Acta virol. **37**: 466–474, 1993

INHIBITION OF HEPATIC EXTRAMEDULLARY HAEMOPOIESIS BY NUCLEOPROTEIN OF HETEROLOGOUS ROTAVIRUS STRAIN IN INFANT MICE

S. S. KANWAR, V. SINGH*, S. K., MEHTA, A. K. MALIK, V. K. VINAYAK, S. R. PAL, S. MEHTA

Postgraduate Institute of Medical Education and Research, Chandigarh-160 012, India

Received July 28, 1992; revised March 30, 1993

Summary. – Infant mice (NMRI strain) showed the inhibition of hepatic extramedullary haemopoiesis by oral inoculation of a 100 ID_{50} dose of EB rotavirus and nucleoprotein of SA-11 rotavirus (serotype 3). The extramedullary haemopoiesis was observed by oral inoculation of surface protein VP7 of SA-11 rotavirus and in control (placebo administered) mice.

Key words: EB (serotype 3) rotavirus; gastroenteritis; infant mice; extramedullary haemopoiesis

Introduction

Rotaviruses are major cause of morbidity and death in young animals and children (Holmes, 1983). The gastroenteritis caused by rotavirus infection in human infants of 6 months to 2 years of age results in approximately 1 million deaths throughout the world (DeZoysa and Feachem, 1985). The rotaviruses belong to the family Reovirideae, are non-enveloped with a diameter of 70–75 nm. They are characterized by the presence of 11 segments of double-stranded RNA (Arias *et al.*, 1982). Rotavirus generally inhabits, multiplies and invades mature villious epithelial cells of the small intestine causing maldigestion and malabsorption with consequent loss of electrolytes into the lumen (Little and Shadduck, 1982; Starkey *et al.*, 1986). The virus shows preferential tissue tropism in the small intestine (Riepenhoff-Talty *et al.*, 1982). The severity of the infection varies in duodenum, jejunum and ileum and perhaps is a serotype-characteristic. The serotype 3 rotaviruses rapidly multiply in the small intestine and are more infectious (Bell *et al.*, 1987). The rotaviruses previously known to inhabit and infect brush border membrane have recently been found to be associated with hepatitis in liver of immunodeficient mice (Uhnoo *et al.*, 1990) and human

*Corresponding author

infants (Kitamoto *et al.*, 1991). Earlier we have reported the inhibition of extramedullar haemopoiesis (EMH) in the liver of infant mice by oral inoculation of EB rotavirus (serotype 3)-homologous strain (Kanwar *et al.*, 1993). In this study we are reporting the inhibition of hepatic-EMH by oral inoculation of nucleoproteins of SA-11 rotavirus (serotype 3).

Materials and Methods

Animals. Ten to twelve day-old inbred mice of NMRI strain were used in the present study. The infants were nursed by their respective dams during the course of experiments.

Virus. Rotavirus strain EB originally supplied by Dr. H. B. Greenberg (California) was serially passaged 11-times in 10–12 day-old infant mice *in vivo* and purified from intestinal homogenate of the infected pups. The ID_{50} titer of the stock virus from the 11th passage (P_{11}) was estimated in a standard way.

Virus purification. Two litters of 10–12 day-old infants (n = 6 each) were orally administered 100 μl of EB rotavirus P_{11} suspension and the animals were sacrificed 3 days post inoculation (p. i.). Their small intestines were dissected out, pooled and homogenized in virus suspension buffer (VSB, 1.5 mmol/l $CaCl_2$ in 100 mmol/l Tris HCl.pH 7.2) at 4 °C. The homogenate was clarified by spinning at 10 000 x g for 30 mins at 4 °C and the supernatant was precipitated with 8.0 % (w/v) of polyethylene glycol (MW 6000, Sigma). This suspension was once agitated, kept undisturbed at 4 °C overnight and then centrifuged at 10 000 x g for 30 mins. The precipitate was collected, suspended in 5 ml 0.5 mol/l sterile NaCl and centrifuged in cold. The collected precipitate was washed twice in 0.5 mol/l NaCl and again reconstituted with 5 ml VSB, extracted with an equal volume of trichlorotrifluoroethane (Sigma) and centrifuged at 3000 x g at room temperature.

