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THE ACTION OF ADENOVIRUS TYPE 3 ON THE REPLICATION, PATHOGENECITY AND IMMUNOLOGICAL PROPERTIES OF HEPATITIS A VIRUS

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Viral hepatitis may be classified into three or more forms including type A hepatitis, type B hepatitis, and a group denoted as non-A non-B which represents viral hepatitis of more causes.

The extensive investigations regarding the etiology of viral hepatitis have pointed out some physical, chemical and biological attributes of the etiological agents of these forms of hepatitis (1, 2, 3, 4, 5, 6, 7, 8).

Some of the HAV characteristics (size, morphology, resistance to physical or chemical agents, experimental infection in animals) revealed by the authors mentioned, were similar or identical to those pointed out by us between 1961—1980 (9, 10, 11, 12, 13, 14, 15).

The identification of adenoviruses from acute hepatitis cases during 1960's — although their etiological role in hepatitis could not be proved — leads us to presume their intervention in hepatitis (16, 17, 18).

Zuckerman (19) has demonstrated in cell cultures of embryonic liver infected with San Carlos viruses, using electron microscopy, two types of viruses: particles with a diameter of approximately 65—70 nm and 30—40 nm, respectively.

The exact nature of these particles could not be precised, although the adenovirus type 3 antiserum neutralised the majority of the strains.

There are some differences between San Carlos and the prototype of the virus.

In the monographs of *Popper & Schaffer* (20) or *Zuckerman* (21) the opinion of some researchers is mentioned that the adenoviruses alone cannot produce a "typical hepatitis" and there might be possible a hybridisation between adenoviruses and the defective particle of hepatitis viruses.

In our results (16, 17, 18) we have noticed the replication of the hepatitis viruses in the presence of adenoviruses in Detroit-6 (VA) and KB cell lines. We have also mentioned that the simultaneous inoculation of adenoviruses and hepatitis viruses in hamsters induced the same histological picture of the liver as in severe human hepatitis cases.

This report summarizes the results of these investigations.

Material and methods

1. *Cell lines.*

In order to isolate the viruses from pathologic products as well as in order to study the simultaneous replication of hepatitis viruses and adeno-3, we have used Detroit-6 (VA) and KB cell lines (23). The growth medium was M 199 medium containing 10 % calf serum, 100 μg/ml. penicillin and 10 μg ml. kanamicyn, and the maintenance medium was M 199 medium with 10 % calf serum and antibiotics, as well as Eagle's mi-

nimal essential medium containing 1 % calf serum.

2. *Viruses*

The virus strains used in our experiments during 13 years (1966—1979) were: R, V, V 6, and 163S in 1962.

208, 226, 258, 270, 272 between 1966—1971

286, P 396, P 400. 439, 440, 441, HB 461 Hs 466,

F 467, 490, 493, Ha 497, F 502. F 501, F 503 between 1971—1977

and F 41, F 42, M—135. F 265, F 365. F 450. F 539 between 1978—1979.

All these strains had been characterised before, according to morphological picture. their resistance to heating, formaldehyde 1 4000, UV rays, ether. and their replication had been also followed up in the presence of HBB (hydroxybenzyl-benzimidazol) 45—90 mg ml and BUDR (5 bromo-2 desoxyuridin) 100 μg ml medium.

All strains except strains 226, and P 396 were characterised as hepatitis A virus (HAV).

Detroit-6 (VA) and KB cell lines were infected with 0.2 ml viral suspension (ID_{50} = 0,2 ml of 10^{-4} dil. virus) and then followed up as to cythopatogenic changes for 14 days.

3. *Simultaneous cultivation of hepatitis viruses and adenovirus type 3.*

In order to elucidate the role of adeno-3 in the replication of hepatites viruses. Detroit-6 (VA) and KB cell lines were infected with adenovirus type 3 (ID_{50} = 0,2 ml from 10^{-6} dilution) and after 30 minutes reinfected with hepatitis virus strains (0,1 ml viral suspension to 1 ml maintenance medium).

