	−	−	−
	10^{-5}				−	−	−	−	−	2+	−	−	−	−	−	−
	10^{-6}				−	−	−	−	−	+	−	−	−	−	−	−
	10^{-7}				−	−	−	−	−	−	−	−	−	−	−	−
Normal serum	10^{-1}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	10^{-2}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Abbreviations: SFV OA=Shope fibroma virus OA; CPVR=cowpox virus LB red; SR=0.5% sheep red blood cells; SSR=0.5% sensitized sheep red blood cells; CIC=0.5% complete indicator cells.

TABLE 2. *Immune hemadsorption with Shope fibroma or cowpox virus infected cell cultures treated with antiviral sera other than poxvirus*[a]

Antisera	Dilution	Cell Culture				
		FL-SFV[b]	FL-CPVR[a]	FL	RK1	FL-Ch V
Anti-Ch V	10^{-1}	—	—	—	—	2+
	10^{-2}	—	—	—	—	2+
	10^{-3}	—	—	—	—	2+
Anti-JEV	10^{-1}	—	—	—	—	
	10^{-2}	—	—	—	—	
	10^{-3}	—	—	—	—	
Anti-SV 40	10^{-1}	—	—	—	—	
	10^{-2}	—	—	—	—	
	10^{-3}	—	—	—	—	
Anti-PYV	10^{-1}	—	—	—	—	
	10^{-2}	—	—	—	—	
	10^{-3}	—	—	—	—	
Normal Serum	10^{-1}	—	—	—	—	—
	10^{-2}	—	—	—	—	—

Abbreviations: Ch V=Chikungunya virus; JEV=Japanese encephalitis virus; SV 40=simian virus 40; PYV=polyoma virus; SFV OA=Shope fibroma virus OA; CPVR=cowpox virus LB red.

a Only complete indicator cell (CIC) were used as indicator cells.

b FL cells were used 24 hr after infection with SFV OA or CPVR.

TABLE 3. *Cross reactions between pox group viruses as revealed by immune hemadsorption (IHAd) and the fluorescent antibody technique (FAT)*

FL cells infected with	IHAd[a]	FAT[b]
Cowpox		
LB red	3+	3+
50	3+	3+
51	3+	3+
58	3+	3+
Amsterdam	3+	3+
Variola		
Harvey	2+	2+
Vaccinia		
Ikeda	2+	2+
Ecuador-Moscow	2+	2+
Ectromelia		
Hampstead	3+	3+
Shope fibroma	+[c]	?

▶

a Anti-CPVR virus serum diluted 10^{-4} was used.

b Live cells were stained with FITC conjugated anti-cowpox virus rabbit serum γ-globulin.

c Anti-CPVR virus serum diluted 5×10^{-1} was used.

TABLE 4. *Test of immune hemadsorption[a] of the surface antigen of the cells themselves, on cells infected with Shope fibroma virus and cowpox virus treated with anti-FL cell rabbit serum*

Antisera	Dilution	Cell culture			
		FL-SFV OA[b]	FL-CPVR[b]	FL	RK1
Anti-FL cell	10^{-1}	3+	3+	3+	−
	10^{-2}	3+	3+	3+	−
	10^{-3}	3+	3+	3+	−
	10^{-4}	3+	2+	3+	−
	10^{-5}	2+	2+	2+	−
	10^{-6}	+	+	+	−
	10^{-7}	−	−	−	−
Normal serum	10^{-1}	−	−	−	−
	10^{-2}	−	−	−	−

Abbreviations: SFV OA=Shope fibroma virus OA; CPVR=cowpox virus LB red.
a Only complete indicator cells (CIC) were used.
b FL cells were used 24 hr after infection with SFV OA or CPVR.