The virus containing upper aqueous phase was retained and centrifuged at 125 000 x g through 40 % (w/v) sucrose cushion at 4 °C for 90 mins (M 60 IEC ultracentrifuge). The virus pellet was finally reconstituted with 2 ml of sterile PBS pH 7.2, and used to infect fresh litters of infant mice.

Preparation of virus antigens. A crude preparation of tissue culture grown (MA-104 cell line) simian rotavirus SA-11 proteins and nucleoproteins (PNP) was prepared by the method of Sripati and Warner (1978). The outer capsid virus polypeptide VP7 was extracted from double capsid rotavirus by the method of Brussow *et al.* (1990). The protein concentration was estimated in each preparation.

Oral inoculation of infant mice. Five litters of 10–12 day-old infant mice were intragastrically administered 100 μl (100 ID_{50}) of EB rotavirus (P_{11}). The pups were kept with their respective mothers. Simultaneously two litters of 10–12 day-old infant mice were given 100 μl of the placebo (prepared from uninfected 10–12 day-old infant mice intestines) and kept away from the infected pups. Three to six infants from placebo or infected regiments were sacrificed on day 1, 3, 5 and 7 p. i., their liver sections were fixed in 10 % formaline saline and tissues were embedded in paraffin blocks. Besides the infected and placebo regiments, one litter of 10 day-old infant mice each was orally administered 6 μg of crude SA-11 rotavirus PNP antigen or SA-11 rotavirus VP7 antigen. Three mice from each group were sacrificed on the 3rd day after antigen intake for study of liver histology.

Histological stainings. 4–5 μm thick liver sections from each infant liver biopsy were stained with Haematoxylin-Eosin or rotavirus-specific immunoperoxidase. For immunoperoxidase staining, the tissue sections were passed through descending concentrations of methanol and brought into PBS. The endogenous peroxidase activity was quenched with 0.3 % H_2O_2 in methanol and the non-specific sites were blocked with 3 % bovine serum albumin (BSA) in PBS. The EB rotavirus in the biopsies was tagged with anti EB rotavirus rabbit hyperimmune serum. Finally, swine anti-rabbit horse radish peroxidase-conjugated swine immunoglobulins (Dakopatts, Denmark), were added as a detector antibody and the enzyme reaction was visualized with diaminobenzedine solution. The counter-staining was done with Haematoxylin for 1 min. The tissue sections were then passed through ascending concentrations of methanol and kept in xylene for 30 mins before mounting.

Results

The EB rotavirus-infected mice became lethargic, developed distention of stomach, haunchback and muscle cramping 2–3 days p. i. The incidence of diarrhoea was maximum 3–4 days p. i. The diarrhoea was self-limiting and resolved by the 6th day p. i. The diarrhoeal mice passed yellowish, watery stools and even the caecum was found to be filled with yellowish fluid on dissection. The infected pups lost 30–40 % of their body weight as a result of dehydration as compared to the control mice.

The Haematoxylin/Eosin stained liver biopsies of control group of 10–20 day-old mice showed presence of EMH. The latter was diffuse and characterized by the presence of large-sized megakaryocytes with simultaneous presence of granulocytes (Fig. 1). All the liver biopsies of control infant mice were positive for EMH. The EB rotavirus infection gradually inhibited EMH in the liver (Table 1, Fig. 2). By the 5th day p. i. EMH was completely inhibited. Furthermore, 25 % to 60 % of the infected liver biopsies revealed also dilatation of central vein.