Cultures were harvested when cell changes occurred. then they were fixed in glutaraldehyde 1,5 % for 1 hour and in osmium tetroxyde according to Millonig technique, then they were embedded in Vestopal W or in a mixture of methyl and butyl methacrylate.

The electron microscopic examinations were made with a TESLA BS 613 microscope.

Some cultures with cytopathogenic changes were submitted to the effects of freezing and thawing for three times, centrifugated, the supernatant being treated with Genetron and then dyalised against polyvinylpyrolidon 20 %. This material was used for serological investigations.

4. *Experimental inoculations*

250 hamsters out of 657 animals used for experimental transmission of hepatitis. had been inoculated with a mixture of hepatitis virus and adenovirus. and with the material obtained after inoculation of cell lines with these two types of viruses, too.

The hamsters were divided into 5 groups:

— the first group of 107 hamsters were inoculated intraperitoneally with 0,5 or 1 ml adenovirus followed by a second inoculation with hepatitis virus (0.2 or 0.5 ml.) harvested from Detroit-6 (VA) or KB cell lines inoculated with hepatitis strains R. V 9. Rc 208. 258. 286, P 400, HB 461, F 490, F 493. F 41. F 42. F 450. HB 530. F 539, H 133. F 265. F 365.

— the second group of 44 hamsters were inoculated with strains 226 and P 396. These strains containing adenovirus particles of 70—90 nm

and 30 nm, whose replication was not inhibited by HBB, were named bu us "normal hybrid of hepatitis viruses".

— 21 animals were inoculated with 0,5 ml. of an "artificial hybrid virus" obtained by simultaneous cultivation of adeno-3 and hepatitis virus (strains RH, 208 H, 442 H, 465 H and F 41 H).

— 27 hamsters were inoculated intraperitoneally with 0,5 or 1 ml suspension of adeno-3 virus.

— the last group including 51 hamsters were used to induce acute hepatitis by inoculation of 0,5 ml. of virus isolated from acute hepatitis cases (strains R, V/9, 208, 256, 286, P 400, F 461, F 490, F 493, F 41, F 42, F 450, F 539, M 133, F 265, F 365).

After 6—8 weeks we checked up the histological and electronoptical changes in the liver of all animals experimentally infected.

5. *Serological investigations* were carried out to prove the role of adenovirus in the replication of HAV.

Thus, 20 serum samples from patients with acute viral hepatitis were investigated for anti-HAV (strain 208), for antibody to "artificial hybrid virus" (strain 208 H) using PHA (Friedman and Benett).

By serum neutralization test we have followed up in serum of 10 patients the dynamics of anti-HAV (strain 208), of anti-adeno 3, and of antibody against strain 208 H.

283 serum samples harvested between 12—18 days of the illeness were checked up for the antibody anti-adeno-3 against the "artificial hybrid viruses" (441 H, 208 H, 466 H), as well as against natural hybrid (P 396), using immunoprecipitation technique.

We have also investigated the presence of viral antigens in patients'serum, using antiserum anti-adeno 3, anti-208 H, anti-466 H and anti-P 396 prepared in hamsters, as well as the levels of antibodies (by PHA) against strains 441 H, 208 H, 286 H, P 400 H obtained by simultaneous cultivation of adeno-3 and virus strains 441, 208, 286, P 400.

Results

1. *Some aspects of the replication of HV in Detroit-6 (VA) and KB cell lines*

Seven to fifteen days after inoculation of cell lines we have noticed a moderate cytopathogenic effect: the appearence of foci of rounding cells and finally the cell detach from the tube walls.

These cytopathic modifications are not similar to those produced by enteroviruses, and sometimes the multiplication of HAV can be proved only by electronmicroscopic examination.

Our first study (1961—1965) revealed that the viral replication took place in cell's cytoplasm. when the viral particles can be arranged in symmetrical ranges or in clusters, having a size of 70 nm and being composed of particles of approximately 15—20 nm. Some of these complex particles were found in the vacuoles of the cytoplasm (10.).

Between 1966—1971, 4 out of 5 strains were identified as HAV and one (strain 226) as adenovirus. These viruses were localized in the cytoplasm as well as in the nucleus, being composed of 30 and 70 nm particles. Their replication was inhibited by HBB (18).

