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A COMPREHENSIVE STUDY OF VIRAL HEPATITIS

Final Progress Report

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SUMMARY

With the assistance of this contract human viral hepatitis and a great variety of other hepatic diseases known are suspected to be caused by viruses that have been investigated. Of these diseases the principal ones have been human neonatal giant cell hepatitis, human cytomegalic inclusion disease and rubella as well as mouse hepatitis and mouse cytomegalic inclusion disease. These human and experimental infections were studied by morphologic and biologic methods in the hope that a better understanding of the mechanism of viral liver injury will lead to better control of human viral hepatitis.

Particular attention has been paid to a study of cell death in the liver by electron microscopy and histochemistry. This led to a comparison of hyaline and lytic necrosis. The possible protective role of the reticuloendothelial system, the harmful effect of cortisone and the role of lysosomes in protecting the cell against some viral infections have also been explored.

A generalized summary of those cooperative investigations in the classification of candidate viral agents has been outlined. The AR-17 strains of Dr. Richtsel formerly of Parke Davis and Dr. Boggs of Chicago, Ill. and the A-2 (CW) plaque virus of the AFIP have not been classified, and only the A-2 plaque virus has had significant reduction in the plaque-forming-units by only the positive reference patient's sera in our investigations.

The Abstract of an Interim Report, dated 22 January 1968, has been included for completeness of this terminal FY68 progress report. It has described the zonal ultracentrifugation attempts to purify the A-2 (CW) plaque virus. Fraction B-18 was significantly inhibited in plaque-forming-units by convalescent sera, but subsequent viral plaque purification steps are still in progress.

Cellular transformation with possible virogenization studies were summarized. The use of radioactive isotopes has been described in an attempt to trace viral propagation in host cells of human embryonic kidney tissue cultures without overt cytopathic effect. Preliminary results have indicated that an apparent viral RNA entity was recovered from one icteric plasma specimen without overt cytopathic effect in either human embryonic kidney or in the LLCMK₂ (continuous rhesus monkey kidney cell line of Hull et al., 1959) tissue cultures.

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Work performed at the Johns Hopkins University School of Medicine

Under this contract a great variety of liver lesions have been studied throughout has been a better understanding of human viral hepatitis in the hope that a better understanding of this disease will lead to better control. In particular, we have tried to elucidate the pathologic and biologic changes in human viral hepatitis by a comparative study of viral liver injury in human and in experimental animals. In man, the following conditions have been studied: A) Infectious and serum hepatitis, B) Neonatal giant cell hepatitis, C) The hepatic lesions of the congenital rubella syndrome as well as, D) Certain other conditions which are possibly, but by no means certainly related to viral infection, such as hepatoma and familial intra-hepatic cholestasis. These human diseases have been compared with certain animal models. The principal one among these has been, E) Viral hepatitis in mice. We have studied the hepatic lesion produced by mouse hepatitis virus (MHV3 and MHV1). Also those produced, F) By the mouse cytomegalovirus which are very similar to those produced by human cytomegalic inclusion disease. We have also studied, G) The effect of endotoxin on the mouse liver since this ubiquitous substance may play a subsidiary role to the virus in human hepatitis.

During the course of these studies, it was noted that some of our experimental viruses also produced effect on organs other than the liver. We, therefore, tried to learn more about the mechanism of hepatocellular injury by comparing the lesions produced in the liver with those produced in other organs by these viruses. In the case of the mouse hepatitis virus, the lesions which we studied were those produced in the lymphoid system and the pancreas and, in the case of mouse cytomegalovirus, we studied particularly the lesion produced in the salivary gland. Finally, we studied the effect of various chemicals on the course as well as the lesions produced by our experimental viral hepatitis. Here again, the aim was a better understanding of viral liver injury and a rational approach to its control.

The pathology of human viral hepatitis in biopsies was studied by light and electron microscopy (1, 2). The liver cell lesions were chiefly of two types: The first consisted of hydropic degeneration leading to lytic necrosis. Electron microscopy showed this to be due to dilatation of the rough endoplasmic reticulum. The second lesion was hyalinization sometimes resulting in one type of acidophilic body. Electron microscopically, this consisted of indistinct organelles and increased electron opacity. The Kupffer cells also showed corresponding lesions. Many were swollen and vacuolated; occasionally they contained glycogenosomes. Necrosis of Kupffer cells resulting in increased electron opacity and presumably corresponding to hyalinization was also observed. Basement membranes, generally discontinuous, were quite common between hepatocytes and endothelial cells. The lateral cell membranes of the hepatocytes often showed some separation with the development of microvilli. Various types of material composed the biliary thrombi. In agreement with most other investigators, no virus particles were ever identified in any of our material.