The rotavirus (serotype 3) specific immunoperoxidase staining performed on liver sections 3 days p. i. was positive for EB rotavirus in 40 % of the tested

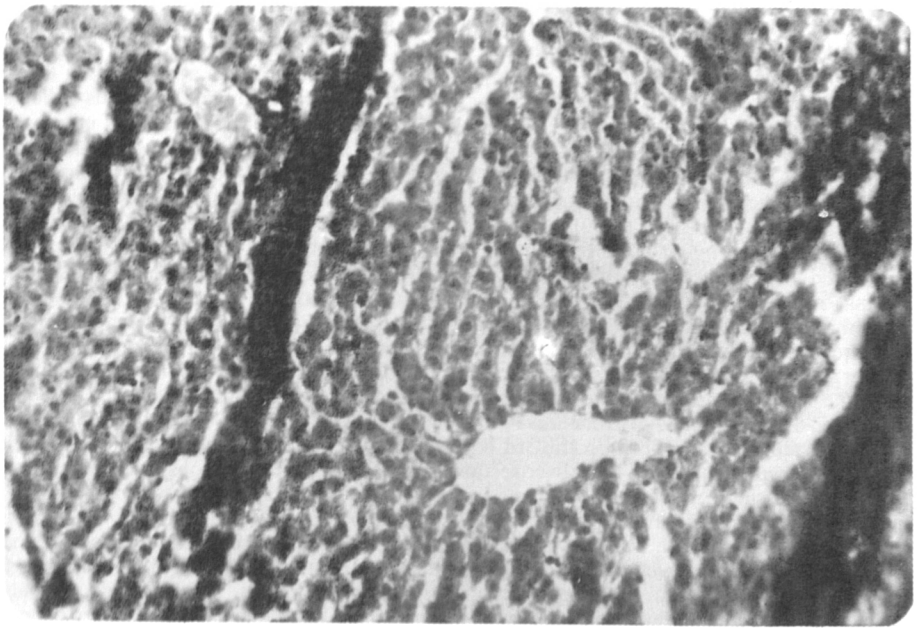

Fig. 1
Inhibition of hepatic EMH by rotavirus infection

Table 1. Effect of EB rotavirus infection on liver histology of infant mice

Biopsy taken (days p. i.)	Haematoxylin/Eosin staining						Immunoperoxidase staining	
	Dilatation of central vein		EMH positive		Hepatocyte degeneration			
	Infected	Control	Infected	Control	Infected	Control	Infected	Control
1	0/6*	0/3	1/6	3/3	1/6	0/3	0/6	0/3
3	3/5	0/3	1/5	3/3	1/5	0/3	2/5	0/3
5	2/6	0/3	0/6	3/3	0/6	0/3	0/6	0/3
7	1/4	0/3	0/4	3/3	0/4	0/3	0/4	0/3

*Ratio of positive to total tested.

Table 2. Effect of SA-11 rotavirus protein VP7 and crude PNP on liver histology of infant mice

Antigen administered	Haematoxylin/Eosin staining			Immunoperoxidase staining
	Dilatation of central vein	EMH positive	Hepatocyte degeneration	
VP7	0/3*	3/3	0/3	0/3
PNP	0/3	0/3	0/3	0/3

*Ratio of positive to total tested.

Biopsies taken 3 days post antigen administration. VP7 antigen was prepared from tissue culture grown virus by EDTA treatment (Brussow *et al.*, 1990). PNP antigen was extracted from tissue culture grown virus with phenol: chloroform:isoamylalcohol (50:48:2) (Sripati and Warner, 1978).