The virus strains isolated in the last period of time (1978—1980) were found by electron microscopy to contain particles with a diameter of 27—42 nm.

The viral agent is ether resistent and is not destroyed by heat at 60 °C for 30', the Genetron has no influence upon their pathogenity and BUDR does not interfere with its replication on KB cell lines.

On the basis of a lent replication, the disappearance of cytopathic modifications when the cultures were successively by-passaged, the high number of incomplete viral particles, we considered that virus a defective one.

2. *Simultaneous cultivation of HAV with adenovirus type 3*

According to the 30 experiments on cell lines, we are able to underline again the fact that adeno-3 acts as efficient helper for the replication of HV isolated by us, and is able to produce the following morphological modific tions: five days after inoculation, the cells become swollen and the cell margins rounded, their size surpassing the magnitude of cells infected with adenovirus. Subsequently the damaged cells begin to detach from the tube walls after 7 days.

The cells infected only by adeno-3, cluster together and after about 4—5 days become rounded. The strains of HAV induced a tardive cytopathic effect (7—15 days) on the cell cultures, characterised by the appearence of small foci of rounding cells.

The simultaneous replication of these two viruses in the nucleus and more rarely in the cytoplasm could be proved by electronoptical examination. In the nucleus of the infected cells we can prove, also by electron microscopic examination, the presence of:

— adenovirus particles of 70—90 nm diameter, with a nonequal or crystal dispersion;

— spherical viral particles of 25—30 nm ranged in a compact mass or with a nonequal dispersion;

— spherical particles of 40—45 nm having irregular margins and without central core (incomplete viral particles?);

— sometimes in the infected cell nucleus, some ovular, spherical particles with irregular margins, having the size of about 120—140 nm, occur.

3. *Experimental inoculation in hamsters*

In 107 hamsters used in this experiment, the simultaneous association of HV and adenovirus type 3 revealed the following liver lesions: cellular necrosis, periportal and parenchymatous infiltration with monocytes, lymphocytes, fibroblasts or even the apparition of connective tissue.

The same histological picture has been produced by the 226 and P 396 virus strains in other groups of 44 hamsters. These strains resemble with adenoviruses, although they contain particles of 30 nm, being probably nonhomogenous mixed strains in the moment of the isolation from patients with hepatitis.

The presence of the connective tissue in hamster's liver inoculated with HAV and adeno-3 was found in 30 %.

The "artificial hybrids" obtained in cell lines by successive passages and inoculated in hamsters induced important modifications of the liver: megalohepatia with rugged surface and thickened Glisson capsule, cell

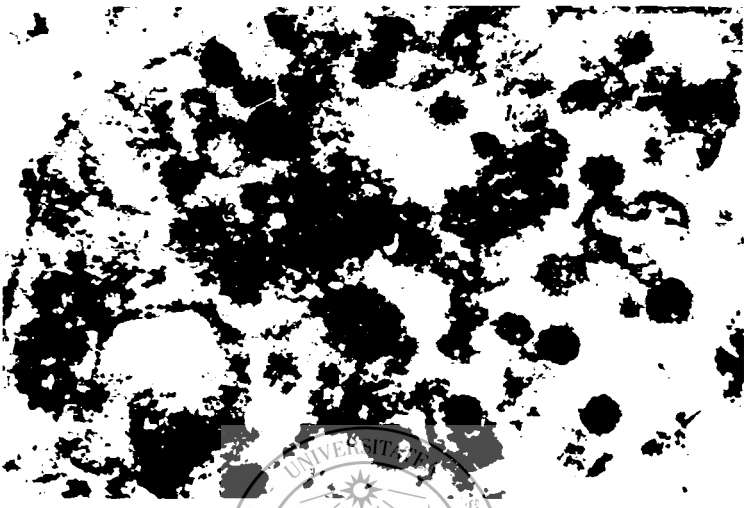

Fig. 1: Hepatitis A virus inside citoplasmatic vacuole. 180.000 ×.