The pathology of human neonatal hepatitis and biliary atresia were also studied since both conditions may well be of viral etiology. The formation of giant cells and of duct like liver cell aggregates was more striking in neonatal hepatitis than in atresia. Acidophilic bodies were also more striking in neonatal hepatitis while periportal inflammation was more characteristic of atresia. Centrilobular cholestasis was more frequent in atresia. These differences, however, were not reliable enough to help in differential diagnosis. Giant cells appeared to be common histologic response of the liver to various types of insult, including particularly viral infection. Portal scarring and bile duct proliferation were typical of well-established extra-hepatic atresia while intralobular fibrosis was characteristic of advanced neonatal hepatitis. It is desirable that patients be studied in the first two or three months of life when the pattern of fibrosis may not yet be clear. In such cases operative cholangiography combined with liver biopsy gives the best chance of making an accurate diagnosis. Excessive extramedullary hemopoiesis was more common in neonatal hepatitis than in atresia and gave some help in making this diagnosis.

The hepatic alterations of twelve patients with congenital rubella were reviewed and studied by electron microscopy and histochemistry (3). The spectrum of hepatic lesions included hyalinization and swelling of hepatocytes, cholestasis, hemapoiesis and in some cases multinucleated giant cells. The picture, therefore, in many cases, was that of neonatal giant cell hepatitis. Cases of extrahepatic or intrahepatic atresia in conjunction with congenital rubella were not seen by us. Many giant cells contained heavy glycogen deposits and showed marked activity of several hydrolytic and oxidative enzymes. The fine structure of the cytoplasmic organelles in both multinuclear and mononuclear liver cells was relatively normal except for cholestasis. The bile canaliculi adjacent to the giant cells appeared reduced.

In experimental mouse hepatitis, focal hepatic necrosis developed within two days of intraperitoneal infection. The foci were composed of hyalinized cells and extended rapidly so that after four days the confluent necrosis was produced which was fatal depending on the dose given. Histochemically the necrotic foci had little enzyme activity, but some perifocal parenchymal cells showed increased activity of some dehydrogenases (4, 5). The necrotic foci appeared largest in sections stained for glucose-6-phosphatase which suggested that glucose-6-phosphatase, an enzyme related to the endoplasmic reticulum, was our most sensitive indicator of this type of hepatic injury. Striking diffuse changes in oxidative enzymatic activity unrelated to focal necrosis also developed. Succinic/dehydrogenase and glucose-6-phosphatase showed a decrease affecting peripheral areas rather more than central areas while reduced DPN diaphorase and cytochrome oxidase were uniformly reduced. However, glucose-6-phosphate dehydrogenase, TPN linked isocitric dehydrogenase and reduced TPN diaphorase showed increased activity in centrilobular zones. Generalized changes effected also the phosphatases. The sinusoidal alkaline phosphatase activity of parenchymal cells increased while their canalicular activity decreased. There was a disturbance of the normal pericanalicular arrangement of parenchymal cells lysosomes and a marked decrease in their staining intensity. The number of

acid phosphatase positive Kupffer cells decreased, but increased again during the recovery phase. These changes suggest that widespread metabolic changes developed in virus infected liver cells. They also show that both Kupffer cells and parenchymal cells are severely damaged. The problem whether Kupffer cells or parenchymal cells were injured first could not be resolved in these experiments.

The lesions produced by mouse hepatitis in the liver were studied by electron microscopy, as well as histochemistry (6, 7). Particular attention was paid to the effect of cortisone. Cortisone not only increased the number and size of the necrotic foci produced by MHV3 virus, but also it altered the histologic appearance of the necrotic parenchymal cells at the light microscopic level. Instead of becoming uniformly hyalinized hepatocytes became markedly swollen and subsequently underwent a lytic type of necrosis. Cytoplasmic virus formation was seen most clearly in hepatocytes of cortisone treated animals. Particles appeared to form in relation to newly synthesized endoplasmic reticulum and acquired an outer envelope by budding through smooth membranes derived from this endoplasmic reticulum. Hyalinization seemed to correspond to two ultrastructural lesions, one of these was the development in hepatocytes of focal cytoplasmic degradation with increased electron opacity and indistinct cytoplasmic organelles. The other, less common one was proliferation of smooth endoplasmic reticulum. Both these alterations were occasionally seen in Kupffer cells. Lytic necrosis in hepatocytes of cortisone treated animals corresponded to marked intracellular edema, striking mitochondrial swelling and the formation of intracellular vacuoles probably derived from dilated endoplasmic reticulum.