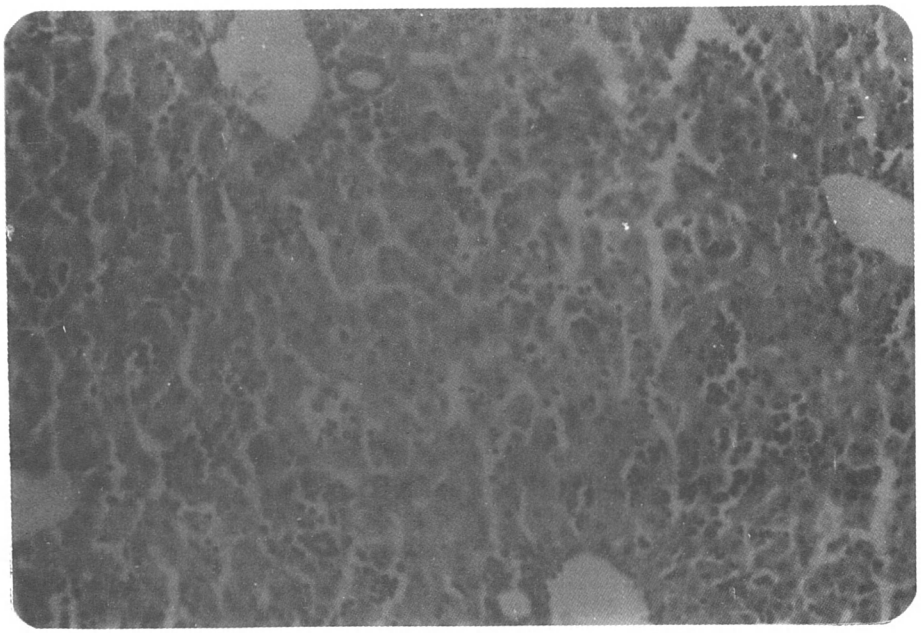

Fig. 1

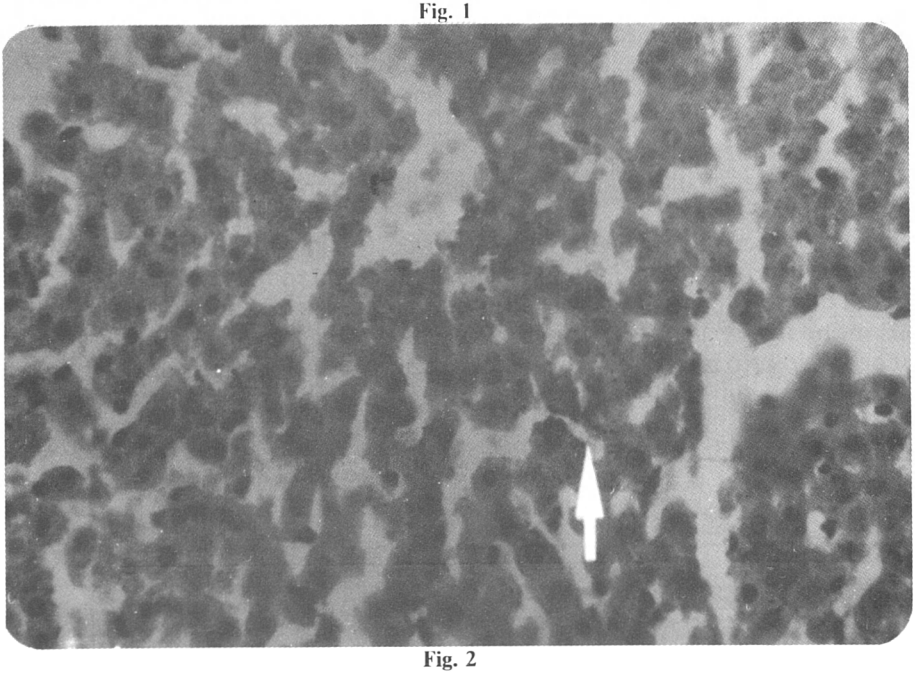

Fig. 2

For legends see page 472

biopsies (Table 1, Fig. 3). Hepatocytes as well as Kupffer cells showed positivity for rotavirus antigen.

The liver biopsies of the animals orally administered with rotavirus outer capsid VP7 polypeptide revealed normal histology and presence of EMH on the 13th day of age as observed in the controls (Table 2). However, the animals given orally an equal amount of crude PNP antigen showed inhibition of EMH on the same day of age. The virus antigen could not be detected in any of the liver biopsies of VP7 or PNP antigen-administered mice by EB rotavirus specific immunoperoxidase staining.

Discussion

The rotaviruses, previously known to multiply exlusively in the small intestinal mature villous enterocytes with or without clinically recognizable diarrhoea, have recently been implicated in liver infection in immunointact and immunodeficient animals (Uhnoo *et al.*, 1990) and infants (Kitamoto *et al.*, 1991). The involvement and demonstration of the rotavirus in the hepatocytes have further complicated the clinical manifestation and pathogenesis of the rotaviral disease. Uhnoo *et al.* (1990) have recently demonstrated that mice (both immunointact and immunodeficient) develop lesions in the liver resembling diffuse hepatitis with focal areas of parenchymal necrosis with two heterologous animal rotavirus strains; RRV (rhesus) and WC3 (bovine) (Uhnoo *et al.*, 1990).