Fig. 2: Adenovirus and viral particles of about 33 nm intranucleos of KB cell line after simultaneous infection with adenovirus type 3 and hepatitis A virus. 120 000 ×.

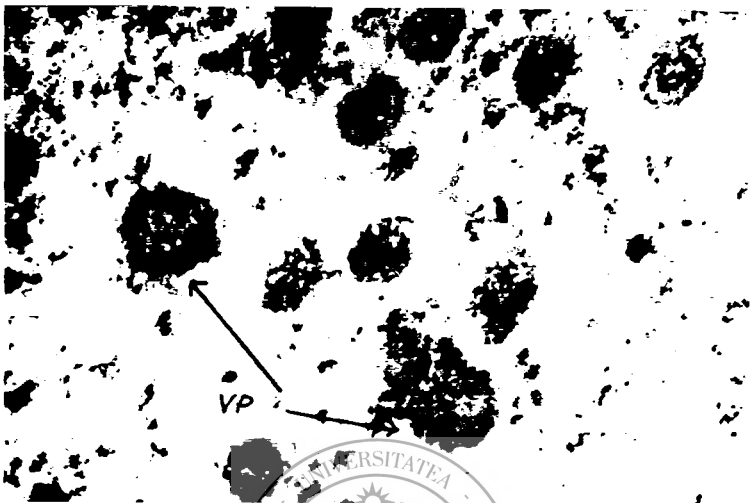

Fig. 3: Adenovirus and large viral particles (VP) in the nucleus of KB cell after simultaneous infection with adenovirus type 3 and hepatitis A virus. 140 000 ×.

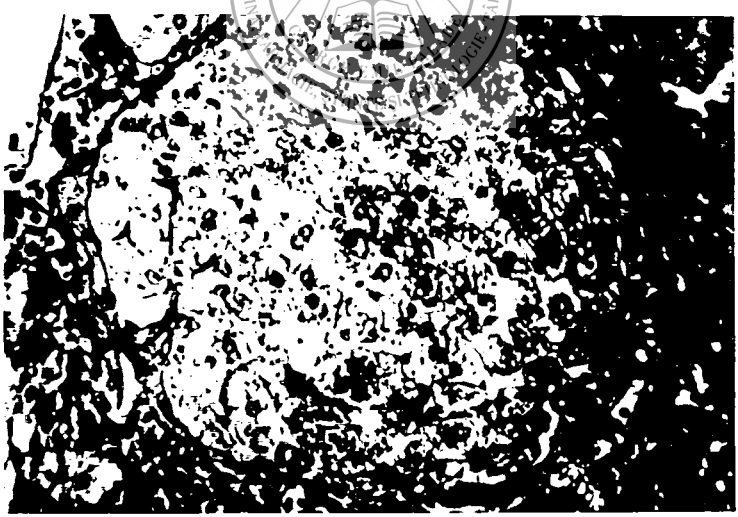

Fig. 4: Pseudolobules after simultaneous infection with adenovirus and hepatitis A virus. Ob. 20 ×, Oc. 10 ×.

necrosis, periportal and parenchymatous infiltration, accumulation of connective tissue.

Adenovirus type 3 induces also histological modifications, but the damage was not so severe as that noticed when we used the "hybrid viruses".

In 68,3 % the viruses isolated fron acute hepatitis cases without association of adenovirus, have induced in hamsters the following modifications of the liver: dislocation of the hepatic trabeculae, activation of the Kupfer cells, accumulations of the bile pigments, periportal or parenchymatous infiltration with lymphocytes, plasmocytes, hystiocytes and fibroblasts.

The most severe hepatic lesions comparable to those from human cirrhosis were observed after the inoculation of hamsters with the M 133 strain isolated from a case of icterus neonatorum.

The electron microscopical investigations of the hamster's liver infected with HAV strains acknowledged the presence of viral particles in the cytoplasm of the cells in the first weeks after inoculation.

4. *Serological investigations*

— Following up the dynamics of specific antibodies against "hybridized hepatitis virus" by PHA technique, we have noticed an increase of their incidence in 75 % of the cases and a decrease in 25 %, in the convalescents.