While liver damage is the best recognized manifestation of infection by mouse hepatitis, we also noted necrosis of the follicles in lymph nodes and spleen (8, 9). These lesions follow particularly infection by MHV3 virus. The lesions were comparable to the hepatic damage in severity, frequency, and probably in importance. The lymphoid necrosis and those cellular changes which may reflect the immune response of the host were studied sequentially by histochemical methods. Particular attention was paid to pyroninophilia, a feature common to the plasma cell series and to germinal center cells. Alkaline phosphatase activity also was carefully evaluated because of its presence in the lymphocytes and to a greater extent in the plasma cells of our animals. The lesions consisted of widespread severe necrosis of lymphoid follicles. Follicular necrosis in the spleen began at the marginal sinus and extended centrally. The lymphocytes of the marginal zone were usually spared and appeared to be transformed into plasma cells together with those lymphocytes of the true follicles which survived the necrosis. The transformation was characterized by an increase in alkaline phosphatase. The lymphoid necrosis seemed attributable neither to liver damage nor to nonspecific stress. In mildly infected animals with undamaged follicles, germinal centers developed in the later stages of the infection. Direct transformation of germinal center cells into plasma cells seemed unlikely.

The severe lymphoid necrosis produced by murine hepatitis virus was prevented by cortisone while the liver lesions were aggravated. (10). Induction of hepatic sinusoidal alkaline phosphatase activity was also inhibited by cortisone. Prevention of lymphoid necrosis and of induction of sinusoidal alkaline phosphatase were highly correlated suggesting that a depression of protein synthesis was a common underlying factor. It was concluded that multiplication of the virus alone did not produce the lymphoid necrosis, but that protein synthesis induced by the infection, presumably an immune reaction, might be primarily responsible for this lesion.

Our studies on cytomegalovirus disease (11,12,13,14,15) showed that the lesions produced by the mouse virus in most respects were an excellent model for the human disease. The development of the mouse cytomegalovirus in the salivary gland and liver was compared by electron microscopy and histochemistry. In the early intranuclear stages of infection more viral cores appeared to be produced than protein coats, while in the later stages this was reversed. Cytoplasmic virus particles persisted almost unchanged in the submaxillary salivary glands for at least 28 days. Viral titers showed relatively little decrease up to 100 days. In the liver, however, cytoplasmic virus particles soon became surrounded by dense material with lysosomal characteristics. Virus particles were rarely seen after the fifth day and the viral titers decreased at the same time. We suggested that the persistence of the cytomegalovirus in the salivary gland may be related to the relatively low lysosomal activity of this organ. The cytoplasmic particles of human cytomegalovirus in human fibroblasts resembled those produced by mouse cytomegalovirus in the mouse liver rather than in the salivary gland.

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Work Performed at the Armed Forces Institute of Pathology

In a generalized summary of progress for those aspects of research conducted at the AFIP under this contract since its initial grant in July of 1961, the following factors have been presented. From the evaluation of viral agents which were forwarded from other investigators in a cooperative study, it was determined that a) the San Carlos agents of Davis, in Arizona, were predominantly type 3 adenoviruses, b) the Brown virus of McKee, in Iowa, was confirmed to be a type 1 reovirus, and c) the A-1 agent of O'Malley, in NIH at Bethesda, Md., was determined by another laboratory to be a Mycoplasma gallisepticum. Inconclusive results have been accumulated work with the AR-17 strain of Rightsel, formerly of Parko Davis and with the AR-17 strain of Boggs in Chicago, Ill. The Willowbrook (WB) virus of Leibhaber and Krugman, of New York, yielded no plaque reduction by patient sera in a cooperative study with the Division of Veterinary Medicine of Walter Reed Army Institute of Research. There has been no cytopathic viral agent recovered from the second series of patients that were described by Conrad in 1965, although icteric and convalescent plasma specimens from ten patients of this second "Korean series" had been distributed to approximately twenty-two separate laboratories. From the first series of military patients who were ill with infectious hepatitis in Korea during the late portion of 1962, the early investigators, Col. T. O. Berge, Dr. Ambhan D. Felsenfeld, and Major (Lt. Col.) J. D. Douglas, recovered type 16 adenovirus from the icteric sera of three patients, and a type 11 adenovirus from urine of one patient. In recent studies, however, re-isolation of these viruses has not been successful. From a survey of incidence rates of a serum-virus neutralizing antibodies against the type 11 and 16 adenovirus, no significant difference could be observed between the series of patients from that of the series of healthy individuals whose sera were collected during the time of a study within the same geographic area.