In our earlier study we have shown the inhibition of hepatic EMH by oral inoculation homologous EB rotavirus and also demonstrated the presence of rotavirus antigen in hepatocytes, Kupffer cells and enterocytes of ileum of mice by immunoperoxidase staining by 3 days p. i. (Kanwar *et al.*, 1993). The infection was rapidly cleared and viral antigen could be demonstrated by 7 days p. i. in the small intestine. Thus it appears that virus migrates from the small intestine after it had replicated within the villous enterocytes and reaches the liver either through the blood circulation or lacteals. In addition to the demonstration of the virus in the hepatocytes, a striking inhibition of the aggregation of haemopoietic cells in the liver was observed in the infected animals. Such an observation has not been hitherto mentioned in any of the earlier studies. It is postulated that the rotavirus (antigen) has an inhibitory effect on the haemopoietic cells while the virus could only be demonstrated for a very short period. Furthermore, such an inhibitory effect might have been carried out by the products of the virus. It may

Fig. 2
Aggregates of megakaryocytes and WBC precursors (EMH positive) in normal liver

Fig. 3
Hepatocytes and Kupffer cells positive (brown coloured areas) for EB rotavirus antigen

also be mentioned that EB rotavirus did not cause hepatitis-like lesions in contrast to what has been demonstrated by Uhnoo *et al.* (1990). This difference may be caused by different rotavirus strains used and/or immune status of the animals.

The inhibitory effect on EMH in the liver appears to have a direct relationship to the presence of virus in the liver reticuloendothelial cells. But how does the rotavirus multiply and infect the hepatocytes without undergoing a proteolytic cleavage (Offit *et al.*, 1986) (trypsinization) of VP4 polypeptide (viral haemagglutinin) is presently unknown. It may be presumed that the virions are activated in the small intestine before reaching the liver. The hepatocytes seem to be competent to display rotavirus-specific binding receptors on their surface but it is yet to be seen whether the nature of rotavirus VP7-specific 300 K receptor (glycoprotein), recently isolated from the infant mouse brushborder membrane (Bass *et al.*, 1991) is similar to those present in the liver of the infant mice. Immunodeficient mice (SCID) develop severe acute diarrhoea with rotavirus shedding that continued indefinitely, but no evidence of extramucosal spread or altered tissue tropism of homologous rotavirus was observed (Uhnoo *et al.*, 1990). Inspite of the high prevalence of rotavirus infections in human infants, extraintestinal localization of this infectious virus has not been previously examined perhaps due to ethical implications. Several reports on elevated aminotransaminases (Kovacs *et al.*, 1986) and a single case of hepatomegaly in children (Tallett *et al.*, 1977) indirectly suggested involvement of liver rotavirus disease. An attempt was also made in the present study to underline the factor(s) responsible for mediating inhibition of hepatic EMH in rotavirus infection. It was observed that the component(s) responsible for EMH inhibition were present both in the virulent EB rotavirus (serotype 3) and crude PNP SA-11 rotavirus (serotype 3) antigen. It was further confirmed that rotavirus outer capsid glycoprotein VP7 does not cease the hepatic EMH. But it is yet to be seen whether the EMH inhibition is mediated by rotaviral haemagglutinin (VP4 polypeptide) or some other viral substance or it is a serotype characteristics of the rotavirus strain. Another possibility may be that the viral nucleic acid (RNA) present in the crude PNP preparation mediates the inhibition of hepatic EMH because the viral RNA does not require "trypsinization" which is indispensable for the infectivity of the live virus.

In the light of foregoing discussion it is evident that the children suffering from rotavirus infections must be kept under close observation. Furthermore, the clinical evaluation and follow up of the patients should include assessment of liver function in rotavirus infections or prospective vaccine trials.