In contrast, the titre of anti-adeno-3 decreases in 45 % the cases in the same period of the illness, and increases in only 20 % of the cases.

— Serum neutralization test revealed in 8 out of 10 persons a rise of antibody anti-hybrid virus during the acute phase of the illness, while the neutralizing antobides against adeno-3 virus after a slight rise decreased.

— Using gel immuno-precipitin technique (IP), antibodies against adeno-3 were found in 12,01 % and antibodies against HAV in 25 %. The low incidence of antibody by IP is due to the fact that serum samples were obtained between 12—18 days of the illness.

— Investigations carried out in the presence of antigens in serum revealed a positivity of HA—AG in 25,86 %, of adenovirus-Ag in 12,14 % and of HBsAg in 6,6 % of cases.

As regards the incidence of specific antibodies, anti-HAV were found in 40,7 %, anti-adeno 3 in 28,5 %. This fact drew attention again to the possible role of adenoviruses in inducing hepatitis.

Discussion

1. According to the experiments made on cell lines, we are able to underline the fact that the virus strains isolated between 1957—1967 as well as those obtained in the last period of time, induced the same alterations on Detroit-6 (VA) and KB cell lines.

As for the replication of the viruses the appearence of cythopathic effect in foci and the instability of this modification in successive passage are characteristic.

That phenomenon is probably due to the presence of a high number of incomplete virions (defective, viral particles without core).

Human hepatitis A virus was reliably and repeatedly propagated in primary explant cell cultures of marmoset liver and in the normal fetal rhesus kidney cell line (FRHK—6) by *Provost* and *Hilleman* (3).

Although no cytopathic changes were observed, the identity of the virus was established in immunofluorescence, immunofluorescence blockade, serum neutralization, immune adherence, radioimmunoassay, immune electron microscopy and marmoset inoculation tests (3).

Recently *Frösner* et al. (7) reported the isolation of HAV directly from human feces on human hepatoma cell line, without cytopathic effects.

Similar results were obtained by *Daemer* et al. (8) using African green monkey kidney cell culture (AGMK) for primary isolation and serial passage of HAV. The virus appears to be cell-associated.

Our results proved also the replication of HBV on Detroit-6 (VA) cell cultures, the maturization of the virion taking place in the cytoplasm after 18 days (22). These results are in agreement with those of *Panouse-Perin* (23).

Concerning the different size of the HAV particles (15—17 nm) our opinion is that it is influenced by the conditions of cultivation and by the composition of the maintenance medium.

In the presence of adeno-3, the HAV replicates both in nucleus and cytoplasm of infected cells. Big particles of 120—140 nm seem to be aggregations of small 15—20 nm particles.

2. The hamsters' receptivity to the simultaneous infection with adenoviruses and HAV, is revealed by the histologic modifications similar or identical with those observed in humans.

The mild hepatic lesions noticed after inoculation of adenovirus or HAV alone and the severe lesions observed in case of administration of both viruses or of "hybrid viruses".

3. The role of adenoviruses in the pathogeny of hepatitis A is revealed by serological researches: the occurrence of antibodies against "hybridizated strains" in high titres during convalescence (24), the presence of adenovirus antigen in 12.14 % at the beginning of the disease, respectively the presence of specific antibodies in 28.5 % of the cases.

Conclusions

1. Using Detrois-6 (VA) and KB cell lines it is possible to follow up the replication of HAV, but its cytopathic effect is not characteristic.

2. The size of viral particles ranges between 15—17 nm, the majority having 27—30 nm.

3. The adenovirus type 3 facilitates the replication of the HAV, the number of the virions in cytoplasm and nucleus being greater than in case of cell infection with A virus alone.

4. After the simultaneous inoculation of hamsters with adenovirus 3 and HAV or with "hibridizated strains", serious lesions of the liver were observed.

5. The dynamics of the increase of antibodies against "hybrid virus", studied in serum samples from acute hepatitis cases, confirms the role of the adenoviruses in the pathogeny of hepatitis type A.

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