TABLE 5. *Comparison of results obtained by immune hemadsorption, the fluorescent antibody technique with unfixed and fixed cells and by Giemsa staining*

Cell culture	Tested for	Hours after virus inoculation									
		2	4	6	8	10	12	14	16	24	48
FL-CPVR	IHAD	+	+	2+	3+	3+	3+	ND	ND	3+	3+
	FAT (Unfixed)	+	+	2+	2+	3+	3+	ND	ND	3+	3+
	"B"	−	±	+	2+	3+	3+	ND	ND	3+	3+
	FAT (Fixed)	−	±	+	3+	3+	3+	ND	ND	3+	3+
RK1-SFV OA	IHAD		−	−	−	+	2+	3+	3+	3+	3+
	FAT (Unfixed)		−	−	−	+	+	2+	2+	2+	2+
	"B"		−	−	−	−	+	2+	2+	3+	3+
	FAT (Fixed)		−	−	−	−	+	2+	2+	3+	3+

Abbreviations: CPVR=cowpox virus LB red; SFV OA=Shope fibroma virus OA; ND=not done; IHAd=immune hemadsorption; "B"="B" type inclusions; FAT=fluorescent antibody technique.

TABLE 6. *Inhibitory effect of drugs on the IHAd and FA reactions in FL cells infected with CPVR*

Drug	Indicator	Time of addition (hrs)									
		−2	−1	0	1	2	3	4	5	6	7
5′-Fluoro-deoxyuridine	IHAd	+	+	+	+	+	+	+	+	+	+
	FAT	+	+	+	+	+	+	+	+	+	+
Cytosine Arabinoside	IHAd	+	+	+	+	+	+	+	+	+	+
	FAT	+	+	+	+	+	+	+	+	+	+
Actinomycin	IHAd	−	−	−	+	+	+	+	+	+	+
	FAT	−	−	−	+	+	+	+	+	+	+
Puromycin	IHAd	−	−	−	−	−	+	+	+	+	+
	FAT	−	−	−	−	−	+	+	+	+	+

Abbreviations and symbols: CPVR=cowpox virus LB red; IHAd=immune hemadsorption; FAT=unfixed fluorescent antibody technique; +=positive reaction of IHAd or FAT; −=negative reaction of IHAd or FAT.

TABLE 7. *Tests for immune hemadsorption of cells infected with cowpox virus and Shope fibroma virus in the presence and absence of cytosine arabinoside (24 hrs after infection)*

Cell culture	Cytosine arabino-side	DNA synthesis[a]		RNA synthesis[a]		IHAd	FAT (Unfixed)	HAd
		"N"	"C"	"N"	"C"			
FL-CPVR	−	−	3+	3+	+	3+	3+	3+
	+	−	−	3+	+	2+	2+	−
RK1-SFV OA	−	−	2+	ND	ND	3+	2+	−
	+	−	−			2+	?	−

Abbreviations: CPVR=cowpox virus LB red; SFV OA=Shope fibroma virus OA; "N"=nuclear; "C"=cytoplasmic; IHAd=immune hemadsorption; HAd=hemadsorption; FAT=fluorescent antibody technique; ND=not done.

a Tested by autoradiography.

3. *Surface antigen of FL cells themselves on cells infected with poxvirus demonstrated by IHAd*

FL cells, RK1 cells, FL cells infected with SFV and FL cells infected with CPVR were treated with anti-FL cell serum and then mixed with CIC. As shown in Table 4, all these cells except RK1 cells gave positive reactions of the same intensity. Thus induction of VICSA of poxviruses does not appreciably affect the surface antigen of the cell itself.

4. *Relationship between the formation of VICSA and inclusion bodies*

The relationship between the formation of VICSA and the synthesis of viral antigens and inclusion body formation was examined by IHAd, FAT and Giemsa staining. As shown in Table 5, VICSA of CPVR was positive by either IHAd or uf-FAT 2 hr after virus infection, but cytoplasmic viral antigen and inclusion bodies were rarely seen at this stage. Ten hr after viral infection, almost all cells gave a strong positive reaction by any of these tech-

niques. VICSA of SFV was positive by either IHAd or uf-FAT 10 hr after infection, but at this time cytoplasmic viral antigen and " B " type inclusions were still rare. Twenty four hr after viral infection, almost all cells gave a strong positive reaction by any of these techniques.

