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DEPARTMENT OF VIROLOGY

GENERAL SUMMARY

Current research activities are mainly concerned with cancer cell biology which ought to be obtainable from cell-virus interaction. For this purpose, the following projects have been undertaken.

1) The mechanism of persistent infection with non-oncogenic viruses, Paramyxovirus : HVJ(Hemagglutinating Virus of Japan = Sendai virus), Orthomyxovirus (Influenza virus), Rubella virus or Herpes simplex virus.
2) Xenogenization of tumor cell membrane antigen by infection with Poxvirus (CPV = Cowpox virus) or Herpes simplex virus (HSV), or by persistent infection with HVJ or Rubella virus.
3) Lysosomal enzyme activities regulated by intracellular cyclic AMP.
4) Transformation of human nasopharyngeal epithelial cells by the Epstein-Barr virus(EBV) in relation to the development of nasopharyngeal carcinoma.
5) Isolation and purification of cleavage enzyme or interferon inducer from bacterial products or extracts.

Persistent infection of cells with non-oncogenic viruses gives us not only a typical model of many chronic viral diseases, but also a good example of viral carcinogenesis. Recently, progress on virus vaccines generally has made a prophylaxie of acute viral diseases themselves more effective except for a few special cases, though reducing of their side effects or new development of more effective ones still remains to be achived. In contrary to this, a number of serious chronic viral diseases has been shown to be caused by chronic, persistent, slow virus infection in the nervous system, cardiac system etc. For example, subacute sclerosing panencephalitis is a result of slow virus infection in children with measles virus; and congenital rubella syndrome with severe anomalies of heart, great vessels, eye defects or neurosensory deafness is derived from rubella infection in pregnancy. Similarly, it might be possible to assume that viral transformation of cells originates from the intracellular persistence of oncogenic virus genome. The first problem to be examined in both chronic viral diseases and carcinogenesis of cells seems here to be what the mechanism is to make the persistence of viral genome stable intracellularly. Following this, initiation of cytopathogenicity, onset of viral diseases, uncontrolled proliferation of tumor cells, death of individuals etc. should be investigated to correlate them respectively. Considering a possible connection of chronic, persistent, slow viral diseases with suggestive human oncogenic viral diseases, we have selected project No. 1 as our main target.

Another general characteristic of the virus-infected cells is their biological alterations caused by the virus infection, regardless of persistent or lytic infection. In the case of lytic

infection, the fate of virus-infected cells is surely occurrence of the cytopathogenic effect(CPE) and death of the cells in the end. However, we believe that the virus genome penetrated into cells could inevitably produce some effects on cellular functions before the appearance of CPE. These possible effects, including the ones by persistently infected virus genome, may be expressed immunologically, biochemically etc.. Using cultured transplantable cells, we have observed them immunologically as a virus-specific formation of cell membrane(surface) antigen(S-ag) and a lowered transplantability induced by cellular immunity *in vivo*. This S-ag newly formed after the virus infection might be made to act as xenogenized antigen *in vivo*, suggesting a possible application of this phenomenon to the immunotherapy of tumors in the future. Concerning this, enhancement or regulation of lysosomal enzymes was found to depend on the intracellular concentration of cyclic AMP. These problems are presented as our Projects No. 2 and 3.

As our Project No. 4, we have attempted to transfer the Epstein-Barr(EBV) genome to human nasopharyngeal cells. EBV is well known to be the most likely virus responsible for the development of human nasopharyngeal carcinoma(NPC). However, the question why EBV cannot generally transform human epithelial cells experimentally still remains obscure, while the transformation of human lymphoblastoid cells is easy. It will be impossible to determine whether this EBV is a causal virus of NPC unless the possible transfer and mechanism of its persistence in human epithelial cells can be revealed. Therefore, our interests in following this EBV come from this point.

During last 5 years, we have developed the Cell Injuring Reaction(CIR) method through the measurement of cellular RNA-proteins, which were made to be released extracellularly by the action of a CIR active substance. Products of *Bacillus cereus*, MS-1063 strain, were purified and shown to have strong CIR activity on tumor cells. However, we could not eliminate undesirable side effects contained in this sample, even after final purification. This made us turn to a new approach to seek other bacterial products which might possibly act on the tumor cell membrane and enable it to be cleaved or exposed. Concerning this cleavage or exposure effect, trypsin or similar proteolytic enzyme treatment of virus particles such as Paramyxovirus and Poxvirus has been shown to result in accelerated growth of the virus. Moreover, tumor cells treated with neuraminidase exhibited enhanced activity causing cellular immunity after *in vivo* transplantation. Similarly, trypsin treatment of contact-inhibited cultured cells is known to cause a release of cells from contact-inhibition. In addition to these cleavage or exposure effects, an interferon inducing activity will also be tested in the same sample. If more active cleavage substances than trypsin or neuraminidase could be isolated, tumor cell growth or its immunological problems might be tested from a slightly different approach. This program is shown as our Project No. 5.