The second group of viral agents which was repeatedly recovered from the icteric phase sera of those patients has been designated A-2, or agent 2. The prototype A-2 (CW) plaque virus has had significant reduction in its plaque-forming-units only by the positive reference patients' sera in our investigations. Characterization studies of the A-3 (CW) plaque virus are still in progress. From preliminary studies it has had the apparent properties of an enterovirus: a) Survived heating at 60° C for 30 minutes and at 56° C for 60 minutes, b) Survived heat at 50° C in the presence of Mg⁺⁺, CA⁺⁺ divalent cations, and c) Survived ether, sodium desoxycholate, Actinomycin D, and a range of pH from 3.9 through 8.9. It has not been significantly reduced in plaque-forming-units by hyperimmune type-specific antibody against the following viruses: types 1,2,11,12,15-19,25,33,34,36, and 59 simian virus, types 3,4,5 simian adenovirus, types 3, 4, and ^{UP5}HIH rhinovirus, types 1-5, 6"-3, 9",12,26,28-32, and 36 echovirus, or types 11, 15,16,22,24,26,27 adenovirus. Guinea pig antiserum prepared against the AR-17 strain from Boggs of Chicago, Illinois has not neutralized the plaque-forming-units of the A-2 (CW) plaque virus. The A-2 (CW) plaque virus has had no toxicity or lethal effect in suckling or adult mice or hamsters nor has it agglutinated a wide variety of erythrocytes. Although it would appear to be an unclassified enterovirus, there are approximately one hundred type-

specific antisera which must yet be screened for possible neutralization and subsequent classification. The results of the plaque reduction tests with only patient sera and plasma have appeared to be highly significant when subjected to profit analyses such that we feel this is a tentatively presumptive test for human infectious hepatitis. As soon as classification attempts with type-specific hyperimmune antibody against a limited but significant number of additional agents, as well as exhaustive isolation attempts for possible mycoplasma contamination, have been completed, this agent with its type-specific antiserum will be released to the Division of Biologic Standards at NIH and to other interested investigators.

Reference is made to Interim Report, dated 22 January 1968. The following Abstract has been included in order to assure completeness of this terminal FY68 progress report:

a. Zonal ultracentrifugation. - The attempts at "banding" in a sucrose density gradient two major fractions from the A-2 (CW) plaque virus, numbered 16 and 18, have been described. Preliminary results have indicated the plaque forming units (pfus) of fraction 16 to be inhibited to significant levels by one normal and one 13-day post icteric sera, but not by two convalescent sera. Virus-like particles of approximate diameters of 200 Angstrom units were observed, and the equivalent specific gravity of the sucrose in this fraction was 1.11. The pfus of fraction of 18 were inhibited to significant levels only by convalescent phase sera from two patients with infectious hepatitis--not by the normal or the 13-day post icteric sera. Virus-like particles of approximate diameters of 430 (with a minimal number of particles of approximate diameters of 200) Angstrom units were observed in fraction 18. The equivalent specific gravity of the sucrose in fraction 18 was 1.12.

Plaque purification procedures of the fraction 18 portion from the sucrose density gradient have been in progress. The full spectrum of characterization studies on the recovered progeny from the plaques have been initiated in order to qualify the purified A-2 (CW) plaque virus as an unclassified but acceptable candidate virus for its release to other investigators in study of its possible etiology for human infectious hepatitis.