5. *Effects of various antimetabolites on the formation of VICSA*

Monolayer cultures of FL cells were infected with CPVR at a multiplicity of infection of approximately 1 to 10 and after adsorption for 1 hr, they were washed three times with Hanks' solution, and incubated at 37 C until 10 hr after infection. At this time VICSA was severe. Then these cells were examined by IHAd and uf-FAT. Compounds were administered at different times from 2 hr before infection to 7 hr after infection and thereafter. The results are shown in Table 6. Induction of VICSA was not inhibited by the presence of FUdR or CA, while viral DNA synthesis was inhibited. Induction of VICSA was inhibited when AM was administered within 1 hr after infection. PM suppressed VICSA formation when administered within 3 hr after infection and thereafter.

6. *Relationship between viral hemagglutinin (HA) and VICSA*

Hemadsorption of chick erythrocytes mediated by viral HA was clearly demonstrated (Fig. 4), while it was not demonstrated on cells infected with SFV, which does not produce HA. In the presence of CA, cells infected with CPVR did not adsorb chick erythrocytes, while VICSA of the cells was clearly demonstrated (Table 7). Therefore VICSA is probably not identical with HA of poxvirus.

DISCUSSION

Virus induced cell surface antigens (VICSA) which react with anti-cowpox virus (CPV) serum and with anti-Shope fibroma virus (SFV) serum were found on cells infected with homologous poxvirus using either immune hemadsorption (IHAd) or the unfixed(uf)-fluorescent antibody technique (FAT). The cross reactivities of anti-CPV serum with VICSA of cells infected with various poxviruses were examined. The serum reacted well with VICSA induced by poxviruses belonging to the subgroup CPV, but poorly with VICSA induced by SFV. The latter reaction was only demonstrated by IHAd, which is a much more sensitive technique than uf-FAT.

Fl cells showing VICSA induced by CPV or SFV also gave a strong positive IHAd reaction using anti-FL cell serum. Thus the antigen on the surface of the host cells themselves is not affected appreciably by virus infection.

The presence of specific antigen on the cell surface (CSA) of tumor cells induced by chemical carcinogen, and on lymphoma cells induced by Molony virus infection have been demonstrated by FAT (Klein et al., 1964; Möller, 1964; Fenyö et al., 1968). CSA on transformed cells induced by SV 40 was demonstrated on fixed preparations using the indirect FAT (Tevethia et al., 1965) and on unfixed preparations (Kluchareva et al., 1967; Diamandopoulos et al., 1968). Irline (1967) and Malmgren et al. (1968) observed CSA in the lytic cycle of mouse cells and hamster cells infected with polyoma virus using uf-FAT. Thus CSA induced by oncogenic viruses has been demonstrated not only in transformed cells induced by viral infection but also in cells of productive viral infection. Hamada and Uetake (1968) observed VICSA on cells productively infected with several oncogenic adenoviruses (adenovirus type 12, 18 and 7) as well as on cells transformed by these viruses. They considered that induction of VICSA may be a common phenomenon in cells infected with oncogenic virus. In this work we found VICSA on cells productively infected with several nononcogenic poxviruses and SFV. The transforming ability of these viruses has never been demonstrated, although SFV is an

oncogenic virus which produces a benign fibroma in rabbits. Recently, VICSA of cells infected with chikungunya virus was demonstrated by Mantani and Igarashi (1971). Therefore, the induction of VICSA seems to be a common phonomenon in cells infected with animal viruses, regardlless of whether these viruses are oncogenic.

Induction of VICSA on cells infected with CPVR and SFV was not inhibited by inhibitors of DNA synthesis, such as cytosine arabinoside (CA) or FUdR. CA, which inhibits virus DNA synthesis, does not inhibit RNA and protein synthesis coded by parental viral DNA in cells infected with poxvirus (Oda and Joklik, 1967). It seems certain that VICSA induced by poxvirus is one of the earliest symptoms of virus infection and is independent of virus multiplication. It could be at least part of the early protein coded by parental DNA of poxvirus.

