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THE FIFTH DR. P. V. GHARPURE ORATION

Hepatitis Virus Infection — Global Concern

BY

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Professor of Pathology, All India Institute of Medical Sciences, New Delhi

Delivered at the Haffkine Institute, Parel, Bombay-400 012, on 24th February, 1981.

ORATION

The Fifth Dr. P. V. Gharpure Oration, delivered at the Haffkine Institute, Parel, Bombay, on
24th February, 1981.

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HEPATITIS VIRUS INFECTION — GLOBAL CONCERN

N. C. NAYAK

Text of P. V. Gharpure Oration delivered at the Haffkine Institute on 24th February, 1981.

Standing before you here I feel justifiably honoured to have been given this opportunity of paying my personal tribute to this great Institute of work and learning and to Dr. P. V. Gharpure, one of the chief contributors to its glorious heritage. I am extremely grateful to the Haffkine Institute, its Governing Body and Director for the honour they have bestowed on me.

Dr. Purshottam Vishwanath Gharpure born in the Victorian era, was one of the pioneers in the field of Virology in this country. Dr. Gharpure, a brilliant student of pathology and microbiology, was the recipient of numerous coveted awards and fellowships during his career as a student at

the Grant Medical College, Bombay. Later on he held the post of the Professor of Pathology & Microbiology at the same college from 1925 to 1951. During the second world war he served in the Indian Army as a major.

Dr. Gharpure was instrumental in establishing the Enterovirus Research Unit of the ICMR. Originally the unit, then known as Polio Research Unit was operating from the Grant Medical College where Dr. Gharpure was employed as Professor. Later on the unit was transferred to Haffkine Institute with an enlarged scope and expanded activities to cover the entire group of enteroviruses. His research work in the field of enteroviruses has received international recognition. It was his initial efforts with the WHO that ultimately led to the establishment of a laboratory for production of Polio vaccine, now with the Haffkine Biopharmaceutical Corporation.

My first contact with Dr. Gharpure was more than two decades back when I was entering the portals of pathology as a special discipline. As a young member of the profession as well as later, one could not but admire Dr. Gharpure's keen and brilliant mind, logical, scientific attitude and a mild, generous disposition. Dr. Gharpure's contributions to medicine in general and to pathology and microbiology in particular have been many and he will always be remembered as one of the doyens in medical sciences in our country.

In paying my personal homage to this great scientist I wish to briefly highlight this morning, some areas of investigative work by my colleagues and myself on hepatitis virus infections, a field that has made an impact on human health and disease possibly unprecedented in history. Man's success in mastering the microbes lies in his ability to combine astute observation, technological skill and logical deduction. As Louis Pasteur said "To be astonished at anything is the first movement of the mind towards discovery". Within one and a half decade of Blumberg's discovery of the Australia Antigen we know that at least three major categories of hepatitis viruses infect man. Knowledge on the structure and biological properties of these viruses, the diseases associated with them, and the means of identifying and possibly preventing infections are accumulating at an unbelievable speed. By using various immune markers it has been possible to relate certain forms of liver diseases with these virus infections.

The B virus having been the first to be discovered, is the one, most extensively studied. Various components and antibodies to each one of these have been identified. Serological studies have shown that the hepatitis B virus (HBV) is a highly successful agent in terms of its symbiosis with the host. Not only does it produce significant acute disease but also a very peculiar, often prolonged asymptomatic carrier state, frequencies of occurrence of which can be extremely high. A recent estimate reveals that in the USA alone about 15,000 individuals suffer from acute hepatitis

B annually. The figure for our own country is certainly several fold more, possibly numbering to millions, though no accurate or even rough estimate is available. The number of asymptomatic carriers, on the other hand, is computed to a staggering figure of 200 millions in the world today, comprising of approximately one-twentieth of the total population. The carrier rates, however, vary from one part of the world to another, being very high to the tune of 15 to 20% in the Far East and Africa, 4 to 10% in India, Southern and Eastern Europe and the USSR and low figures ranging from 0.1 to 0.7% in the UK, Western and Northern Europe, USA, Canada, South America and Australia.