These works were supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture,

Japan and by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

ABSTRACT

(7) The mechanism of persistent infection with non-oncogenic viruses, Para-myxovirus: HVJ (Hemagglutinating Virus = Sendai virus), Orthomyxovirus (Influenza virus), Rubella virus or Herpes simplex virus.

1) Specific modification of cell membrane in spontaneous and viral transformation *in vitro*.

T. Yamada, J. Tanaka, R. Shimizu, M. Hatano and S. Odajima

2) Characteristics of temperature-sensitive variant of HVJ derived from stable HVJ carrier cultures.

O. Morita and M. Hatano

Since our first report on persistent infection with HVJ in 1970, we have been able to establish many HVJ carrier cultures. However, their establishment is always accompanied by some troubles such as a long period of more than 2-3 months of irregular results. These troubles have been solved completely by an application of a temperature-sensitive(ts) variant (HVJts) instead of the wild type (HVJo).

Using plague assay on VERO cells in the presence of trypsin and CIU (Cell Infectious Unit) assay on the same cells, the existence of infectious viruses in various cells persistently infected with HVJ was first proved. As shown in Table, this ts variant (HVJpits), even after the usual passages in developing chick embryos at 32C (HVJpits-e), could not grow significantly at higher temperature more than 37C. This characteristic of intracellular viruses in the HVJ carrier cells seemed to cause that their infectivities are usually negative at 37C up to the present time. At 35C, these ts variants showed less than half the growth of that seen at permissive temperature (32C). No difference in antigenicity was found among these variants and wild type viruses.

In HeLa cells infected with HVJpits at 35C, little cytopathic effect (CPE), and mild and transient release of lysosomal enzymes from lysosome into the cytoplasm occurred. Furthermore, HVJpits induced no production of interferon(IF) in HeLa cells and easily led the cells to the establishment of persistent infection. On the other hand, HVJo caused strong CPE with activated cytoplasmic release of lysosomal enzymes. The production of IF in HeLa cells by the HVJo infection depended on multiplicity of infection(m.o.i.). This appeared to make it hard for the cells to establish a persistent infection with that virus, especially at m.o.i. of 10, because a large amount of IF produced may possibly result in the inhibition of intracellular replication of the viruses and finally the elimination of HVJ genome from the host cells. For the establishment of persistent infection with HVJo, a low m.o.i. of 0.1 was used effectively following long subculturing of HVJo-infected cells.

Viruses re-isolated from new carrier cells established with

HVJo were not ts variant, showing a replication in VERO cells at 37C. When these HVJo carrier cells without CPE were submitted to repeated subculturing with more than 40 passages, more than 90% of their intracellular viruses were proved to be ts variant instead of wild type virus, suggesting a possible change in HVJo to ts variant intracellularly. The possibility of coexistence of a ts variant in the original HVJo and its selective establishment of a carrier state or subsequent selection to the stable ts carrier cells during long subcultures should be made clearer in the future. Comparative examination of reverse transcriptase activity in both HVJo and HVJts is now in progress as the next problem.

Similar easy establishments have been recently observed in the case of Rubella virus carrier cultures and persistent infection with Influenza virus or Herpes simplexvirus type 2 is also going on.

Total yields of HVJo, HVJpits or HVJpits-e in VERO cells at different temperatures

Viruses	Temperature (℃)	Total Virus Yields*		Ratio (infectivity)
		HA/ml	Infectivity	
HVJo	32	16	2.1×10^4	1
	35	64	4.0×10^5	20
	37	512	2.9×10^6	140
	39	1024	9.8×10^6	467
HVJpits	32	128	1.5×10^5	1
	35	64	7.0×10^4	0.47
	37	8	3.2×10^2	0.0021
	39	4	2.5×10^1	0.00017
HVJpits-e	32	256	1.4×10^6	1
	35	256	6.0×10^5	0.43
	37	32	1.3×10^4	0.009
	39	16	7.0×10^0	0.000005

* Virus yield, CIU/ml, at 24 hours after the infection (1.0 CIU/cells) in the presence of trypsin, 0.6μg/ml.

(8) Xenogenization of tumor cell membrane antigen by infection with poxvirus (Cowpox virus = CPV) or Herpes simplex virus (HSV), or by persistent infection with HVJ or Rubella virus.

1) Lowered transplantability of cultured tumor cells by persistent infection with paramyxovirus.

T. Yamada and M. Hatano.

2) Effect of infection with trypsin-treated cowpox virus or persistent infection of cells with HVJ (Sendai virus) on production of cowpox virus-infected cell surface antigen.