When actinomycin (AM) was added to infected cells 1 hr after infection, VICSA formation was not supressed, possibly because at this time informational RNA for VICSA formation had already been synthesized. AM is known to combine with DNA (Kawamata and Imanishi, 1960; Rauen et al., 1960) and to inhibit over 95% of all RNA synthesis at the concentration used in this experiment (Reich et al., 1961; Hurwitz et al., 1962). Jungwirth and Joklik (1965) demonstrated that the activity of early enzymes in the cytoplasm began to increase within 1.5 hr after infection of HeLa cells with vaccinia virus and these early enzymes have remarkably long lives. The production of these enzymes is mediated by virus induced messenger RNA which is transcribed from the viral genome and is detectable in the cytoplasm within 1–1.5 hr after infection (Becker and Joklik, 1964).

Puromycin (PM) inhibits VICSA formation when added to infected cells within 3 hr after infection. PM which is known to inhibit protein synthesis selectively (Yarmolinsky and de la Harn, 1959; Rabinovitz and Fisher, 1962), suppresses over 90% of the protein synthesis of L cells within 30 min (Kaku and Kamahora, 1964). The synthesis of early enzymes is induced about 1.5 hr after infection, and is arrested at any time during the initial period of infection on addition of PM (Jungwirth and Joklik, 1965). Our results suggest that VICSA may be newly synthesized protein which is translated within 3 hr after viral infection and that its synthesis its mediated by newly synthesized RNA transcribed from viral DNA within 1 hr after infection.

Vogel and Shelokov (1958) and Fujio (1962) demonstrated simple HAd of chicken erythrocytes on cells infected with vaccinia virus which can produce hemagglutinin. We also demonstrated the same phenomenon on cells infected with CPVR. However HAd of chicken erythrocytes on cells infected with CPVR in the presence of CA was negative, whereas IHAd remained positive. Simple HAd of chicken erythrocytes was negative on cells infected with SFV, which does not produce hemagglutinin. These results show that VICSA induced by poxvirus is not related with hemagglutinin. Studies on the relationship between poxvirus-induced antigens other than hemagglutinin and VICSA induced by poxviruses are in progress.

REFERENCES

Becker, Y. and W. K. Joklik. 1964. Messenger RNA in cells infected with vaccinia virus. Proc. Nat. Acad. Sci. U. S. 51: 577–585.

Black, P. H., W. P. Rowe, H. C. Turner and R. J. Huebner. 1963. A specific complement-fixing antigen present in SV40 tumor and transformed cells. Proc. Nat. Acad. Sci. U.S. 50: 1148–1156.

Defendi, V. 1963. Effect of SV40 virus immunization on growth of transplantable SV40 and polyoma virus tumors in hamsters. Proc. Soc. Exp. Biol.

Med. 113: 12–16.

Defendi, V., B. Ephyussi and H. Koprowski. 1964. Expression of polyomainduced cellular antigen(s) in hybrid cells. Nature 203: 495–450.

Beichman, G. I. and T. E. Kluchareva. 1964. Immunological determinants of oncogenesis in hamsters infected with SV40 virus. Virology 24: 131–137.

Diamandopoulos, G. Th., S. S. Tevethia, F. Rapp and J. F. Enders. 1968. Development of S and T antigens and oncogenicity in hamster embryonic cell lines exposed to SV40. Virology 34: 331–336.

Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science 130: 432–437.

Eddy, B. E., G. E. Crubbs and R. D. Young. 1964. Tumor immunity in hamsters infected with adenovirus type 12 or simian virus 40. Proc. Soc. Exp. Biol. Med. 117: 575–579.

Fagraeus, A. and A. Espmark. 1961. Use of a ' Mixed haemadsorption ' method in virus-infected tissue cultures. Nature 190: 370–371.