Our own interest in the relationship of HBV infection to chronic and fatal liver diseases was initiated almost at the same time when Prof. Shikata was reporting on orcein positivity of the surface component of the virus (HB s Ag). The studies I am going to describe now were carried out by a young intern, Dr. Rajiv Sachdeva during her period of elective posting and by Dr. Asha Dhar. We initially posed the following two questions to ourselves: (1) if the formalin fixed antigen can be stained by a non-specific histochemical reaction like orcein staining, can it also be identified by more specific immuno-histochemical tests? (2) If the answer to (1) is "yes", can one define the degree of specificity and reliability of the technically simpler orcein stain? Using conventional formalin-fixed paraffin-embedded liver tissue, some of these stocked for several years, it was possible to

clearly localize and identify the HBsAg by indirect immunofluorescence and indirect-immuno peroxidase staining reaction. Also, complete locational and morphologic identity was seen between material stained by these specific immunological methods and by orcein. Since immuno-staining revealed only the surface coat of the virus this seemed to be the only component stainable by orcein. Applying identical immunohistochemical reactions after using specific antibody to the core component of virus B (HBcAg) we could again successfully demonstrate this antigen in the nuclei of hepatocytes in conventional formalin-fixed paraffin embedded tissue. Both components of the virus showed complete randomness in distribution within the liver and inside the liver cells. This latter finding made it apparent that detection or otherwise of HBV components in a sample of liver tissue would depend entirely on the quantum of material examined. We later confirmed this possibility by comparing positivity rates between needle biopsy, wedge biopsy and autopsy material as well as between increasingly larger amounts of tissue examined for the virus. These findings helped us to draw the following conclusions. Application of these immunological techniques and orcein staining to material stored for decades in hospitals and laboratories and available for examination of relatively large samples from each specimen would provide fairly conclusive evidence on the association or otherwise of hepatitis B virus in a variety of liver diseases.

We next proceeded to test the hypothesis that hepatocellular cancer (HCC — primary liver cell cancer) and certain

forms of hepatic cirrhosis in man are associated with hepatitis B. virus infection. One of the important and first requirements to ascribe causal role for a virus to a tumour is association between the two both at epidemiological and at biological levels. Sufficient indications for the former are already available. Parts of the world where carrier rates for HBV are high show an extremely high incidence of HCC as well as of non-alcoholic cirrhosis of liver. In fact, the frequencies of occurrence of these fatal liver diseases in different parts of the world can be directly correlated with the respective HBsAg carrier rates. Also, HCC almost universally occurs in a liver that is cirrhotic as well, particularly of the non-alcoholic variety. With this background of epidemiological informations it would be necessary to establish a biological association between the virus and these forms of liver disorders. Serological marker studies have shown evidence of continuing infection by HBV in significant numbers of these categories of patients as compared to controls. We, therefore, wanted the following questions to be answered while trying to identify viral components in liver tissue: (1) Is there a significant association between the virus on one hand and HCC and non-alcoholic cirrhosis on the other? (2) If so, how strong are these associations? Initially we collected from different parts of the country 50 cases of hepatocellular carcinoma, 58 of cirrhosis and 54 of miscellaneous liver disorders, the last named serving as controls. The staining procedures were rigidly controlled and particular attention was given to screen adequate amounts of tissue in each case in order to overcome false

negative results due to sampling errors referred to earlier. HBsAg was detected in 94% of HCC, 71% of cirrhosis and only 2% of control livers while HBcAg was found in 22%, 31% and none respectively. Both antigens were present in 9 of 41 HCC, 12 of 39 cirrhosis and none of 25 controls. HCC was associated with cirrhosis in the majority (92%) and antigen positive cirrhosis had a higher chance of harbouring HCC than antigen negative disease. In 4 cases HBsAg was seen inside occasional tumour cells though no tumour showed HBcAg. We subsequently examined a much larger number of livers removed at autopsy, 904 to be precise, through the aegis of the International Agency for Research on Cancer as coordinator in an international collaborative programme. While this material showed patterns of positivity in HCC and in non-alcoholic cirrhosis more or less identical to those in our initial investigation, an additional feature that emphasized the association between HBV infection and these two liver disorders was the fact that a further correlation was observed between the positivity rates and the risk of developing HCC. In other words, livers obtained from high and intermediate cancer areas showed much higher frequencies of positivity for HBsAg as compared to those from low risk areas. Also, the commonest histological variant of HCC, namely the trabecular type was most frequently associated with HBsAg in the liver. In this material again, livers with cirrhosis not only tended to be more frequently positive for HBsAg but also, in the latter's presence harboured the tumour more often than in its absence. We concluded that not only is there a significant asso-

ciation between hepatitis B virus, HCC and cirrhosis but also that these associations seem to be very strong indeed. We surmised that infection with HBV may lead on to the development of hepatocellular carcinoma through more than one pathway and that cirrhosis may provide a rich soil for the development of this fatal tumour.