J. Tanaka and M. Hatano

Previously, we found a lowered transplantability of HVJ carrier tumor cells in the hamster. This lowered transplantability was shown to be due to the enhanced induction of cellular immunity *in vivo* caused by xenogenized antigen of cell membrane. A similar phenomenon is now observed in Rubella virus carrier tumor cells. In an attempt to make cell membrane antigen xenogenize, we have also used formation of a virus-specific cell surface antigen(S-ag) by CPV or HSV infection.

In these tests, some tumor cells reproducibly gave the high formation of CPV S-ag, but other cells did not, suggesting the dependency of S-ag forming ability on the cell species specificity. The low incidence of CPV S-ag formation in such tumor cells was remarkably enhanced in the presence of trypsin without significant effect on the virus adsorption. On the other hand, the HVJ carrier cultures were described as having cellular ability causing increased multiplication of other viruses or enhanced cellular repair activity. Therefore, a possible effect of persistent infection with HVJ, especially the temperature-sensitive(ts) variant, was examined to discover whether it makes the low S-ag producing tumor cells convert to high producing ones or not.

The formation of CPV S-ag in the HVJ carrier cultures was strongly enhanced at 32-35C, when compared to that in parent cells. Temperature shifts from 32-35C to 37C for these carrier cultures reduced this once enhanced formation, making them equivalent to the parent cells as shown in Table.

In a comparison of intracellular CPV growth at 35C, the eclipse phase and one step growth of CPV in the HVJ carrier cells occurred within a much shorter period, while the CPV adsorption was the same as that on parent cells. In the coruse of artificial degradation of the CPV outermembrane by detergent: NP-40 *in vitro*, the CPV infectivity was made to increase early and decrease later. The meaning of this early increase *in vitro* reaction is unknown. However, it may be due to a partial degradation of the CPV outermembrane, which enables the CPV to be adsorbed on the sensitive assay cells more easily and respond to the cellular uncoating activities more rapidly with subsequent rise in infectivity. These infectivity changes occurred more rapidly and markedly when the CPV were reacted with crude cell extracts from the HVJ carrier cells cultured at 32-35C. Furthermore, the S-ag forming ability of HVJ carrier cells in the presence of Actinomycin D or Puromycin was not lowered so much as that of the parent cells at the permissive temperature of ts variant.

The results indicate that persistent infection with HVJ, especially ts variant, makes the cells promote the first uncoating stage and concomitantly the subsequent intracellular step of CPV growth, resulting in enhanced formation of S-ag. This CPV

S-ag together with the one of HVJ or Rubella virus carrier tumor cells is now being investigated to find out whether or not it is capable of inducing cellular immunity after *in vivo* transplantations as the xenogenized antigen of tumor cell membrane.

Effect of culture temperature of THEL and its HVJ carrier cells on S-antigen formation by CPV

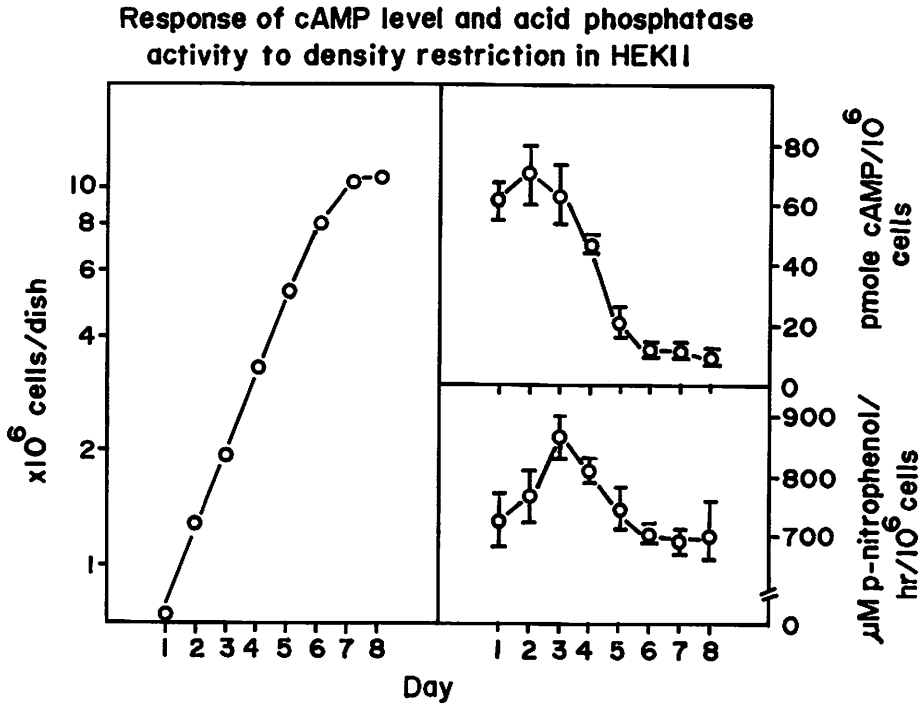
Cells	S-antigen positive cells (%)[a] cultured at			
	32℃[b]	(ratio)	37℃[b]	(ratio)
THEL	29.0	(1.0)	32.2	(1.0)
THEL-HVJ	71.4	(2.4)	28.9	(0.8)
THEL(HVJpits)	79.4	(2.7)	33.9	(1.0)
THEL(HVJpits-e)	69.7	(2.4)	33.3	(1.0)
THEL(HVJo)-38	57.2	(1.9)	36.2	(1.1)
VERO	82.0	(2.8)	85.2	(2.6)