Fenyö, E. M., E. Klein, G. Klein and K. Swiech. 1968. Selection of an immunoresistant Moloney lymphoma subline with decreased concentration of tumor-specific surface antigens. J. Nat. Cancer Inst. 40: 69–89.

Fujio, Y. 1962. Detection of hemaggulutinin production of vaccinia virus by hemadsorption. Biken J. 5: 109–111.

Habel, K. and B. E. Eddy. 1963. Specificity of resistance to tumor challenge of polyoma and SV40 virus-immune hamsters. Proc. Soc. Exp. Biol. Med. 113: 1–4.

Habel, K., F. Jensen, J. S. Pagano and H. Koprowski. 1965. Specific complement-fixing tumor antigen in SV40-transformed human cells. Proc. Soc. Exp. Biol. Med. 118: 4–9.

Ham, R. G. 1965. Clonal growth of mammalian cells in a chemically defined synthetic medium. Proc. Nat. Acad. Sci. U.S. 53: 288–293.

Hamada, C. and H. Uetake. 1968. Specific surface antigen on cells transformed or infected with adenovirus. Ann. Rept. Inst. Virus Res., Kyoto Univ. 11: 69.

Hellström, I. and H. O. Suögrem. 1967. In vitro demonstration of humoral and cell-bound immunity against common specific transplantation antigen(s) of adenovirus 12-induced mouse and hamster tumors. J. Exp. Med. 152: 1105–1118.

Hellström, I. and K. E. Hellström. 1969. Studies on cellular immunity and its serum-mediated inhibition in molony-rirus-induced mouse sarcomas. Int. J. Cancer 4: 587–600.

Huebner, R. J., W. P. Rowe, H. N. Turner and W. T. Lane. 1963. Specific adenovirus complement-fixing antigens in virus-free hamster and rat tumors. Proc. Nat. Acad. Sci. U.S. 50: 379–389.

Hurwits, J., J. J. Rurthe, M. Malamy and M. Alexander. 1962. The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of the enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by actinomycin D and proflavin. Proc. Nat. Acad. Sci. U.S. 48: 1222–1230.

Irlin, S. L. 1967. Immunofluorescent demonstration of a specific surface antigen in cells infected or transformed by polyoma virus. Virology 32: 725–728.

Jungwirth, C. and W. K. Joklik, 1965. Studies on " Early " enzymes in HeLa cells infected with vaccinia virus. Virology 27: 80–93.

Kaku, H. and J. Kamahora. 1964. Giant cell formation in L cells infected with active vaccinia virus. Biken J. 6: 299–315.

Kato, S., M. Takahashi, S. Kameyama and J. Kamahora. 1959. A study on the morphological and cyto-immunological relationship between the inclusion of variola, cowpox, rabbit pox, vaccinia (variola origin) and vaccinia IHD and a consideration of the term " Guarnieri body ". Biken's J. 2: 353–365.

Kato, S., Aoyama and J. Kamahora. 1963 a. Autoradiography of tissue of mice infected with ectromelia virus using ^{3}H-thymidine. Biken J. 6: 9–16.

Kato, S., M. Takahashi, H. Miyamoto and J. Kamahora. 1963 b. Shope fibroma and rabbit myxoma viruses. I. Autoradiographic and cytoimmunological studies on " B " type inclusions. Biken J. 6: 127–134.

Kato, S., H. Miyamoto, M. Takahashi and J. Kamahora. 1963 c. Shope fibroma and rabbit myxoma virses. II. Pathogenesis of fibroma in domestic rabbits. Biken J. 6: 135–143.

Kawamata, J. and M. Imanishi. 1960. Interaction of actinomycin with deoxyribonucleic acid. Nature 187: 1112–1113.

Kawamura, A. Jr. 1969. Fluorescent antibody technique and their application. Univ. of Tokyo Press (Tokyo).

Khera, S. S., A. Ashkenazi, F. Rapp and J. L.

Melnick. 1963. Immunity in hamsters to cells transformed in vitro and in vivo by SV40. Tests for antigenic relationship among the papovaviruses. J. Immunol. 91: 604–613.