Another requirement for proving virus as an etiological agent in cancer is to demonstrate a direct target cell transformation by the virus itself. There are essentially two ways of looking at this aspect of the problem, firstly to look for neoplastic features in cell transformed by the virus and secondly to demonstrate virus production by human tumour cells which grow *in vitro* like cancer cells while maintaining features of the cell of origin. We were lucky in being able to establish and maintain a human hepatocellular carcinoma cell line which releases hepatitis B virus surface antigen to the medium. This work was largely carried out by my colleague Dr. P. K. Das. The cell line, referred by us as DELSH-5 was derived from a HBsAg sero- and tissue-positive patient subjected to operative wedge biopsy of the liver in which the cells not only resemble hepatocytes on light and electron microscopic examination but also possess biosynthetic markers of the latter like albumin and alpha feto protein. They have an aneuploid karyology with the major modal chromosome number being 61, and have been releasing moderate amounts of HBsAg following the few initial passages. Immuno-histochemical techniques as well as electron microscopy on the other hand, have consistently failed to

demonstrate any virus component inside the cell. We have never been able to show HBcAg either inside the cell or in the medium. With the availability of cell lines like this it would be possible to study several aspects of hepatitis B virus-liver cell interaction with particular reference to the oncogenic potential of the virus. In fact, some recent studies have indicated that parts of the viral genome are incorporated in the chromosomes of these tumour cells and seem to be responsible for the coding of the RNA protein representing HBsAg. Certain other criteria still need to be fulfilled in order to finally say with definitiveness that HBV does induce hepatocellular cancer in man.

One other aspect of hepatitis virus infection that has interested us is the pathogenesis of the liver cell injury in acute and chronic hepatitis. There is almost no doubt that the virus itself, and this is particularly obvious in case of the B virus, has no cytopathic effect. Carriers often contain a very large load of HBV components inside the liver cells without exhibiting any significant liver injury. Even when one detects the viral components in livers of chronic hepatitis, cirrhosis or HCC the individual cells that contain the virus frequently show no evidence of injury or inflammatory cell reaction whatsoever. Compelling evidence is available now to indicate that out of the two limbs of immune reaction mounted by the host against the virus the cellular rather than the humoral one plays a major role in the pathogenesis of hepatocytic injury. Indeed it is this immune killing of the infected liver cell that is the body's principal attempt at eliminating the

virus. Lymphocyte is the predominant reactant cell in the livers of acute hepatitis, often seen in close contact with infected cells while *in vitro* tests have demonstrated heightened cellular immune reactions mediated by lymphocytes against purified hepatitis B virus exhibited as blast transformation or killing of antigen coated cells. However, very recent studies reveal that the specificity of liver cell killing is not determined by recognition of hepatitis B viral components by sensitized lymphocytes. It is thus possible that these effector cells identify either some constituents of the normal cell membrane or some new or altered antigens expressed in the infected liver cell.

In this connection a great deal of interest has recently been shown in the so-called "liver specific protein" (LSP), a substance or substances which appear to be specific to the liver cell membrane and can be referred to as liver membrane protein (LMP). In our laboratory Dr. Jain, Dr. Panda and Miss. Pranava have attempted to examine the role of immunological reaction against LSP (or LMP) in liver cell injury, both in human patients of hepatitis as well as in experimental models. Antibodies to heterologous LSP was looked for by indirect immunofluorescence test on normal rabbit or rat hepatocytes and by hemagglutination of sheep red blood cells coated by purified rat LSP in sera of patients suffering from acute hepatitis taken at different phases of illness. Cell-mediated cytotoxicity was assessed by studying killer action of lymphocytes from these patients and from controls on isolated and short-term cultured rat liver cells.