Acknowledgements. This study was supported by a Research Fellowship (9/141(33)/88 EMR-I) from CSIR, New Delhi, India, given to S. S. K. and forms a part of Ph. D. thesis of S. S. K.

References

Arias, C. F., Lopez, S., and Espejo, R. T. (1982): Gene protein products of SA-11 simian rotavirus genome. *J. Virol.* **41**, 42–50.

Bass, D. M., Mackow, E. R., and Greenberg, H. B. (1991): Identification and partial characterization of rhesus rotavirus binding glycoprotein on murine enterocytes. *Virology* **183**, 602–610.

Bell, L. M., Clark, H. F., Brien, E. A., Kornstein, M. J., Plotkin, S. A., and Offit, P. A. (1987): Gastroenteritis caused by human rotaviruses (serotype 3) in a suckling mouse model. *Proc. Soc. exp. Biol. Med.* **184**, 127–132.

Brussow, H., Bruttin, A., and Marc-Martin, S. (1990): Polypeptide composition of rotavirus empty capsids and their possible use as a subunit vaccine. *J. Virol.* **64**, 3635–3642.

DeZoysa, I., and Feachem, R. G. (1985): Intervention for the control of diarrhoeal diseases among children. *Bull. WHO* **63**, 569–583.

Holmes, I. H. (1983): Rotaviruses. In W. K. Joklik (Ed.): *The Reovirideae,* Plenum Publishing Corp., New York.

Kanwar, S. S., Singh, V., Malik, A. K., Vinayak, V. K., Komal, H. S., Pal, S. R., Mehta, S., and Mehta, S. K. (1993): Hepatic involvement in rotavirus gastroenteritis in a homologous murine model. *Immunol. inf. Dis.* **3**, 77–81.

Kitamoto, N., Raig, R. F., Matson, D. O., and Estes, M. K. (1991): Comparitive growth of different strains in differentiated cells. *Virology* **184**, 732–737.

Kovacs, A., Chan, L., Hotrakitya, C., Overturf, G., and Portnoy, B. (1986): Serum transaminase elevations in infants with rotavirus gastroenteritis. *J. Pediatr. Gastroenterol. Nutr.* **6**, 873–877.

Little, L. M., and Shadduck, J. A. (1982): Pathogenesis of rotavirus infection in mice. *Infect. Immun.* **38**, 755–763.

Offit, P. A., Blavat, G., Greenberg, H. B., and Clark, H. F. (1986): Molecular basis of rotavirus virulence: Role of gene segment 4. *J. Virol.* **57**, 46–49.

Reed, L. J., and Muench, H. (1938): A simple method of estimating fifty percent end points. *Am. J. Hyg.* **72**, 493–497.

Riepenhoff-Talty, M., Lee, P., Carmody, P. J., Barrett, H. J., and Ogra, P. L. (1982): Age dependent rotavirus - enterocyte interactions. *Proc. Soc. exp. Biol. Med.* **170**, 146–154.

Sripati, C. E., and Warner, J. R. (1978): Isolation, characterization and translation of mRNA from yeast. *Meth. cell. Biol.* **20**, 61–80.

Starkey, W. G., Collins, J., Wallis, T. S., Clarke, G. J., Spencer, A. J., Haddon, S. J., Osborne, M. P., Candy, D. C. A., and Stephen, J. (1986): Kinetics, tissue specificity and pathological changes in murine rotavirus infection of mice. *J. gen. Virol.* **67**, 2625–2634.

Tallett, S., MacKenzie, C., Middleton, P., Kerzner, B., and Hamilton, R. (1977): Clinical, laboratory and epidemiologic features of a viral gastroenteritis in infants and children. *Pediatrics* **6**, 217–222.

Uhnoo, I., Riepenhoff-Talty, M., Dharakul, T., Chegas, P., Fisher, J. E., Greenberg, H. B., and Ogra, P. L. (1990): Extramucosal spread and development of hepatitis in immunodeficient and normal mice infected with rhesus rotavirus. *J. Virol.* **64**, 361–368.