a) Observation after 8 hours in cells infected or superinfected with CPV
b) Cultured for 72 hours before CPV infection and maintained at respective temperatures after CPV infection

(9) **Lysosomal enzyme activities regulated by intracellular cyclic AMP (cAMP): Relationship between intracellular cAMP and lysosomal enzyme activity in human embryonal kidney cells.**
H. Ogura, J. Tanaka and M. Hatano

Relating to the observations of lysosomal enzyme activities in the HVJ carrier cells and CPV-infected cells, this problem was extracted. Addition of dibutyryl-cAMP(B2cAMP) or cAMP to the culture medium caused human embryonal kidney cells a significant elevation of enzyme activities, while deprivation of these substances from the medium resulted in lowering of enzyme activities. In the case of addition of B2cAMP, enzyme activities reached to maximum elevation within 3 days, while deprivation produced rapid lowering within 12 hours. No significant effects on cell growth or cellular protein content were found in these cloned fibroblast-like cells at an effective drug concentration of 0.5-1.0 mM. Optimal addition of epinephrine or isoproterenol known as an activator of cellular adenylate cyclase resulted in an accumulation of intracellular cAMP and subsequent elevation of lysosomal enzyme activity similar to that caused by B2cAMP. For this enhancement, *de novo* synthesis of RNA or protein seemed to be essential, as shown by the experimental effect of pre-treatment of cells with Actinomycin D or cyclohexamide. Similar results to these experiments were also found in other cloned human epithelial kidney cells derived from the same origin.

Measurement of cellular cAMP levels under logarithmic cell growth clearly demonstrated its close correlation to lysosomal enzyme activity (Figure). This regulated elevation or lowering of enzymes(acid phosphatase, acid protease and β-glucuronidase) was observed to occur only in the lysosomal fraction and not in the soluble fraction. Further problems on such regulation in virus-infected cells, regardless of either persistent or lytic infection, are now being studied to reveal a possible difference between viral CPE or carrier state and cell viability.



(10) Transformation of human nasopharyngeal cells by Epstein-Barr virus (EBV) in relation to the development of nasopharyngeal carcinoma (NPC).

1) Transfer of EBV genome from EBV-carrying human lymphoblastoid cells to human epithelial cells.

M. Furukawa and M. Hatano.

2) Experimental studies on the viral etiology of nasopharyngeal carcinoma related to Epstein-Barr virus infection.

M. Furukawa

Using 2-27-Ad cells, which we established from human adenoid tissues, transfer of EBV genome to them from EBV-carrying human lymphoblastoid cells, P3HR-1 or EB3 cells, was carried out. These human nasopharyngeal epithelial cells showed high frequency of EBV genome transfer in the cell-fusion technique mediated by UV-irradiated HVJ, while low frequency in other human epithelial cells derived from lung or kidney. The result

indicates that EBV really infects human nasopharyngeal epithelial cells and produces in them the formation of EBV-specific nuclear antigen(EBNA) detected by fluorescent antibody staining. The next problem to make clear will be how the EBV genome transfered could be maintained persistently in these nasopharyngeal cells and cause the cell transformation *in vitro*.

(11) **Isolation of cleavage enzyme or interferon inducer from bacterial products.**

1) The effect of trypsin on the formation of virus-specific surface antigen in cowpox virus-infected cells.

J. Tanaka and M. Hatano

2) Effect of oral bacterial extracts on growth of Echo-6 virus in human intestine cells.

S. Amano, K. Tamai, K. Miyamoto, S. Fukuda and M. Hatano.

In our previous report concerning the CFV S-ag formation, we had observed something like a cleavage effect of trypsin on the CPV-infected cells. Thereafter, we screened anaerobic oral bacteria having this cleavage activity on cultured tumor cells together with normal human cells. This experiment resulted in the finding of biological activity like an interferon inducer of the *Lactobacillus* strain, instead of cleavage activity. These bacterial extracts significantly inhibited Echo-6 virus multiplication in human intestine cells pre-treated with it, without marked cell damage. Purification of this active substance is now in progress, in addition to the re-examination of its cleavage effect on tumor cells.