Klein, E. and G. Klein. 1964. Antigenic properties of lymphomas induced by the Molony agent. J. Nat. Cancer Inst. 32: 547–568.

Kluchareva, T. E., K. L. Shachanina, S. Belova, V. Chibixova and G. I. Deichman. 1967. Use of immunofluorescence for detection of specific membrane antigen in simian virus 40-infected non-transformed cells. J. Nat. Cancer Inst. 39: 825–832.

Malmgren, K. A., K. K. Takemoto and P. G. Carney. 1968. Immunofluorescent studies of mouse and hamster cell surface antigens induced by polyoma virus. J. Nat. Cancer Inst. 40: 263–268.

Mantani, M. and A. Igarashi. 1971. Immunofluorescent studies on the location of viral structural components and surface antigen in FL cells infected with chikungunya virus. Biken J. 14: 131–140.

Merchant, D. J., R. H. Kahn and W. H. Murphy, Jr. 1960. Handbook of cell and organ culture. Burgess Publishing Co. Minneapolis, U.S.A.

Milgrom, F., K. Kano, A. L. Barron and E. Witebsky. 1964. Mixed agglutination in tissue culture. J. Immunol. 92: 8–15.

Miyamoto, H. and S. Kato. 1968. Immune hemadsorption by cells infected with poxviruses. Biken J. 11: 343–353.

Möller, G. 1964. Effect on tumor growth in syngeneic recipients of antibodies against tumor-specific antigens in methylcholanthrene-induced mouse sarcomas. Nature (London) 204: 846–847.

Oda, K. and W. K. Joklik. 1967. Hybridization and sedimentation studies on "Early" and "Late" vaccinia messenger RNA. J. Mol. Biol. 27: 395–419.

Rabinovitz, M. and J. M. Fisher. 1962. The dissociative effect of puromycin on the pathway of protein synthesis by Ehrlich ascites tumor cells. J. Biol. Chem. 237: 477–481.

Rapp, F., S. S. Teventhia and J. L. Melnick. 1966. Papovavirus SV40 transplantation immunity conferred by an adenovirus-SV40 hybrid. J. Nat. Cancer Inst. 36: 703–708.

Rauen, H. M., H. Kersten and W. Kersten. 1960. Z7r Wirkungsweise von Actinomycinen. Hoppe-Seyler's Z. Physiol. Chem. 321: 139–147.

Reich, E., R. M. Franklin, A. J. Shatkin and E. L. Tatum. 1961. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science 134: 556–557.

Sabin, A. B. and M. A. Koch. 1964. Source of genetic information for specific complement-fixing antigens in SV40 virus-infuced tumors. Proc. Nat. Acad. Sci. U.S. 52: 1131–1138.

Shelokov, A., J. E. Vogel and L. Chi. 1958. Hemadsorption (Adsorption-Hemagglutination) test for viral agents in tissue culture with special reference to influenza. Proc. Soc. Exp. Biol. Med. 97: 802–809.

Tevethia, S. S., M. Katz and F. Rapp. 1965 a. New surface antigen in cells transformed by simian papovavirus SV40. Proc. Soc. Exp. Bio. Med. 119: 896–901.

Tevethia, S. S. and F. Rapp. 1965 b. Demonstration of new surface antigens in cells transformed by papovavirus SV40 by cytotoxic tests. Proc. Soc. Exp. Biol. Med. 120: 455–458.

Tevethia, S. S., L. A. Couvillion and F. Rapp. 1968. Development in hamsters of antibodies against surface antigens present in cells transformed by papovavirus SV40. J. Immunol. 100: 358–362.

Ueda, Y., M. Ito and I. Tagaya. 1969. A specific surface antigen induced by poxvirus. Virology 180–182.

Yarmolinsky, M. and G. L. de la Harn. 1959. Inhibition of puromycin of amino acid incorporation into protein. Proc. Nat. Acad. Sci. U.S. 46: 1020–1030.