Our results showed high levels of cytotoxicity in 39 of 57 patients, the values ranging from 1% to 68% as compared to 1% to 13% in controls. Mean cytotoxicity during the first three weeks after jaundice was $35.5\% \pm 12.8$ in contrast to $5.6\% \pm 4.5$ in the controls. Cytotoxicity values even at the onset of the jaundice was higher in all patients when compared to the upper limit of controls. These high toxicity started reducing after 3 weeks and touched near normal at 6 weeks and later. Serial studies done in 14 patients showed an identical pattern and 1 out of 3 patients who continued to show high levels of cytotoxicity after 6 weeks, one later developed chronic active hepatitis. HBsAg seropositivity did not relate with cytotoxicity. Also, these values did not relate to the serum transaminase and bilirubin levels. The two tests for humoral immune response against LSP correlated well to the extent of 80.5%. Significant positivity was seen only by the 4th week after onset of jaundice and by 6 weeks and later a decline in the titre of antibodies and in the number of patients showing detectable antibody levels was noted. Serial studies on antibodies again showed identical pattern. Since it is well known that by the time the patient of acute hepatitis presents in the hospital, hepatocellular degeneration and necrosis is not only well established but often is on the decline, the exact implication of the immune reaction findings are difficult to assess. It is possible that higher levels of cytotoxicity may have been manifest in the earlier prodromal phase of the disease. It is also possible that the immune reactions observed in our patients to LSP may represent an epiphenomenon unrelated to initial hepatocellular damage.

We have recently studied the immunological response of mice to LSP with essentially three questions in mind. Firstly, what is the type of immunological response mounted against LSP ? Secondly, does liver injury and inflammation of the type seen in acute or chronic hepatitis occur in the sensitized animals ? And lastly, is one or more immunological response related to the liver injury ? Purified rat LSP was injected in one or two doses to batches of mice and development of cellular and humoral immunity were tested at variable intervals initially extending upto 8 weeks but in subsequent experiments to 16 weeks. Delayed type hypersensitivity (DTH) measured by foot pad swelling following a small challenge dose and cellular cytotoxicity by lymphocytes on hepatocytes both in autologous and homologous *in vitro* systems served as parameters of cell mediated immune reaction. Humoral immunity was tested by looking for antibodies through precipitation reactions and hemagglutination of sheep red blood cell coated by purified rat LSP. Significant DTH compared to the controls developed in the sensitized animals at 2, 3 and 4 weeks 24 and 48 hours after challenge. Not only was there obvious swelling of the test foot pad as compared to the control injected by buffer alone, but also the challenge tissue was densely infiltrated by lymphocytes, histiocytes and neutrophils. In the autologous system significant cytotoxicity was elicited in the sensitized animal only, while in the homologous system no cytotoxicity was observed when sensitized hepatocytes were put with control animal's lymphocytes though some degree of cytotoxicity was apparent when control hepatocytes were

incubated with lymphocytes from sensitized animals. These experiments were designed to eliminate possible interference of HLA incompatibilities in these test systems. The cytotoxicity appeared somewhat later than the DTH at 4th week, gradually increased through 6th and 8th week and attained a maximum by the 14th week, plateauing at 16th week. DTH on the other hand reduced by 6th week.

Examination of the liver showed focal infiltration by lymphocytes and a few histiocytes in the initial phases without any significant liver cell damage but at 14 and 16 week almost all animals showed distinct ballooning degeneration, necrosis and severe diffuse inflammation of the liver by lymphocytes, phenomena in many ways reminiscent of the acute hepatic damage in human liver. While the findings in this study are extremely interesting and challenging, the observations are still preliminary and need further substantiation. It, however, appears that immunological response against LSP may be crucial in the pathogenesis of liver injury of acute and chronic hepatitis.

It is apparent that while great strides have been made in the understanding of infections by hepatitis viruses, a lot remains to be explored. In the words of the late John F. Kennedy: Life is unfair. No sooner do you get one problem solved than another one, often worse, rises up to take its place. The rates at which humans get infected by the different types of hepatitis viruses is appalling. Hepatitis A virus infects

almost all individuals throughout the world by the age of 20 years. The B virus is phenomenal in its existence and infective potential, entering through many routes into its human host. The non-A non-B viruses now constitute the most important agent for post-transfusion hepatitis in the West. A very large number of acute hepatitis occurring every day the world over and undoubtedly a large share of chronic hepatitis, cirrhosis and hepatocellular cancer are most likely related to these virus infections. When world figures for HCC are computed, being a frequent cancer in the more populous part of the world, this cancer, a very fatal one, possibly comes out as the number one cancer in the human male. Obviously the concern regarding infection by hepatitis viruses is grave and is universal. Luckily, new light is being thrown on many aspects of these infections every day, more is likely to be learnt about each one of these viruses, particularly the newer ones like non-A non-B virus. The vaccine against hepatitis B virus is almost ready for mass application. Very current reports indicate that it is not only effective but also entirely safe providing in addition a wide time range for protection. There is then light at the end of the tunnel and preventive measures, understanding of the basic pathogenesis of the various lesions produced by the viruses, and application of newer therapeutic regimens would certainly be able to save to a great measure the human race from this particular menace.