b. Cellular transformation with possible virogenization. - Since the A-2 (CW) plaque virus had appeared to have neither an overt cytopathic effect nor plaque forming activity under an agar overlay in human embryonic kidney (HEK) tissue culture, it was employed as a positive infective control in a parallel study with three icteric phase and one normal sera as inocula into the subcultured monolayers from previously trypsinized monolayers of primary HEK. After approximately eleven weeks of study, the experiment was inadvertently terminated. It has been resumed and was in the fifth week of study at this writing. Cellular alterations had begun to form only in those flasks of HEK monolayers which had received the A-2 (CW) plaque virus and in two of the three icteric phase sera--not in the negative control passages. In those HEK flasks which had received the A-2 (CW) plaque virus, a series of coarse-striated multiple layers of cells had formed with markedly thick cytoplasmic and polarized types of extensions. In contrast, two of the three flasks of HEK, which had received the icteric phase sera from

patients with infectious hepatitis, a limited cellular degeneration of the cell sheets had occurred with formation of isolated and multiple layered clusters of HEK cells which had apparently lost the normal property of contact inhibition. ADDENDUM to Interim Report. Plaque purification procedures from the B-18 fraction have not been successful to date. Chromatographic and density gradient analyses have been initiated. The transformation studies are in progress with primary human embryonic kidney and with a continuous cell line of Psy (cloned porcine kidney from Inoue and Ogura) tissue cultures.

It had been observed, as noted above, that the A-2 (CW) plaque virus, having been propagated under an overlayer of agar in African green monkey kidney or the LLCMK2 (Hull), would not form plaques (under an overlayer or agar) or an overt CPE in primary tissue cultures of human embryonic kidney (HEK). However, in the HEK cells the A-2 (CW) plaque virus had replicated with the formation of increased quantities of plaque-forming units (pfus) when titrated from inocula that consisted of infected and trypsinized HEK cell sheets under an agar overlayer in the indicator system of LLCMK2 cells.

In every instance, the mechanisms of action actinomycin D reduced the incorporation of ^3H -Uridine in the production of cellular RNA, which was in evidence to an enhanced degree when the inocula had had actinomycin D present during the previous absorption of the inocula to the human embryonic kidney (HEK) cell sheets. From graphs depicting radioactivity, it would appear that the ^{14}C -2-Uridine had been incorporated as viral RNA without an overt CPE in the HEK host cells, which had actinomycin D in the maintenance medium. A portion of the infective viral RNA was duplicated, and it was detectable primarily by the uptake of ^{14}C -2-Uridine and a secondary precursor isotope of ^3H -Uridine by the formation of new viral RNA within a subsequent cell system of LLCMK2 cells. This was demonstrated substantially by the type 2 reovirus, and, with diminishing quantities, by the icteric plasma of patient MJ and by the A-2 (CW) plaque virus. There were no detectable levels of radioactivity for ^3H -Uridine or ^{14}C -2-Uridine, in the presence or absence of actinomycin D, from cell sheets which had formed in stationary tube cultures from minimal (1.0 ml) quantities of the whole-cell inocula of HEK cells. Thus it was concluded that the production of new viral RNA, as measured by the uptake of both ^{14}C -2-Uridine and ^3H -Uridine, had occurred mainly in the LLCMK2 tissue cultures.

Although a CPE was observed in the indicator system of LLCMK2, when infected with HEK whole-cell-inocula previously inoculated with type 2 reovirus and A-2 (CW) plaque virus, there was an enhanced proliferation of the LLCMK2 and of the inoculum of HEK cells which had appeared to have absorbed a viral RNA entity from the icteric plasma of patient MJ.

The recovery from the icteric plasma of an apparent viral RNA entity, which is defective in the concept of not producing CPE in both HEK and in a secondary cell system of LLCMK2 was unexpected. Most certainly these studies will be substantiated, and studies of its relationship to the confirmed cases of human infectious hepatitis will be presented.

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13. ABSTRACT With the assistance of this contract human viral hepatitis and a great variety of other hepatic diseases known are suspected to be caused by viruses that have been investigated. Of these diseases the principal ones have been human neonatal giant cell hepatitis, human cytomegalic inclusion disease and rubella as well as mouse hepatitis and mouse cytomegalic inclusion disease. These human and experimental infections were studied by morphologic and biologic methods in the hope that a better understanding of the mechanism of viral liver injury will lead to better control of human viral hepatitis. Particular attention has been paid to a study of cell death in the liver by electron microscopy and histochemistry. This led to a comparison of hyaline and lytic necrosis. The possible protective role of the reticuloendothelial system, the harmful effect of cortisone and the role of lysosomes in protecting the cell against some viral infections have also been explored. A generalized summary of those cooperative investigations in the classification of candidate viral agents has been outlined. The AR-17 strains of Dr. Richtsel formerly of Parke Davis and Dr. Boggs of Chicago, Ill. and the A-2 (CW) plaque virus of the AFIP have not been classified, and only the A-2 plaque virus has had significant reduction in the plaque-forming-units by only the positive reference patient's sera in our investigations.			

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